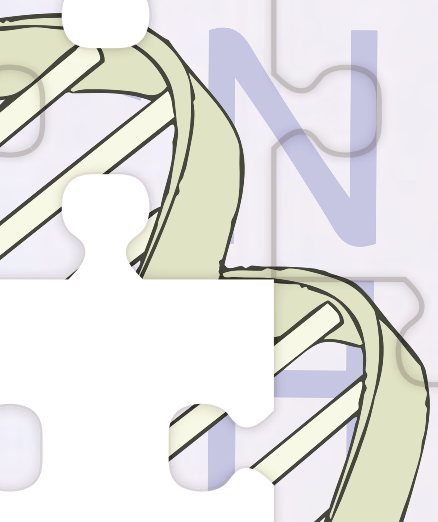
Mechanisms of Autoinflammation and Therapy Targets in Gout

Tania Octavia Crișan



MECHANISMS OF AUTOINFLAMMATION AND THERAPY TARGETS IN GOUT

TANIA OCTAVIA CRIȘAN

Colofon

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Mechanisms of Autoinflammation and Therapy Targets in Gout

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Tania Octavia Crișan

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Promotoren

Prof. dr. L.A.B. Joosten
Prof. dr. M.G. Netea

Manuscriptcommissie

prof. dr. P. Pickkers
prof. dr. dr. P.C.M. van de Kerkhof
prof. dr. N. Busso (Centre hospitalier universitaire Vaudois, Lausanne, Zwitserland)

To my Biology teachers,

Dedic această teză profesoarelor mele de biologie
care m-au împins de la spate spre acest domeniu până când totul a mers de la sine.

Doamnei profesoare Violeta Sarca
Doamnei profesoare Ana Vlad

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Adapted from "Factors modulating the inflammatory response in acute gouty arthritis". Curr Opin Rheumatol. 2017 Mar;29(2):163-170.

INTRODUCTION

The immune system is a complex machinery that protects the human body in response to a very wide range of possible aggressors that threaten its homeostasis, such as infectious pathogens, toxins, but also sterile stimuli found within the body (1). Sophisticated recognition systems allow the immune system to discriminate between self and non-self antigens and to fine tune immune responses (2). The possibilities of the immune system to cause damage to self are tightly controlled, but exist nevertheless. Our individual variability in immune function determines the susceptibility to immunological diseases (3). Moreover, variation in metabolite concentrations are closely linked to our inflammatory reactions, abnormal levels of metabolic products are revealed to function as triggers in immunologic diseases (4-6). With expanding insight into the pathogenesis of common diseases, the role of inflammation is becoming central even for prevalent adult diseases such as diabetes, cardiovascular diseases, gout or metabolic syndrome (7).

Autoinflammatory diseases

Autoinflammatory diseases are caused by an activation of the innate arm of the immune response, leading to an inflammatory cascade that provokes tissue damage. The concept of autoinflammation has been proposed in 1999 when gene variants of the innate immune system have been proven to be involved in a periodic fever syndrome etiology (the TNF receptor–associated periodic syndrome, TRAPS) (8). Knowledge of autoinflammation has expanded considerably and the spectrum of autoinflammatory diseases includes many mendelian diseases caused by rare mutations in innate immune genes (9) (Figure 1). Nevertheless, common polygenic disorders with a complex determinism, both genetic and environmental are increasingly being proven to have an autoinflammatory pathogenesis (9, 10) (Figure 1). Such examples are gouty arthritis, calcium pyrophosphate arthritis (CPP arthritis), inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (9, 10). Moreover, low grade chronic inflammation diseases such as atherosclerosis, obesity and type 2 diabetes mellitus (T2DM) are now also considered to have an autoinflammatory component mediated by the innate immune response through proinflammatory cytokines (9). This is different than the previously known category of autoimmune diseases known to be caused by deregulations involving the adaptive immune system, through autoreactive lymphocytes or autoantibodies (11). However, the branches of the immune response cannot be fully separated, therefore immunological diseases can share components of both types of responses (12)(Figure 1).

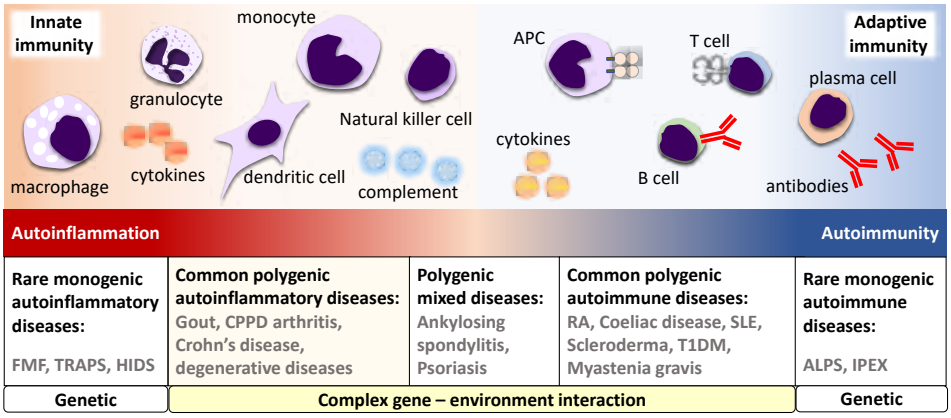

FIGURE 1. Continuous spectrum of immunological diseases ranging from autoinflammatory to autoimmune determinism.

The immune system functions through the interaction of two branches with interconnected cell types and effector molecules. Briefly, the innate immune system is based on the inflammatory and phagocytic capacities of granulocytes, monocytes or macrophages, while the adaptive arm of the immune response follows antigen presentation by antigen presenting cells (APCs) and consists in lymphocyte activation or autoantibody secretion by plasma cells. Dysregulation of any of these two branches causes damage to self through the specific innate or adaptive processes, or a combination of both. Diseases mediated by innate immunity are called autoinflammatory, and diseases where the adaptive immune response plays the main role are called autoimmune. Rare monogenic diseases have a strong genetic determinism due to mutations in genes involved in the immune response. Common polygenic diseases have a complex determinism, based on genes and environment interaction: a genetic susceptibility is usually present, but the disease only becomes phenotypically validated in synergizing environmental conditions. Gout is highlighted as part of the autoinflammatory group of diseases with a high prevalence and multifactorial etiology. FMF, Familial Mediterranean Fever; TRAPS, TNF receptor–associated periodic syndrome; HIDS, Hyper IgD Syndrome; CPPD, Calcium Pyrophosphate Deposition; RA, Rheumatoid Arthritis; SLE, Systemic Lupus Erythematosus, T1DM, Type 1 Diabetes Mellitus, ALPS, autoimmune lymphoproliferative syndrome; immune dysregulation, polyendocrinopathy, enteropathy,X-linked. Figure based on *(6-8)*.

IL-1β – A central mediator in autoinflammation

After the discovery that some autoinflammatory syndromes are caused by mutations in inflammasome components and caspase-1 (13), the role of interleukin-1 (IL-1) became crucial in autoinflammation and nowadays it has major pathogenetic and therapeutic implications in a very broad range of diseases (14, 15).

IL-1 is a very potent proinflammatory cytokine, with a tightly regulated production mechanism and very low circulating levels. There are two major IL-1 proteins, IL-1α, found mostly intracellularly and functioning as alarmin from dying cells, and IL-1β, that is secreted by monocytes or macrophages upon a two-step stimulation process (16, 17). IL-1β production is initially induced in response to stimuli that signal via receptors to induce cytokine transcription. However, IL-1β is translated as precursor protein and needs to be activated through protein cleavage by caspase-1. Caspase-1, in turn needs to be activated in protein platforms called inflammasomes (18). Therefore, a second stimulation that induces

inflammasome formation and procaspase-1 cleavage is needed to complete the activation and release of active IL-1β (Figure 2).

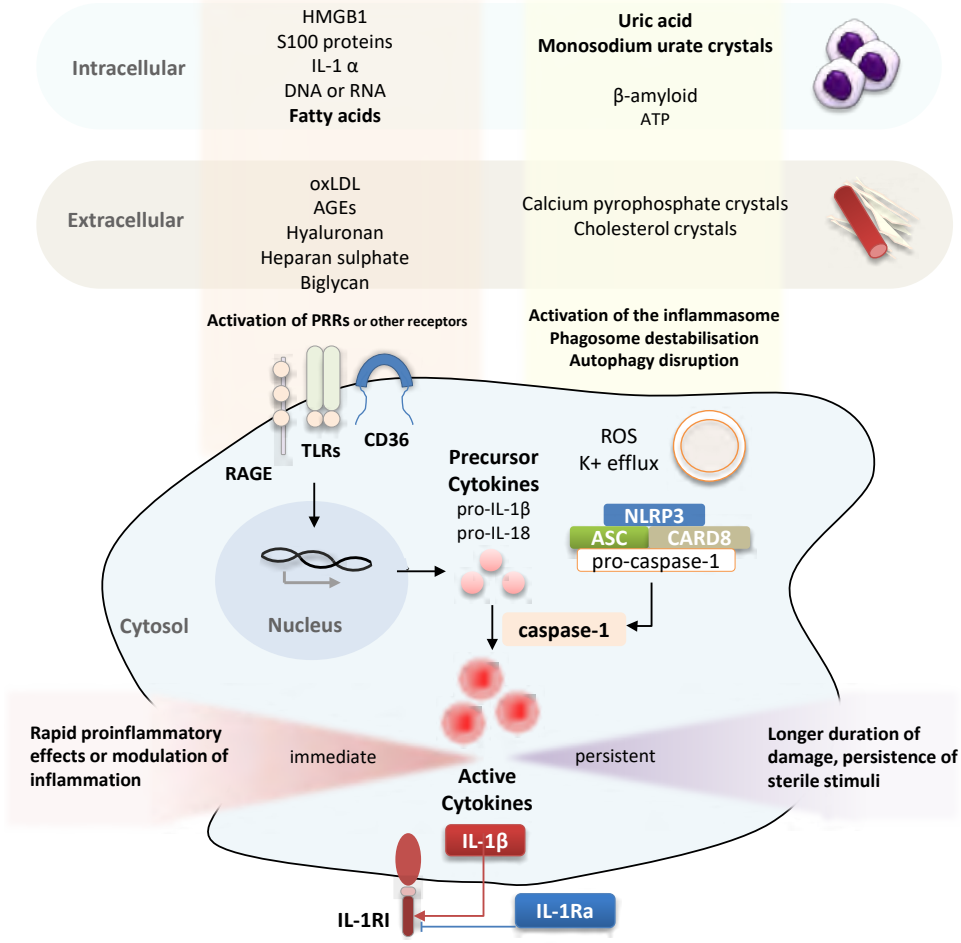

FIGURE 2. The IL-1 activation mechanism model with an emphasis on sterile stimuli that are involved in common autoinflammatory diseases.

Damage to tissues is accompanied by the release of intracellular molecules or by the formation of alarmins in the extracellular environment. These molecules signal via classical PRRs or other receptors and induce cytokine transcription (such as pro-IL-1β or pro-IL-18), whereas others can activate inflammasomes (in this scheme, NLRP3) to produce bioactive cytokines. The proinflammatory effects of DAMP signaling have the role to rapidly clear aggressors, either infectious or sterile. In the case of chronic damage and persistent exposure to sterile inflammatory stimuli, persistent damage can occur in the host due to hyperinflammation, therefore patients develop autoinflammatory disease phenotypes. The scheme gives examples of stimuli studied in relation to IL-1β activation. Upper rows list alarmins that originate in the intracellular space, examples such as fatty acids, uric acid and monosodium urate crystals (MSU) have been highlighted. Other stimuli originate in the extracellular environment and can signal similarly through the innate immune recognition apparatus. Some stimuli (such as fatty

acids), activate receptors located on innate immune cells and induce proinflammatory cytokine transcription and translation. Other stimuli (such as MSU crystals) can change the intracellular status of the cell, either by destabilizing the cell housekeeping machinery, by inducing damage, stress and reactive oxygen production, and by culminating in inflammasome activation. Inflammasomes lead to production and release of active cytokines, mainly IL-1β, that can further stimulate cytokine production in an autocrine or paracrine manner. Inflammation caused by IL-1β is modulated by the natural antagonist IL-1Ra (IL-1 receptor antagonist) that limits hyperinflammation by competing for the IL-1receptor type I (IL-1RI) binding site. DAMP, damage associated molecular pattern; HMGB1, high-mobility group box 1; oxLDL, oxidized low density lipoprotein; RAGE, advanced glycation end product; AGER, receptor for advanced glycation end products; CD36, cluster of differentiation 36; ROS, reactive oxygen species; NLRP3, NACHT, LRR and PYD domains-containing protein 3; ASC, apoptosis-associated speck-like protein containing a CARD; CARD8, caspase recruitment domain 8.

Patients with autoinflammatory disorders are known to have higher levels of IL-1 as compared to healthy controls, but most remarkably, their pathology significantly improves in response to anti-IL-1β therapy (14, 19). This is not only the case in rare autoinflammatory syndromes but is expanding in clinical usefulness in common disorders such as cancer, heart disease and refractory gouty arthritis (7). In gout, for instance, patients that are sub-optimally treated due to adverse effects or who exhibit recurrent arthritis attacks, show a strong and prolonged reduction in symptoms and flare prevention when treated with IL-1 blockade therapy such as anakinra (20), rilonacept (21) or canakinumab (22).

In common autoinflammatory diseases, the triggers of autoinflammation are not rare genetic defects that impair cytokine production, but rather a genetic predisposition to a higher response that becomes pathogenic in specific environmental conditions. For instance, Crohn's disease has been genome wide associated to a genetic variation in autophagy related gene *ATG16L1* that was proven to affect inflammasome activation and IL-1β production (23). Gout is genetically associated to uric acid transporter variation, but also genes involved in inflammation are starting to be studied in relation to the gouty phenotype and an association has been observed for *TLR4* (24, 25). Next to a genetic susceptibility, metabolic triggers that are modified in diseases can function as sterile inducers of inflammation. For example, in hyperglycemia, proteins become modified by glycosylation forming advanced glycation end products (AGEs) and both glucose levels or AGEs are studied as inflammatory stimuli (26). In atherosclerosis, LDL particles can be oxidized and contribute to inflammation and plaque formation (27). In gout and hyperuricemia, uric acid crystalizes to monosodium urate (MSU) crystals that have activator roles on the inflammasome (28).

A schematic overview of how sterile stimuli drive inflammation in common adult disorders, is depicted in Figure 2 with an emphasis on IL-1β production and immediate or persistent consequences. Studying the mechanisms of IL-1β induction is important for understanding the risk and identifying new therapy targets factors in inflammatory diseases.

Gout and hyperuricaemia – The current knowledge on an old disease

Acute gout is an ancient form of arthritis characterized by debilitating joint inflammation, commonly occurring in the first metatarsophalangeal joint. Gout was clinically described in antiquity, it was associated to a rich way of life and has for centuries shaped societies through its high frequency among famous personalities or kings, and through its presence in art and satire (29). The statements about gout made by Hippocrates, that date back to ancient Greek times, are some the oldest insights into the clinical description of gout, which are still true today (30):

"Eunuchs do not take the gout, nor become bald"

"A woman does not take the gout, unless her menses be stopped"

"In gouty affections, inflammation subsides within 40 days"

"Gouty affections become active in spring and in autumn"

Some of the most crucial contributions to understanding the etiology of gout were made by Garrod in the 19th century who described for the first time that uric acid is higher in gout patients and that crystals of urate cause gouty inflammation (31). It took another few decades before another seminal study described the presence of uric acid crystals in the synovial fluid of patients with gout (32).

Despite the long history and the known pathogenesis of gout, its prevalence is rising at an alarming rate and currently affects 1-4% of the population in North America and Europe (33, 34).

Mechanistically, as illustrated in Figure 2, essential for the development of gouty arthritis is the deposition of monosodium urate (MSU) crystals, which form when the blood is saturated with uric acid. MSU crystals act as a danger signal, activating the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome (28). This activates caspase-1, an enzyme capable of converting pro-interleukin-1β (pro-IL-1β) to mature IL-1β by proteolytic cleavage. Only in the presence of a secondary stimulus, like Toll-like receptor ligands or free fatty acids, pro-IL-1β is produced and can promptly be activated via cleavage by caspase-1 (35). The subsequent release of bioactive IL-1β is the key event for the development of acute gouty arthritis.

Despite general knowledge that the progression from soluble uric acid to MSU crystals and generation of inflammation is the causal mechanism of gout, some questions remain open in relation to the clinical observations related to gout and hyperuricemia. First, hyperuricemia is a relatively common metabolic condition, with only a minority of 10-15% of hyperuricemia patients developing symptomatic gout (36). The serum urate levels that define hyperuricemia are still largely debated based on relevance and clinical usefulness,

studies use values ranging from 6 to 7 mg/dL (0.36-0.42 mmol/L) as cutoff (36), while a recent recommendation suggests 6 mg/dL should be considered as common reference point (36). MSU deposition occurs because of uric acid supersaturation, but still a considerable proportion of patients (ranging from 16 to 82% as reviewed in (37, 38)) developed deposits without experiencing symptoms. Finally, under the current considerations, we refer to gout only when inflammatory events take place in the presence of MSU deposits (38). It is therefore also epidemiologically observed that neither hyperuricemia nor MSU deposits are sufficient to trigger full blown inflammation. This implies that additional susceptibility factors are necessary to promote gout progression and studies are required to understand what makes it that patients with similar backgrounds experience different autoinflammatory outcomes.

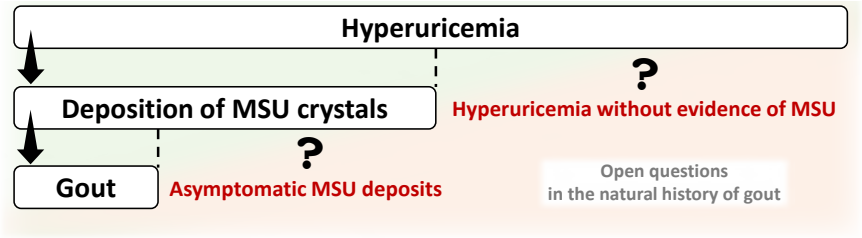

FIGURE 3. Schematic representation of decreasing proportions of individuals that manifest inflammation in gout despite the presence of well-known risk factors.

The natural history of gout follows a series of "check-points" in progression which are well-known and straightforward: hyperuricemia results in MSU crystal deposits, which in turn determine the appearance of gouty inflammation and symptoms (left green area). However, most hyperuricemic patients will not develop gout. More interestingly, an important proportion of patients display MSU deposits without showing any signs of inflammation. These clinical observations indicate that other factors are needed to explain the inherited or environmental additional susceptibility determinants of the gout phenotype.

The treatment of gout is based on urate lowering therapy and anti-inflammatory therapy: the first is based on compounds that reduce the formation of uric acid (e.g., xanthine oxidase inhibitors, such as allopurinol) or increase its excretion (e.g., uricosurics, such as benzbromarone) with the aim of maintaining serum uric acid levels to a target that reduces risk of symptoms; the latter is based on compounds that treat inflammation once an attack ensues (colchicine, steroids, non-steroid anti-inflammatory drugs, anti-interleukin-1 therapy) (39). However, adverse effects are possible, compliance to therapy is consequently affected, and gout is often a poorly controlled disease (39).

Although our knowledge on gout pathogenesis has expanded tremendously in the past decades, gout is still gaining ground and it is essential to get more insight into this complex metabolic disease.

New insights into uricase loss

the culprit for the formation of MSU crystals is hyperuricemia. The largest contribution to the elevated susceptibility to hyperuricemia and gout in humans comes from the fact that uricase, the enzyme responsible for the breakdown of uric acid to allantoin, is inactive. Recently, Kratzer et al. demonstrated that the uricase gene was subject to several mutations, which gradually reduced its enzymatic activity, before the eventual pseudogenization events that rendered the gene completely inactive (40). Tan et al. found that these events coincided with changes in the URAT1 transporter gene (41). The URAT1 transporter that was present in our ancestors 77 million years ago was a low affinity but high capacity transporter. Around 27 million years ago, the gene had mutated stepwise to a form of URAT1 that is characterized by a high affinity for uric acid but low transport capacity. Tan et al. show that the changes in URAT1 allowed for an increase in and better control of plasma urate levels. The persistence of the mutations in uricase and URAT1 indicates that increasing serum uric acid levels was a key event for the survival of the apes.

There are multiple theories about the possible evolutionary advantage provided by the loss of uricase. Some decades ago, it was proposed that uric acid acts as a scavenger for free radicals, protecting our ancestors from oxidative stress-induced damage (42). Another hypothesis postulates that uric acid stimulated intellectual evolution due to its structural similarity to caffeine and theobromine (43). Much more recently, Johnson and Andrews proposed a new hypothesis based on Neel's thrifty gene hypothesis (44-46). They argue that early hominoids first migrated to Eurasia during a period of global cooling and experienced periods of famine due to fruit being unavailable in the winter months. The loss of uricase may have aided the apes to turn fructose from fruits into fat tissue to survive these periods of scarcity. Unlike glucose, fructose leads to accumulation of triglycerides and uric acid. Uric acid was shown to induce hepatic lipogenesis through mitochondrial stress (47) .Additionally, uric acid-dependent inhibition of AMP-dependent protein kinase (AMPK) not only further inhibits beta-oxidation and stimulates fat accumulation (48), but also stimulates gluconeogenesis (49). According to Johnson and Andrews, uric acid protected ancient apes from starvation by increasing fat stores and maintaining glucose levels in the blood, but may currently underlie the metabolic syndrome pandemic.

Soluble uric acid: The prime suspect

Although many studies highlighted a strong association between uric acid and metabolic syndrome (50-52), there is also evidence in support of causative role of soluble uric acid. Liu et al. assessed the effects of allopurinol in addition to conventional treatment in hyperuricemic type II diabetes patients. They found that allopurinol treatment led to a larger reduction in uric acid, triglycerides, insulin resistance, and blood pressure, and led to a more significant improvement of renal function than conventional treatment (53).

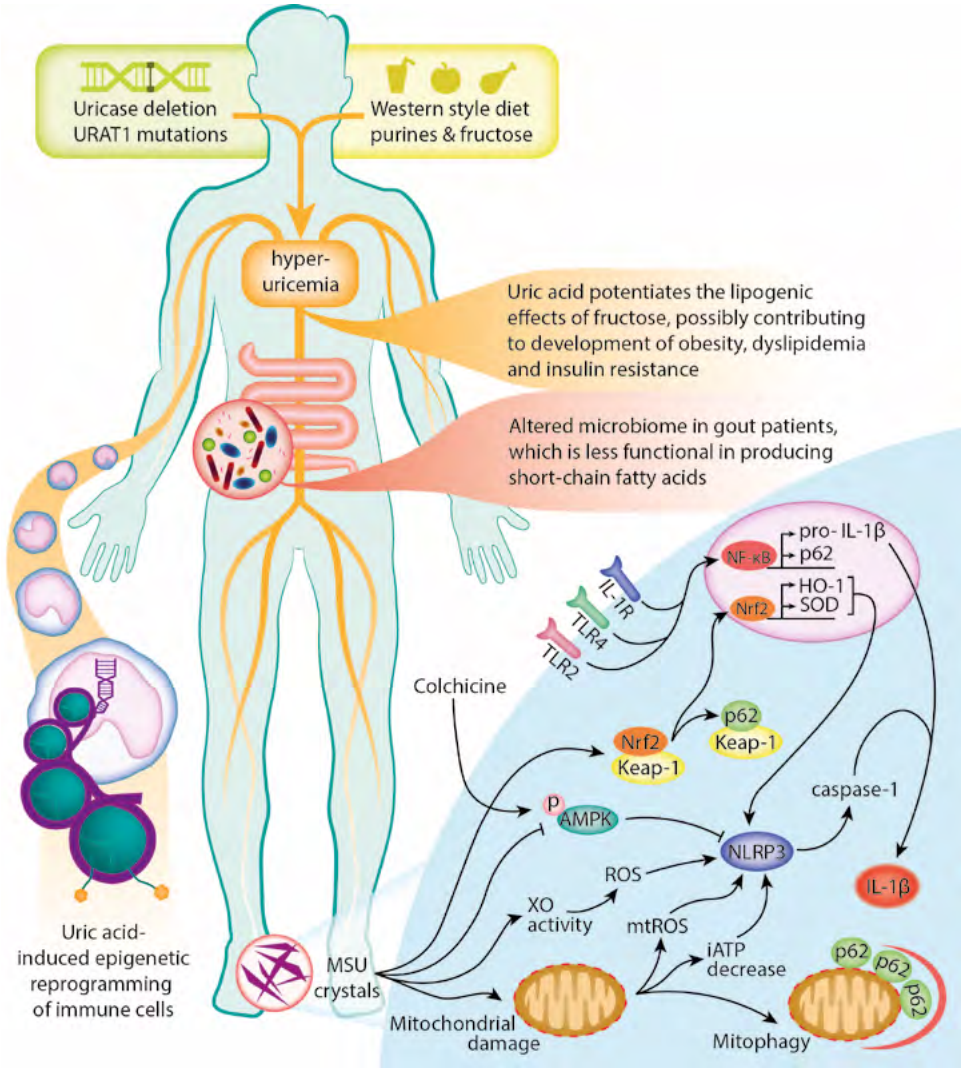

FIGURE 4. Intracellular effects of soluble uric acid and monosodium urate crystals.

Genetic mutations in uricase and URAT1, as well as current purine- and fructose-rich Western diets dramatically increase human susceptibility to development of hyperuricemia. High levels of uric acid can induce epigenetic reprogramming of monocytes, leading to a more pro-inflammatory state. An altered microbiome in gout produced less anti-inflammatory short-chain fatty acids. In the cell, monosodium urate (MSU) crystals induce many different effects, such as mitochondrial damage, up-regulation of xanthine oxidase (XO) activity, production of reactive oxygen species (ROS), decrease of intracellular ATP (iATP), inhibition of AMP-dependent protein kinase (AMPK), and nuclear translocation of Nrf2. All these effects have been shown to induce activation of the NLRP3 inflammasome, and subsequent activation of caspase-1 and pro-IL-1β. Expression of pro-IL-1β expression can be induced through signaling via Toll-like receptors (TLR) or the IL-1 receptor (IL-1R).

In line with this, a pilot study by Takir et al. reports that lowering uric acid with allopurinol in hyperuricemic subjects decreased fasting glucose levels, insulin levels, insulin resistance, and circulating CRP (54). Madero et al. found that allopurinol treatment in overweight prehypertensive subjects resulted in lower systolic blood pressure compared to a placebo group (55). These results suggest that uric acid may cause systemic inflammation and play a role in the development of obesity, diabetes and renal insufficiency. However, there is a need for larger and longer prospective trials with urate lowering therapy to confirm this.
The effects of soluble uric acid on systemic inflammatory responses were also studied *in vitro* (56). A 24-hour pre-incubation of PBMCs with soluble uric acid significantly increases IL-1β and IL-6 production in response to Toll-like receptor ligands alone and in combination with monosodium urate crystals. This coincides with a significant decrease in IL-1Ra production, the endogenous antagonist of IL-1β. The pro-inflammatory effects of uric acid could be restored with MTA, a histone methyltransferase inhibitor, indicating that the responses are likely to be epigenetically regulated (Figure 4). Importantly, this study provides a first mechanistic insight into the immune-modulatory properties of soluble uric acid that could potentially link hyperuricemia to metabolic and autoimmune diseases.

Gut microbiome and metabolites

Recently, a study by Guo et al. identified an altered microbiome in gout patients in China (Figure 4) (57). With a model based on the 17 microbe genera that were differentially represented in the gut of gout patients, gout could be predicted in a validation cohort with an accuracy of 88.9%. This could represent a future tool for the early diagnosis of gout, although validation in bigger cohorts and in different populations is a first necessity.
Interestingly, Guo et al. report that the gout patient gut microbiome is characterized by a significantly impaired microbial butyrate synthesis. Underrepresentation of butyrate-producing bacteria in the gut microbiome has been observed previously in type 2 diabetes patients (58, 59) and beneficial effects of butyrate have been shown with monosodium urate stimulation experiments *in vitro* (60). Butyrate is a potent inhibitor of class I histone deacetylases (HDACs) and inhibits monosodium urate-induced cytokine production in PBMCs from healthy donors and crystal-proven gout patients (60). We envisage, however, that the potential use of sodium butyrate for treatment of systemic inflammatory diseases such as gout is limited due to the high concentration needed for HDAC inhibition. Similar cytokine-suppressive effects were observed with more specific synthetic HDAC inhibitors at low dose (60), which may represent a more targeted treatment option. Therefore, there is a need for the development of safe, highly specific, and orally active histone deacetylase inhibitors.
Vieira et al. used a murine model to assess the effects of the gut microbiome, high fiber diet, and short-chain fatty acids in gouty arthritis. Interestingly, they observed that the

inflammatory response to an intra-articular injection of MSU crystals was dependent on microbial production of short-chain fatty acids in the gut. Adding acetate to the drinking water of germ-free mice restored this response. In addition, *in vitro* production of reactive oxygen species (ROS), caspase-1 activation and IL-1β secretion was dependent on GPCR43, a short-chain fatty acid receptor with highest affinity for acetate (61). In contrast, the same group reported that treatment with oral acetate, propionate and butyrate in mice promoted resolution of MSU-induced inflammation by inducing neutrophil death in the joints. Acetate was shown to accelerate caspase-mediated neutrophil apoptosis in a GPCR43-dependent manner (62). These studies indicate that gut microbiome and short-chain fatty acids are of vital importance in tuning the inflammatory response to MSU-crystals in mice. It remains to be determined how this translates to the human immune responses.

Intracellular sensors

The NLRP3 inflammasome is crucial for the initial IL-1β activation in response to MSU crystals (28), although there is still much debate on the mechanism (Figure 4). Recently, Nomura et al. demonstrated that MSU crystals induce K^+ efflux, leading to a loss of mitochondrial membrane potential and decreased intracellular ATP levels. This increased IL-1β production independently of MSU crystal-induced mitochondrial ROS production (63). Ives et al. found increased xanthine oxidase (XOR) activity in response to NLRP3 activators. Pharmacological XOR inhibitors or XOR knockdown inhibited MSU-induced IL-1β. This effect was dependent on cytoplasmic XOR-derived ROS as well as on mitochondrial PI3K-AKT-mTOR signaling and ROS production (64).

The role of mitochondrial ROS in MSU-induced IL-1β production is also of relevance in the context of autophagy. Zhong et al. reported that NF-κB negatively regulated NLRP3 activation by increasing the expression of p62, an important autophagy receptor. Upon stimulation with NLRP3 activators, p62, LC3-II and Parkin were recruited to damaged mitochondria, initiating their clearance via autophagy (mitophagy). The authors therefore propose that NF-κB-induced p62 expression negatively regulates NLRP3 activation by inhibiting the release of DNA and ROS from damaged mitochondria (65). Conversely, Jhang et al. proposed that p62 accumulates in case of MSU crystal-induced lysosomal disruption and impairment of autophagy. They showed that MSU crystals caused p62 to bind the kelch-like ECH-associated protein 1 (keap-1), which normally acts as a transcriptional repressor by binding to nuclear factor E2-related factor 2 (Nrf2), a transcription factor involved in oxidative stress responses. The release of Nrf2 from keap-1 facilitated its translocation to the nucleus and induced transcription of heme oxygenase-1 and superoxide dismutase, which was shown to be required for MSU-induced NLRP3 activation (66). Another study by Kim et al. observed a positive feedback loop between MSU-induced ROS production and p62 expression, both mediating an increase in caspase-mediated apoptosis and IL-1β

production (67). Thus, the NRLP3 inflammasome can be negatively regulated by p62 when damaged mitochondria are effectively removed by mitophagy, whereas excessive p62 can also stimulate NLRP3 activation through Nrf2-mediated transcription and ROS production.
A second intracellular sensor important for the pathogenesis of gout is AMP-activated protein kinase (AMPK, Figure 4). Wang et al. recently demonstrated that MSU crystals inhibit AMPK activity and AMPKα knockout mice showed a significantly increased inflammatory response in an MSU crystal air pouch model. Furthermore, colchicine was shown to induce AMPK phosphorylation *in vitro*, identifying a new molecular target of this ancient medicine against gout (68). Although it is of importance to validate these findings in human cells and patient cohorts, they do provide a rationale for further research to explore the possibility of using AMPK activators to treat gouty inflammation.

Extracellular processing

During an attack of acute gouty arthritis, enormous amounts of neutrophils are recruited to the joint. Without any control, neutrophils would sustain the inflammatory response endlessly and damage the surrounding tissues. However, even without treatment gouty arthritis is self-limiting. In a study by Cumpelik et al., neutrophil-derived phosphatidylserine (PS)-expressing ectosomes were shown to play a role in the resolution of the inflammation by binding the PS receptor MerTK, and inducing expression of suppressor of cytokine signaling (SOCS) 3. Independent of MerTK, the ectosomes induced tumor growth factor β (TGF-β) secretion (69)(Figure 5).
Another neutrophil-specific process is the formation of neutrophil extracellular traps (NETs). Schauer et al. reported that NETs aggregate when there is a high density of infiltrating neutrophils, as occurs in the synovial fluid during acute gout. Besides extracellular DNA, these aggregated NETs were shown to contain proteins from neutrophil granules, such as serine proteases, which could degrade secreted cytokines and limit the inflammation. In contrast, when the number of neutrophils is too low to induce aggregated NETs, the process of NETosis promotes inflammation (70) (Figure 5). Recently, it was described that NET formation in response to MSU crystals is dependent on signaling via receptor-interacting protein kinase (RIPK)-1, RIPK-3, and mixed lineage kinase domain-like (MLKL) (71). The authors conclude that these signaling molecules may be interesting new therapeutic targets to prevent pro-inflammatory NET formation. However, it is crucial to approach this with care as inhibiting NETosis might also exacerbate inflammation through prolonged presence of neutrophils.
Importantly, neutrophil-derived serine proteases such as proteinase-3, neutrophil elastase, and cathepsin-G can cut precursor IL-1β, which is released upon cell death, very close to the caspase-1 cleavage site. In this way, secretion of neutrophil serine proteases mediates caspase-1-independent IL-1β activation (72, 73). A natural inhibitor of neutrophil serine

proteases is alpha-1-antitrypsin (AAT). Recently, we reported on the therapeutic effects of a newly developed AAT-Fc fusion protein. *In vitro* this compound was shown to inhibit PR3-mediated activation of pro-IL1β and increased IL-1Ra production. Moreover, human AAT transgenic mice were protected from MSU-induced gouty arthritis (74) (Figure 5). Besides treatments inhibiting caspase-1 activation, this is a very promising additional treatment option to limit caspase-1-independent IL-1β activation by infiltrating neutrophils.

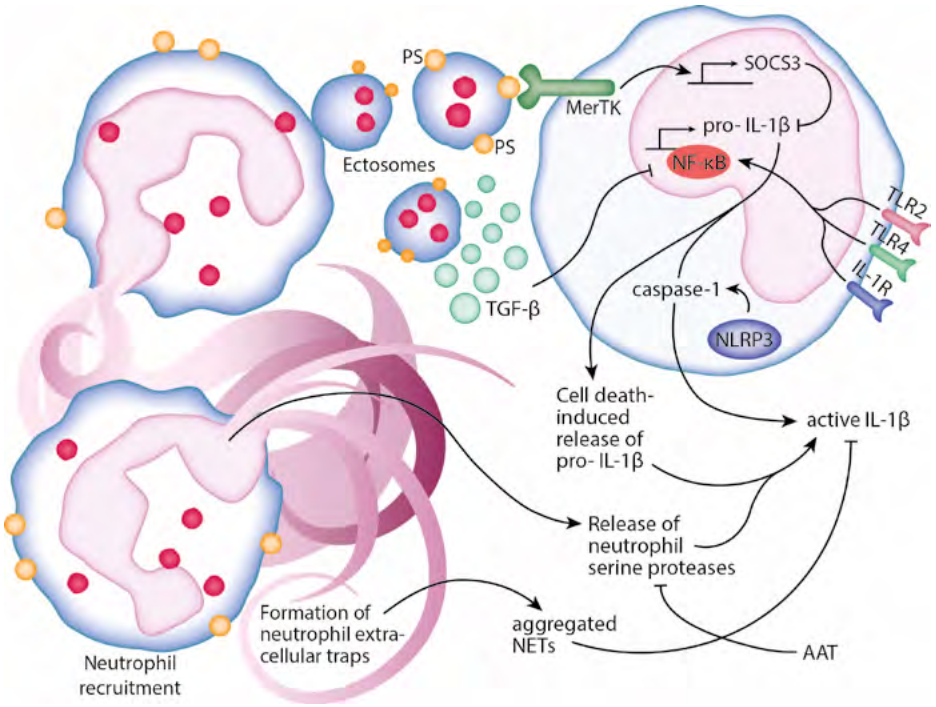

FIGURE 5. Neutrophil-mediated effects in acute gouty arthritis.

Recruitment of neutrophils in the first place serves to promote the inflammation. Upon formation of neutrophil extracellular traps (NETs) the serine proteases are released that can activate pro-IL-1β, which is released upon cell death. Alpha-1-antitrypsin (AAT) inhibits serine protease-mediated IL-1β activation. In contrast, in case of high density of neutrophils, their NETs aggregate and have an anti-inflammatory function by capturing and inactivating pro-inflammatory mediators. Neutrophils have another role in resolution of the inflammation by secreting ectosomes. These express phosphatidylserine (PS), which can bind the MerTK receptor and induce the expression of suppression of cytokine signaling 3 (SOCS3).

Concluding remarks

Here, we highlighted some of the important advances in the field of gouty arthritis. New light was shed on the importance of soluble uric acid. Based on recent results and new theories, urate-lowering therapy may prove to be a crucial aid in preventing not only gout,

but also hyperuricemia-induced renal failure, hypertension, insulin resistance, and systemic inflammation in the future. Another important finding was a gout-specific microbiome, which might represent a new tool for the early diagnosis of gout or an increased risk of gout. New studies on the role of microbiome-derived short-chain fatty acids in gout warrant further research to the role of GPR43 in humans, as well as the possible therapeutic effects of highly specific histone deacetylase inhibitors.

The first step in acute gouty arthritis is the MSU-induced intracellular activation of NLRP3 and caspase-1. This was reported to be mediated for a large part through mitochondrial damage, highlighting the importance of restoring the cellular energy balance and mitochondrial homeostasis in acute gout. Furthermore, AMPK was proposed as a potential new therapeutic target in gout. Finally, the crucial role of neutrophils was discussed. Neutrophil-derived ectosomes were found to mediate anti-inflammatory effects, whereas NETs can have both pro- and anti-inflammatory properties. Neutrophil serine proteases play an important role in activating IL-1β, thus promoting the pro-inflammatory feedback loop. A new AAT fusion protein may have potent effects in limiting inflammation in gout patients. These recent advances not only provide more insight in the pathogenesis of gout, but also propose several important new treatment targets. Additionally, asymptomatic hyperuricemia possibly has much wider implications than just representing a risk factor for gout.

GENERAL OUTLINE OF THE THESIS

Gout is an IL-1β mediated disease characterized by prolonged hyperuricemia that leads to MSU crystal deposits and arthritis attacks. The scope of this thesis was to investigate the mechanisms and possible therapeutic targets behind the autoinflammatory episodes observed in patients with gout and hyperuricemia.

In **Chapter 2** of this manuscript, we initially investigate the general modulatory effects of the autophagy process on the production of proinflammatory cytokines in human cells. Using complementary methods to both inhibit and induce autophagy, we demonstrate important effects on the production of IL-1 β and TNF. Moreover, unlike the situation in murine cells, these effects are exerted at the level of gene transcription, rather than inflammasome activation. This study made a link between autophagy inhibition and higher IL-1β production with implications for autoinflammation.

As defects in autophagy genes have been revealed to increase susceptibility also to Crohn's disease, in **Chapter 3,** we further showed that inhibition of the autophagy process leads to increased pro-inflammatory cytokine responses in human primary immune cells when stimulated with a ligand for NOD2 (Nucleotide-binding oligomerization domain-containing protein 2). By this, we could also link genetic variation in *ATG16L1* (Autophagy related 16 like

1) with higher production of pro-inflammatory cytokines in carriers, and this could play a role in the chronic inflammation observed in Crohn's disease.

In **Chapter 4,** we presented data that primary cells freshly isolated from gout patients depict an enhanced cytokine production capacity compared to healthy volunteers when stimulated with TLR ligands and in synergy with MSU crystals. This study added another proof that MSU crystals alone are insufficient to drive IL-1β production and that second stimuli synergize with MSU crystals for proinflammatory cytokine production.

In **Chapter 5,** we investigated the modulatory effects of hyperuricemia on the production of proinflammatory cytokines in human cells. Using data from healthy volunteers and gout patients, we attempted to observe whether uric acid itself can act as a priming agent on human mononuclear cells and whether this has consequences on the inflammatory responses to gout-related stimuli. We describe a process through which hyperuricemia facilitates IL-1β production in peripheral blood mononuclear cells (PBMCs) while at the same time it down-regulates IL-1 receptor antagonist (IL-1Ra). We therefore conclude that the shift in the IL-1β/IL-1Ra balance produced by PBMCs upon high uric acid exposure contributes to the enhanced states of chronic inflammation observed in hyperuricemic patients and is highly relevant for the pathogenesis of gouty arthritis.

In **Chapter 6,** we studied the mechanism through which uric acid priming works in monocytes *in vitro.* We generated transcriptomic data to extract pathway candidates for the shift in IL-1β/IL-1Ra production and further performed experiments to investigate the mechanistic leads. We showed that uric acid induces phosphorylation of AKT (protein kinase B) and PRAS40 (proline rich AKT substrate 40 kDa) in monocytes and that this coincided with inhibition of autophagy in uric acid primed cells.

In **Chapter 7,** we explored whether uric acid treatment can induce epigenetic reprogramming and whether this could be the basis for the sustained effects observed after uric acid priming. Using a pharmacological library of epigenetic inhibitors followed by specific experiments in vitro and in vivo, we obtained clues that histone methylation could be involved in uric acid priming but we were not able to observe enrichment among the histone marks tested. However, we report interesting results for DNA methylation in a cohort of patients with hyperuricemia compared to normouricemia and validation of these targets is warranted.

In **Chapter 8,** we examined the potency of butyrate in limiting the MSU-induced cytokine production in PBMCs from healthy donors and crystal-proven gout patients. We present evidence that butyrate suppresses cytokine production due to MSU and synergizing fatty acids via inhibition of class I HDACs (histone deacetylases). Due to their anti-inflammatory potency at low concentrations, synthetic HDAC inhibitors may be a novel effective treatment for gouty arthritis in the future.

In **Chapter 9,** we used recombinant human alpha-1-anti-trypsin (AAT)-IgG1 Fc fusion protein (AAT-Fc), to investigate its properties for suppressing inflammation and interleukin (IL)-1β production in a mouse model of gouty arthritis. We show that AAT-Fc has very potent effects in reducing inflammation and enforce the concept that targeting inflammasome-independent IL-1β activation is beneficial in inflammatory diseases mediated by rich neutrophil influx.

In **Chapter 10,** we describe our current insights on the ability of the innate immune system to build immunological memory, process known as "trained immunity". We also focus on the hypothesis that endogenous alarm signals, or damage-associated molecular patterns, can also act as stimuli that induce long-term functional reprogramming in cells of the innate immune system. We describe new and old evidence of persistent effects of DAMPs in driving inflammation and enforce the concept that the influence of tissue-derived signals is critical in adjusting the magnitude and type of immune response built by the host.

In **Chapter 11,** we summarize the results presented in this thesis, and we discuss the implications and future perspectives.

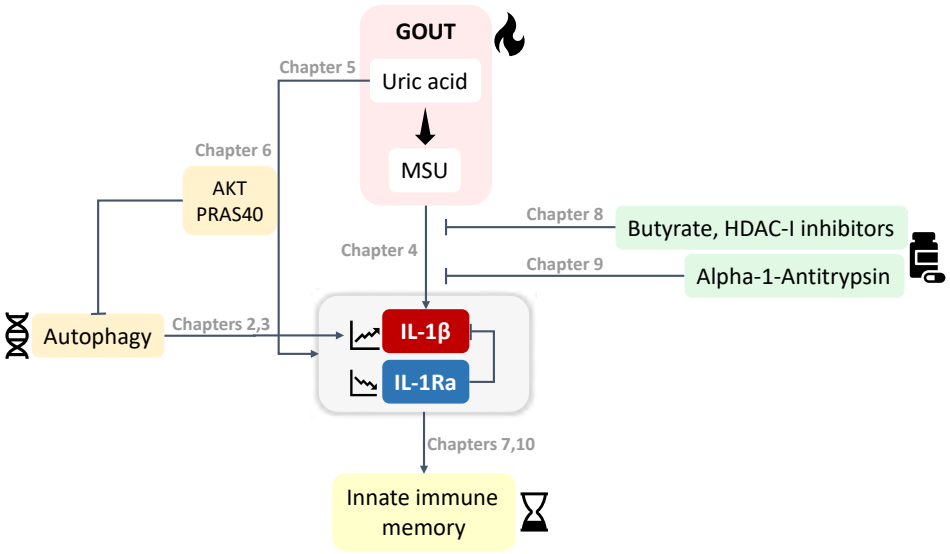

FIGURE 6. Schematic overview of the thesis.

Main research ideas of chapters introduced in this thesis are schematically represented and interconnected. Please see text for details.

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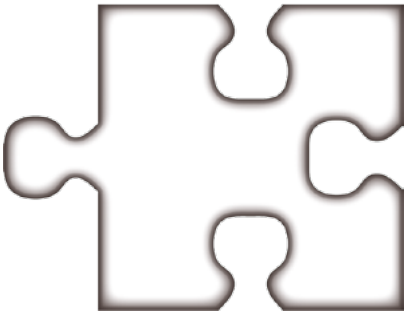

INFLAMMASOME-INDEPENDENT MODULATION OF CYTOKINE RESPONSE BY AUTOPHAGY IN HUMAN CELLS

Crişan TO
Plantinga TS
van de Veerdonk FL
Farcaş MF
Stoffels M
Kullberg BJ
van der Meer JW
Joosten LA
Netea MG

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SUMMARY

Autophagy is a cell housekeeping mechanism that has recently received attention in relation to its effects on the immune response. Genetic studies have identified candidate loci for Crohn's disease susceptibility among autophagy genes, while experiments in murine macrophages from ATG16L1 deficient mice have shown that disruption of autophagy increases processing of IL-1β and IL-18 through an inflammasome-dependent manner. Using complementary approaches either inducing or inhibiting autophagy, we describe modulatory effects of autophagy on proinflammatory cytokine production in human cells. Inhibition of basal autophagy in human peripheral blood mononuclear cells (PBMCs) significantly enhances IL-1β after stimulation with TLR2 or TLR4 ligands, while at the same time reducing the production of TNFα. In line with this, induction of autophagy by starvation inhibited IL-1β production. These effects of autophagy were not exerted at the processing step, as inflammasome activation was not influenced. In contrast, the effect of autophagy on cytokine production was on transcription level, and possibly involving the inhibition of p38 mitogen activated protein kinase (MAPK) phosphorylation. In conclusion, autophagy modulates the secretion of proinflammatory cytokines in human cells through an inflammasome-independent pathway, and this is a novel mechanism that may be targeted in inflammatory diseases.

INTRODUCTION

Autophagy is a conserved mechanism for degradation of defective organelles and long-lived proteins, that plays an important role in the homeostasis of the cell by recycling cytoplasmic cargo for aminoacid and energy re-use [1]. Autophagy comprises three main processes – chaperone-mediated autophagy, microautophagy and macroautophagy. The latter, henceforth referred to as autophagy, is characterized by the sequestration of cytosolic proteins and organelles into double-membrane vesicles called autophagosomes. These autophagosomes maturate through fusion with lysosomes, a process that will eventually lead to the breakdown of the protein content [2]. Through these effects, autophagy has been demonstrated to be a biological response of the cell in stressful situations, traditionally during starvation and growth factor deprivation, ensuring the degradation of old structures with the purpose of sustaining the essential anabolic processes of the cell [3]. Consequently, through its main roles in survival and housekeeping, the process of autophagy has gained relevance in the context of human pathologies like neurodegenerative diseases [4], cancer [5], lysosomal diseases [6], and ageing [1].

In addition to its role in cell survival, autophagy is emerging as a process of high importance for the host defense, influencing both the innate and adaptive immune responses [7]. This role is exerted at three levels [8]: direct involvement in engulfment and removal of intracellular pathogens [9, 10], facilitation of the MHC class II antigen presentation [11], and support of the T-lymphocyte development and survival for optimal protective immune responses [12, 13]. Whether the involvement of autophagy in the immune processes is due to the autophagic mechanism itself or to independent effects of autophagy-related genes [14], is still a matter of debate.

Genetic studies [15-17] have identified allelic variants of the autophagy genes *ATG16L1* (autophagy related 16-like 1) and *IRGM* (immunity related GTP-ases, M) as important risk factors for Crohn's disease, an autoinflammatory disease characterized by severe chronic inflammation of the gut mucosa [18, 19]. One possible explanation for the involvement of defective autophagy in Crohn's disease inflammation couples the risk alleles in *ATG16L1* and *NOD2* (nucleotide-binding oligomerization domain containing 2) to an impaired clearance of microorganisms [20, 21]. The persistence of bacteria in the mucosa could induce an inflammatory reaction, leading to the clinical features of Crohn's disease. An alternative explanation has also been proposed by Saitoh *et al.*, who demonstrated that the disruption of autophagy in *ATG16L1*-deficient murine macrophages enhances the LPS-induced IL-1β production through an inflammasome-dependent pathway [22]. However, whether autophagy has similar inhibitory effects in human cells is not known.

In the present study, we assessed the effect of autophagy on the production of proinflammatory cytokines in human cells. By stimulating human peripheral blood mononuclear cells (PBMCs), we show that the inhibition of autophagy increases IL-1β

production after stimulation with TLR2 or TLR4 ligands. These effects were specific for the modulation of IL-1β secretion, while TNFα production was significantly reduced by agents that inhibited autophagy. In contrast to murine macrophages, these effects on human cells were exerted at the transcriptional level, rather than at the level of the inflammasome.

RESULTS

Western blot assessment of autophagy marker LC3-II shows that starvation induces, whereas 3MA treatment inhibits, the autophagic process

Microtubule associated protein 1 light chain 3 (LC3) is one of the autophagy-related proteins involved in the direct formation of the autophagosome. Through conjugation with phosphatidylethanolamine, the cytosolic LC3-I is transformed to LC3-II which is then bound to the autophagosome membrane, being indicative of autophagic activity inside the cell [23]. In order to verify the modulation of autophagy using the typical induction method by starvation and the inhibition method using the pharmacological inhibitor 3-Methyl Adenine (3MA) in the human PBMCs system used in this study, Western blotting of the two LC3 fractions was performed. Freshly isolated human PBMCs treated with the starvation medium EBSS (Earle's Balanced Salt Solution) showed a markedly increased level of LC3-II compared to RPMI controls. Moreover, when 3MA was added, the LC3-II fraction was decreased (Figure 1), attesting to the validity of the classical autophagy methodologies in human PBMCs.

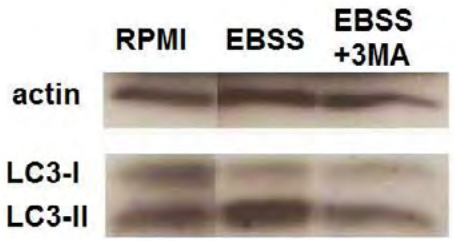

FIGURE 1. Assessment of LC3 I and II levels in PBMCs under starving conditions or pharmacological treatment with 3MA.

Human PBMCs were pre-incubated for 1 hour at 37°C in either RPMI, starvation medium (EBSS) or EBSS with 3MA (10 mM) in which inhibitors of lysosomal fusion have been added: Ammonium chloride 20mM and Leupeptine 100μM. This was followed by 3 hours stimulation with culture medium, LPS (10 ng/ml) or Pam3Cys (10 μg/ml) prepared in the corresponding media (RPMI or EBSS). Cells were lysed and western blot of LC3 fractions I and II has been performed.

Inhibition of autophagy enhances IL-1β production while decreasing TNFα in human PBMCs

To assess its effect on the cytokine production, autophagy was inhibited using 3MA, a blocker of the Beclin-1 complex that regulates the initiation of autophagy. PBMCs treated with 3MA showed a significant higher IL-1β secretion after stimulations with TLR2 or TLR4 ligands (Figure 2A). In contrast, TNFα production was diminished in cells conditioned with 3MA compared to controls (Figure 2B). No specific trend for the effect of 3MA on IL-10 production was observed (Figure 2C).

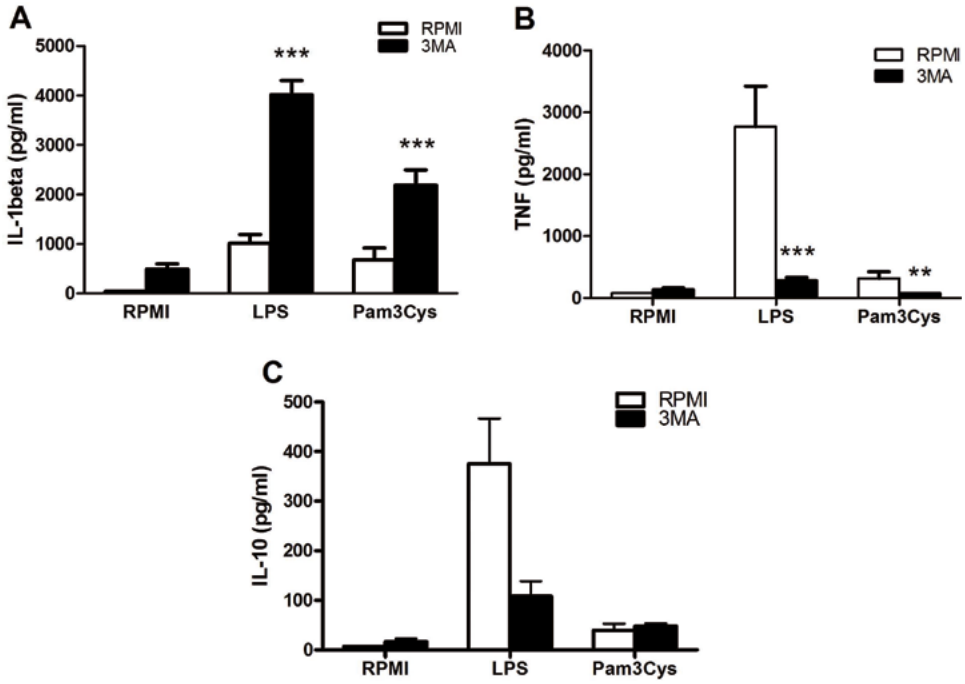

FIGURE 2. Modulation of inflammatory cytokine production by autophagy inhibition.

Freshly isolated human PBMCs were pre-incubated for 1 hour at 37°C in culture medium in the presence or absence of 3MA (10 mM) and afterwards stimulated with culture medium, LPS (10 ng/ml) or Pam3Cys (10 µg/ml). After 24 hours incubation, IL-1β (A), TNFα (B) and IL-10 (C) were measured in the supernatant by specific ELISA. Data are presented as means ± SEM of cells harvested from 15 volunteers, **p<0.01, ***p<0.001.

The modulation of IL-1β and TNFα production is regulated at the transcriptional level

To identify the level in the cytokine production which is modulated by 3MA, we assessed its effect on transcription and processing of IL-1β. IL-1β mRNA levels were increased in human

PBMCs in the presence of 3MA (Figure 3A). Similarly, RT-PCR for TNFα mRNA revealed that the inhibitory effect of 3MA on TNFα production starts with the decrease of the TNFα transcription rate (Figure 3B). On the other hand, western blot analysis of caspase-1 did not show an increased caspase-1 activation (p35 fragment) by 3MA compared to controls (Figure 3C).

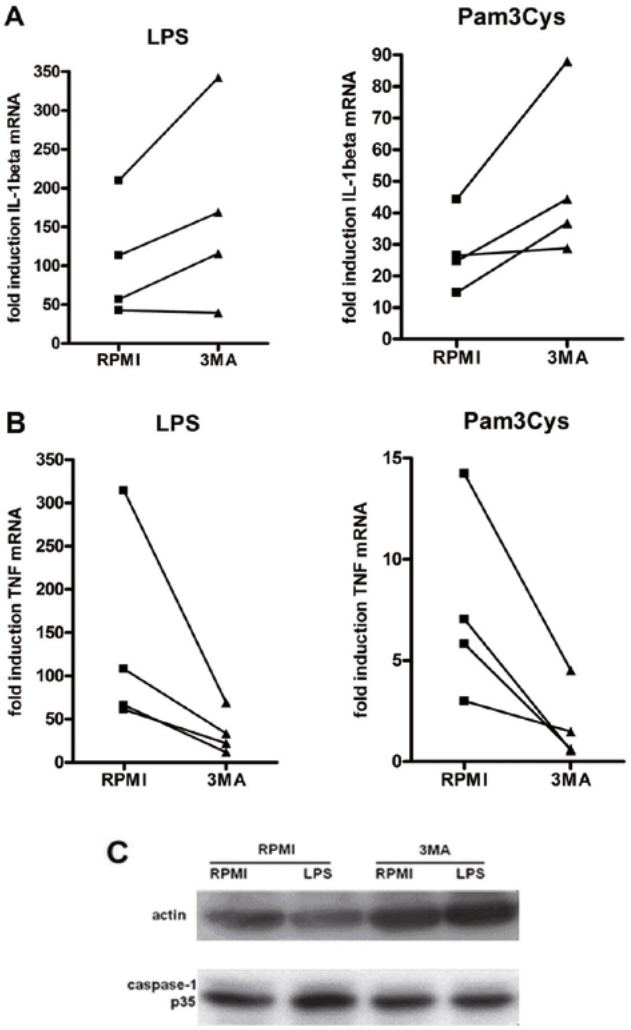

FIGURE 3. The effects of 3MA on transcription and processing of inflammatory cytokines.

Cells pre-treated for 1 hour with culture medium with or without 3MA (10 mM) were stimulated for 4 hours with RPMI, LPS (10 ng/ml) or Pam3Cys (10 µg/ml). RT-PCR was performed and relative levels of IL-1β and TNFα (B) mRNA were determined in 4 volunteers. (C) Western blot of p35 caspase-1 after 1 hour pre-incubation with or without 3MA (10 mM in RPMI), followed by 2 hours stimulation with LPS (10 ng/ml). The picture is representative for results obtained from 6 volunteers.

3MA has an inhibitory effect on the ATP-dependent release of IL-1β

It is known that the inflammasome activator ATP (adenosine triphosphate) is a potent stimulator of IL-1β processing and release [24]. In experiments aiming to assess how this effect is influenced in the circumstances of autophagy inhibition, control PBMCs showed a significantly higher increase (5.73 fold) of secreted IL-1β when exposed to ATP then in samples treated with 3MA (2.25 fold increase) (Figure 4).

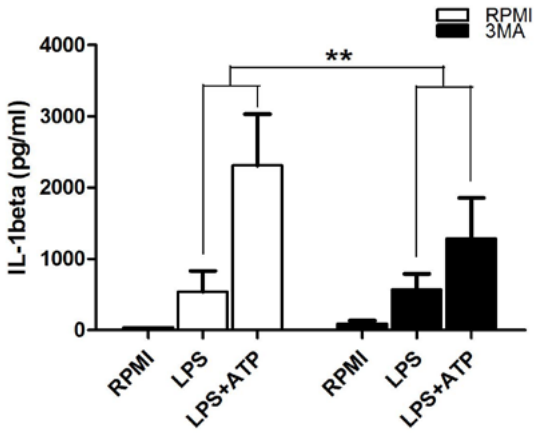

FIGURE 4. The influence of 3MA on the ATP-dependent release of IL-1β .

PBMCs were pre-incubated for 1 hour in the presence or absence of 3MA and were stimulated for 4 hours with LPS (10 ng/ml). After the stimulation, supernatants were discarded and refreshed with RPMI or with RPMI containing 1mM ATP and cells were incubated for another 15 min. Data are shown as mean ± SEM of supernatant IL-1β levels obtained in 8 volunteers, **p<0.01.

Induction of autophagy by starvation inhibits IL-1β transcription

In an additional set of experiments, we pursued to verify whether consistent findings with those revealed when inhibiting autophagy could be seen in the context of autophagy induction, typically obtained during starvation. In line with the effects observed by the experiments using 3MA, starvation reduced the transcription of IL-1β after LPS or Pam3Cys stimulation of cells (Figure 5A). Furthermore, these effects of starvation were reversed by 3MA, demonstrating the involvement of autophagy in the effects of starvation (Figure 5B). In contrast to its effect on IL-1β, starvation inhibited TNFα mRNA, but 3MA decreased even further TNFα transcription. This observation is consistent with the experiments showed earlier that 3MA reduces the TNFα gene transcription.

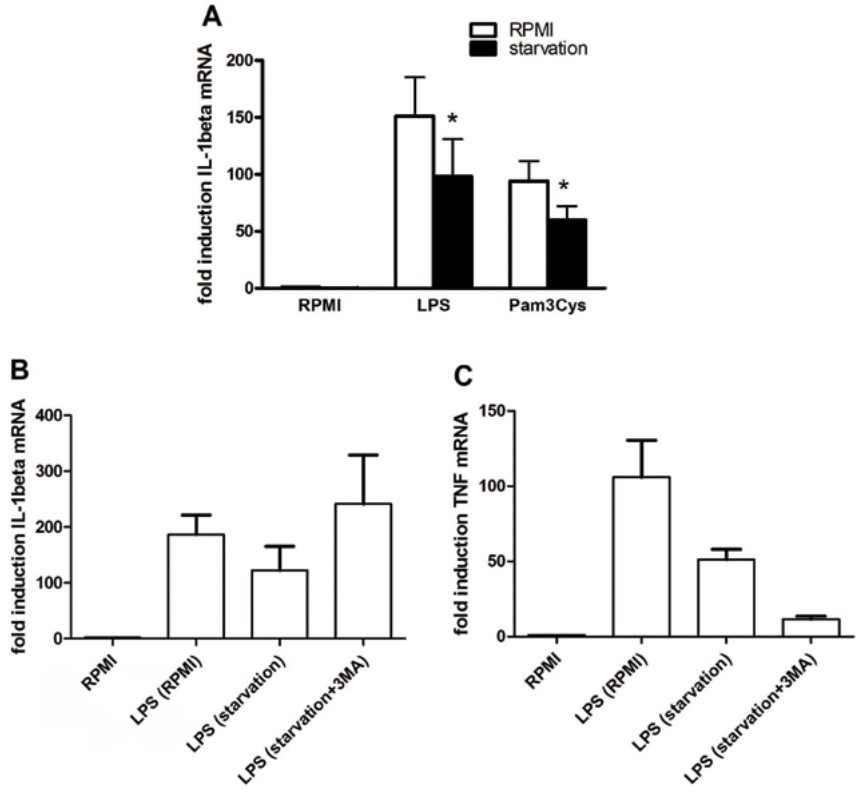

FIGURE 5. The effects of starvation on mRNA levels of inflammatory cytokines.

Cells pre-incubated for 2 hours in either starvation medium (Earle's Balanced Salt Solution) or RPMI were stimulated for 4 hours with medium, LPS (10 ng/ml) or Pam3Cys (10 μg/ml) prepared respectively in starvation medium or RPMI. Subsequently, RT-PCR was performed and IL-1β mRNA levels are depicted as mean ± SEM of cells harvested from 6 volunteers, *$p<0.05$ (A). RT-PCR results of IL-1β (B) and TNFα (C) mRNA levels in cells pre-incubated for 2 hours with RPMI, starvation medium or starvation medium and 3MA (10 mM) followed by 4 hours stimulation with RPMI or LPS (10 ng/ml). Results are shown as mean ± SEM of data obtained in 4 volunteers.

The role of MAP kinases for the modulatory effects of autophagy inhibition by 3MA

Mitogen-activated protein kinases (MAPKs) such as ERK1/2 (activated by MEK), JNK or p38 are important intracellular mediators of the stimulation of pro-inflammatory cytokine production during innate immune responses [25]. In order to decipher the mechanism through which autophagy inhibition modulates cytokine transcription, we investigated the effects of MAPK inhibition on the 3MA-dependent variation of cytokine production. Cells pre-treated with inhibitors of MEK, JNK or p38 in the presence or absence of 3MA have further been stimulated with LPS and Pam3Cys and cytokine production is depicted in Figure 6 (panels A and B for IL-1β; C and D for TNFα).

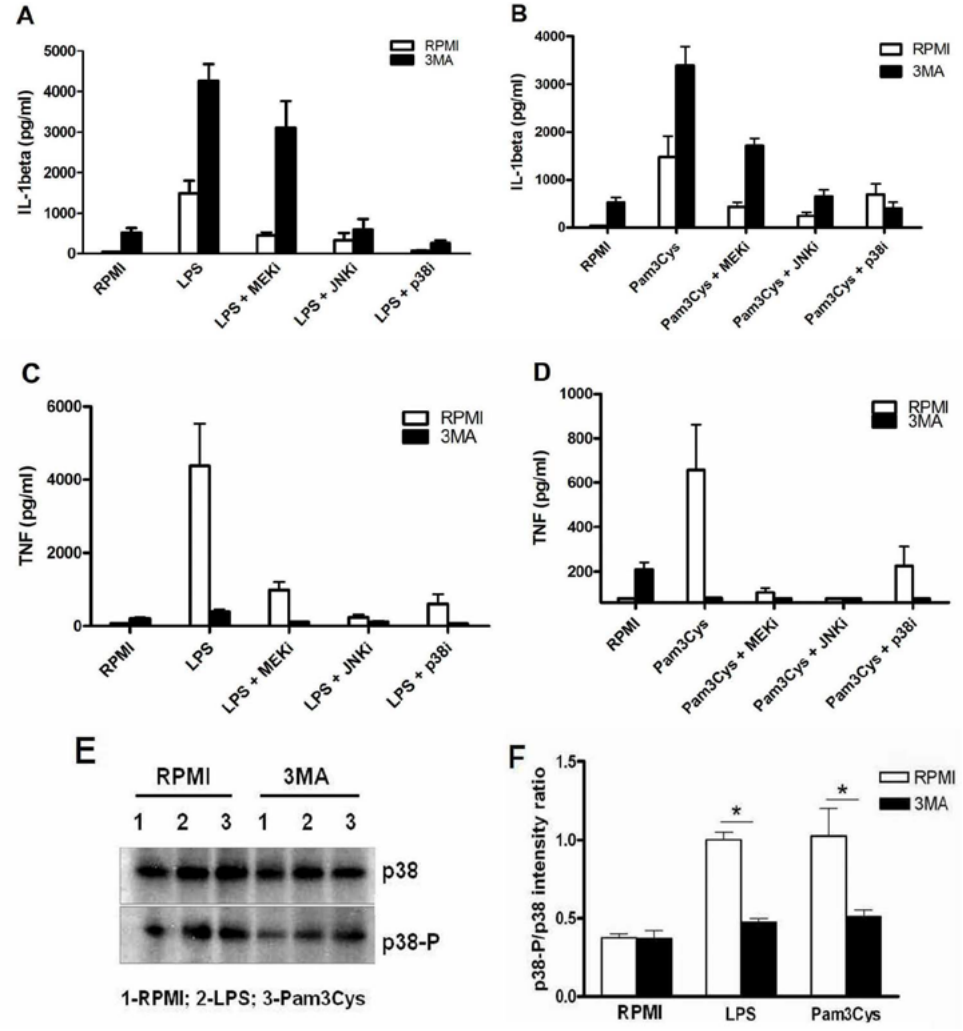

FIGURE 6. MAPK influence on the 3MA-dependent modulation of IL-1β and TNFα production.

PBMC samples conditioned for 1 hour in either medium or in the presence of MAPK inhibitors: MEK inhibitor (5 μM), JNK inhibitor (20 μM) or p38 inhibitor (1 μM) were subjected to 1 hour treatment with RPMI or 3MA (10 mM), followed by stimulation with RPMI, LPS (10 ng/ml) or Pam3Cys (10 μg/ml). After 24 hours incubation, specific ELISA was performed to determine the level of IL-1β in response to LPS (A) and Pam3Cys (B) stimulations, same as for TNFα (panels C and D, respectively). Data from 6 volunteers are shown as mean ± SEM. (E) Western blot of total and phosphorylated p38 in cells pre-treated for 1 hour with RPMI or 3MA (10 mM) and subjected to 1 hour stimulation with RPMI, LPS (10 ng/ml) or Pam3Cys (10 μg/ml). (F) Quantification of the effect of 3MA on the phosphorylation of p38.

With all inhibitors, except p38i, 3MA had similar consequences on the cytokine production, consisting in increasing IL-1β and decreasing TNFα secretion after both LPS and Pam3Cys stimulation. Nevertheless, when using p38i, lower IL-1β concentrations were measured in

samples treated with 3MA after Pam3Cys stimulation than in controls. Consequently, in order to test the hypothesis of p38 being involved in the effects of 3MA, we performed Western blots of total and phosphorylated (active) p38 which revealed that in samples treated with the autophagy inhibitor, the phosphorylated fraction of p38 is lower than in controls, after TLR4 or TLR2 stimulations (Figure 6E). Furthermore, the slight induction of TNFα and IL-1β by 3MA alone was shown to be dependent on p38 MAPK signaling (3MA: IL-1β 665±135 pg/ml, TNFα 215±45 pg/ml and 3MA+p38i: IL-1β 20±10 pg/ml, TNFα 40±15 pg/ml; $p<0.05$ for both IL-1β and TNFα).

DISCUSSION

Earlier studies linked autophagy to innate immunity as a defense system against invading pathogens in nonphagocytic cells [26]. Later it has been also demonstrated that autophagy regulates pathogen-associated molecular pattern (PAMP) recognition by facilitating TLR stimulation in response to viral antigens [27]. Subsequently, it has been discovered that components of the autophagy machinery are used for intracellular degradation of bacteria and MHC-II dependent activation of specific immunity [9]. These effects could prove very relevant, as genetic polymorphisms of the autophagy genes *ATG16L1* and *IRGM* are associated with Crohn's disease [15-17]. In addition, deletion of the autophagy gene *ATG16L1* in mice resulted in inflammasome activation and more severe experimental colitis [22]. However, the effect of autophagy on IL-1β production was demonstrated only in murine cells, and no information was available whether similar effects may be exerted in human cells.

In the present study, using a complementary approach by both inhibition and induction of autophagy as verified using the LC3-II marker, we show that autophagy has strong inhibitory effects on IL-1β production. These results add to the increasing body of evidence showing that basal autophagy in physiological conditions has important modulatory effects on inflammation [22, 28]. However, in contrast to murine cells, autophagy did not inhibit inflammasome activation in human PBMCs, and its effects were the consequence of the modulatory effect on transcription of cytokine genes. These data point out once again that inflammasome activation and regulation differs greatly in human PBMCs and murine macrophages, as inflammasome activation is constitutive in human monocytes, but not in mouse macrophages [29]. Subsequently, in human PBMCs, caspase-1 activation is not increased by autophagy inhibition. In addition, the assessment of ATP/inflammasome-dependent release of IL-1β shows that this is not increased in the presence of 3MA compared to controls. This would suggest that although the cytokine release appears to be slightly inhibited on the short term (15 minutes after ATP addition), transcriptional regulation is sufficient to determine the higher levels obtained in longer stimulations. These findings confirm that the increase of IL-1β production during autophagy inhibition is determined by

the activation of transcription and is not due to the influence on the subsequent steps of processing by caspase-1 or secretion from the intracellular compartment.

In the attempt to point out to the mechanism underlying the effect of autophagy on gene transcription, we have tested the importance of several MAPK-dependent mechanisms and observed that the only condition that reversed the stimulatory effects of 3MA was the p38 inhibitor SB202190. As there is evidence of this inhibitor also influencing Rip2 activation [30], the observed effect might also be involving other p38-related MAP kinases in addition to p38. However, while these results have led us to the hypothesis that p38 activation might be important for the effect of 3MA on IL-1β, this was not confirmed by Western blot analysis, as phosphorylated p38 (active form) was decreased in 3MA treated samples. However, in line with earlier evidence showing that p38 phosphorylation is positively linked to the process of autophagy [31, 32], 3MA treatment led to inhibition of p38 phosphorylation and this could, at least in part, represent a pathway of autophagy effect on TNFα release. In summary, while the inhibitory effects of 3MA on p38 phosphorylation can explain its effect on TNFα production, this cannot be the cause of the potentiation of IL-1β transcription, and further studies are needed to decipher this aspect.

Recently, two new studies have also reported that the peptidoglycan receptor NOD2, whose gene is considered a susceptibility locus for Crohn's disease, is linked to autophagy by recruiting ATG16L1 at the site of the microbial entry within the infected cell [21], directing microbial engulfment in the autophagosome and inducing antigen presentation [20]. These latest two studies represent the connection between the two genetic pathways that induce susceptibility to Crohn's disease: NOD2 and autophagy. Crohn's disease is a chronic inflammatory reaction in the gut mucosa, and recent studies strongly suggested a role of autophagy in this process. On the one hand, autophagy seems to be crucial for the homeostasis of Paneth cells. Mice with a defective ATG16L1 display abnormal Paneth cells with a lower amount of granules containing antibacterial defensins, while in turn displaying an increased IL-1β production [28]. These effects could lead to bacterial persistence in the mucosa and overwhelming inflammation. The studies by Cooney *et al.* [20] and Travassos *et al.* [21] show for the first time that a decreased autophagy in humans can lead to decreased bacterial clearance, while our study provides evidence that diminished autophagy in human cells (e.g. through pharmacological inhibition as shown here, or through genetic mutations in Crohn's disease) could lead to uncontrolled IL-1β production.

The differential findings in mice and humans concerning the modulation of IL-1β by autophagy, transcription in humans and caspase-1 activation in mice, remains to be elucidated. One possible explanation lies in the different cell populations studied: monocytes in our study in humans versus macrophages in the mouse studies. We have previously shown clear differential regulation of the caspase-1 inflammasome in monocytes (constitutive activation) versus macrophages (inducible activation) [29]. Another source

of difference could be species specificity, an aspect which in terms of the autophagy-inflammation interaction is still largely unknown. This is an aspect which certainly deserves attention in future studies, and the present study represents an important first step in this direction.

In conclusion, this study is the first attempt to investigate the modulation of inflammatory cytokines by the process of autophagy in humans. We have shown that disruption of autophagy has an important impact on inflammatory cytokine modulation, determining a remarkable and specific increase of IL-1β secretion, while decreasing TNFα production. We demonstrate that the inflammasome activation is not influenced in human PBMCs and that the changes are exerted at the gene transcription level. Although the p38 MAPK pathway seems to be linked to the autophagy process and our findings may explain the effects on TNFα production through modulation of p38 phosphorylation, this is probably not the mechanism of the observed higher IL-1β mRNA levels in the circumstances of autophagy inhibition. Further assessment of the mechanisms through which inflammation is regulated by autophagy may bring new opportunities for the understanding and treatment of autoinflammatory disorders such as Crohn's disease.

MATERIALS AND METHODS

Reagents

3-Methyl Adenine (3MA) was purchased from Sigma (St. Louis, MO). LPS (*E. coli* serotype 055:B5) was purchased from Sigma. Synthetic Pam3Cys was purchased from EMC Microcollections (Tubingen, Germany). Anti-actin (A2066) antibody was purchased from Sigma; anti-human caspase-1 p10 (sc515) antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA); anti-human/mouse LC3B antibody (NB600-1384) was purchased from Novus Biologicals (Cambridge, UK); anti-human total p38 antibody (CS9212) and phosphorylated p38 antibody (Phospho p38 MAPK Thr180/Tyr182 Antibody, CS4511) were purchased from Cell Signaling (Danvers, MA). oxATP was purchased from Sigma. SB202190 p38/Rip2 MAPK inhibitor (p38i); SP600125, JNK1/2/3 inhibitor (JNKi) and U0126 MEK1/2 inhibitor (MEKi) were purchased from Superarray Bioscience Corporation (Bethesda, MD). In experiments using pharmacological inhibitors, control cells were treated with an equivalent concentration of vehicle (0.1% DMSO).

PBMC isolation and stimulation

Peripheral blood was harvested from the antecubital vein of healthy volunteers, after obtaining informed consent. The PBMC fraction was obtained by differential centrifugation over Ficoll-Paque (Sigma). Cells adjusted to $5x10^6$ cells/ml were suspended in culture medium RPMI (Roswell Park Memorial Institute) 1640, supplemented with 50 μg/ml gentamicin, 2

mM L-glutamine and 1 mM pyruvate. All cytokine induction experiments were performed in duplicate wells. Cells were pre-incubated for 1 hour at 37°C in culture medium or in the presence of 3MA (10 mM). After the pre-treatment, RPMI (negative controls), purified LPS (10 ng/ml) or Pam3Cys (10 µg/ml) was added to the cells. In separate experiments, cells were first incubated for 1h with RPMI, MEK inhibitor (5 µM), JNK inhibitor (20 µM) or p38 inhibitor (1 µM) before performing the steps described above. After 24 hours, the supernatants were collected and stored at -20°C until assayed.

To investigate the effect of 3MA on the ATP-dependent IL-1β release, cells pre-treated for 1 hour with RPMI with or without 10 mM 3MA were stimulated for 4 hours with LPS (10 ng/ml or 1 µg/ml). After the stimulation, supernatants were discarded and refreshed with RPMI containing 1mM ATP, after which the cells were incubated for another 15 min. The LPS-dependent IL-1β production during the first 4 hours, and the ATP-dependent IL-1β secretion after the additional 15 minutes, was assessed in the supernatant.

Autophagy induction was performed in adherent monocytes using starvation medium, Earle's Balanced Salt Solution, EBSS (Invitrogen, Carlsbad, California). PBMCs were incubated for 1 hour after which the supernatant containing the non-adherent lymphocytes was discarded. Adherent cells were pre-treated for 2 hours with starvation medium. Subsequently, cells were stimulated for 4 hours with LPS or Pam3Cys prepared in starvation medium. In parallel, controls were given the same treatment using RPMI. After 4 hours, supernatants were discarded and TRIzol Reagent was added to the cells which were subsequently frozen and stored at -80°C until assayed. In different experiments, 3MA was also used in combination with starvation medium to investigate whether the effects of starvation are reversed by 3MA. Both experiments involving stimulation or inhibition of autophagy were performed with cells isolated from the same healthy volunteers. However, in some experiments in which not all experiments could be performed with blood collected from the same volunteers, studies were done in cells collected from additional healthy volunteers.

Cytokine measurements

Cytokine concentrations were determined using specific sandwich ELISA kits for IL-1β, TNFα (R&D Systems), and IL-10 (Sanquin).

RT-PCR

Samples stimulated for 4 hours at 37°C were treated with TRIzol Reagent (Invitrogen) and total RNA purification was performed according to manufacturer's instructions. Isolated RNA was subsequently transcribed into complementary DNA using iScript cDNA Synthesis Kit (Bio-Rad) followed by quantitative PCR using the Sybr Green method. The following primers were used in the reaction: IL-1β forward 5'-GCCCTAAACAGATGAAGTGCTC-3'

and reverse 5'-GAACCAGCATCTTCCTCAG-3', TNFα forward 5'-TGGCCCAGGCAGTCAGA-3' and reverse 5'-GGTTTGCTACAACATGGGCTACA-3', β2-microglobulin forward 5'-ATGAGTATGCCTGCCGTGTG-3' and reverse 5'-CCAAATGCGGCATCTTCAAAC-3' (Biolegio). Results are shown as fold increases in mRNA levels in stimulated samples compared to controls. Quantitative PCR for cytokines was used especially in short-term induction of autophagy experiments using starvation medium, in which long-term incubation led to a high percentage of cell death, and in which ELISA cytokine measurements in the supernatants were not able to provide an appropriate assessment of cytokine stimulation.

Western blot

For western blotting of actin, caspase-1 and p38 MAPK (total and phosphorylated), $5x10^6$ cells were lysed in 100 µl lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 40 mM β-glycerophosphate, 50 mM sodium fluoride, 200 µM sodium orthovanadate, complete mini EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche)). The homogenate was stored at -20°C. When needed, samples were thawed, then centrifuged for 10 min at 14,000 rpm, and the supernatant was taken for western blotting. Equal amounts of protein were subjected to SDS-PAGE electrophoresis using 12% polyacrylamide gels at a voltage of 100V. After separation, proteins were transferred to polyvinylidene fluoride (PVDF) membrane using the dry blotting method (iBlot™, Invitrogen). The membrane was blocked with 5% (w/v) milk powder in Tris-buffered saline/Tween 20 (TBST) for 1 hour at room temperature followed by incubation over night at 4°C with the primary antibody 1:500 in 5% (w/v) BSA/TBST (5% bovine serum albumin / TBST). After overnight incubation, the blots were washed three times with TBST and incubated with HRP-conjugated anti-rabbit antibody at a dilution of 1:5000 in 5% (w/v) milk powder in TBST for 1 hour at room temperature. After washing three times with TBST the blots were developed using ECL Plus Western Blot Detection Reagents (Amersham Biosciences, Buckinghamshire, UK). For western blotting of LC3, an amount of $10x10^6$ cells were lysed after being cultured in media containing the inhibitors of lysosomal fusion, ammonium chloride,20 mM, and leupeptine,100 µM. After protein electrophoresis in 15% polyacrylamide gel, the transfer was performed on nitroglycerine membranes using the wet blotting method (Bio-Rad) and was followed by blocking, incubation with first and then second antibody, each time using 5% (w/v) milk powder in Tris-buffered saline/ Tween 20 (TBS-T). After washing 3 times with TBS-T, blots were developed using the Super Signal® West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Quantitative assessment of band intensity was performed by Image Lab statistical software (Bio-Rad, CA, USA).

Statistical analysis

The differences were analysed using Wilcoxon signed rank test and were considered statistically significant at a p-value<0.05. Data are shown as cumulative results of levels obtained in all volunteers (means $\pm$ SEM).

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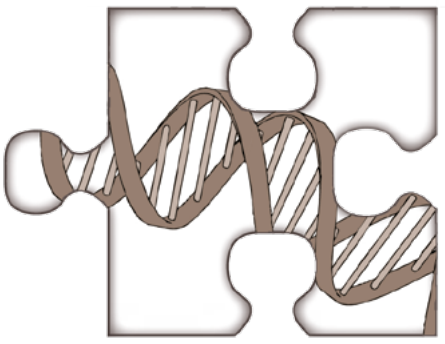

CROHN'S DISEASE ASSOCIATED ATG16L1 POLYMORPHISM MODULATES PRO-INFLAMMATORY CYTOKINE RESPONSES SELECTIVELY UPON ACTIVATION OF NOD2

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Crişan TO
Oosting M
van de Veerdonk FL
de Jong DJ
Philpott DJ
van der Meer JW
Girardin SE
Joosten LA
Netea MG

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SUMMARY

Objective

Autophagy has recently been shown to modulate pro-inflammatory cytokine production and to contribute to antigen processing and presentation through the major histocompatibility complex. Genetic variation in the autophagy gene *ATG16L1* has been recently implicated in Crohn's disease pathogenesis. The mechanisms underlying this association are not yet known, although experimental models suggest an inhibitory effect of autophagy on IL-1β responses. Here, the effect of *ATG16L1* genetic variation on cytokine responses has been assessed in humans.

Design and setting

Peripheral blood mononuclear cells from healthy individuals and Crohn's disease patients with different *ATG16L1* genotypes were stimulated with ligands for TLR2, TLR4 and NOD2, with or without the autophagy inhibitor 3-MA. Induction of cytokine production and related factors were measured at the mRNA and protein level. Furthermore, protein levels of ATG16L1 were assessed by Western blot.

Results

The present study demonstrates that cells isolated from individuals bearing the *ATG16L1* Thr300Ala risk variant, that it is shown to affect ATG16L1 protein expression upon NOD2 stimulation, display an increased production of the pro-inflammatory cytokines IL-1β and IL-6, specifically after stimulation with *NOD2* ligands. In contrast, no differences were found when cells were stimulated with TLR2 or TLR4 agonists. These findings were confirmed in two independent cohorts of volunteers and in a group of Crohn's disease patients. The increased production could be ascribed to increased mRNA expression, while processing of pro-IL-1β by caspase-1 activation was not affected. The effect of the *ATG16L1* polymorphism was abrogated when autophagy was blocked. Conclusions: The present study is the first to link the ATG16L1 polymorphism with an excessive production of IL-1β and IL-6 in humans, which may explain the effects of this polymorphism on the inflammatory process in Crohn's disease.

Keywords

autophagy, ATG16L1, pro-inflammatory cytokines, NOD2

INTRODUCTION

Eukaryotic cells are equipped with a machinery for collecting and degrading cellular constituents, designated as cellular self-digestion or autophagy (1). In general, activation of autophagy is triggered by conditions that are threatening for cell survival, including nutritional starvation and hypoxia, and is an important strategy to prevent cell death. Upon activation, cellular components such as cytoplasmic organelles and long-lived proteins are captured into double-membraned vesicles called autophagosomes that fuse with lysosomes for the degradation of its contents (2). Recruitment of autophagy has also been implicated in host defence to intracellular bacteria that can escape from innate recognition by membrane receptors upon engulfment, such as Mycobacterium tuberculosis (3;4), Salmonella typhimurium (5) and adherent-invasive Escherichia coli (6) and which is designated as xenophagy (7). In this respect, after the fusion of lysosomes with autophagosomes that have encapsulated bacteria, the bacteria are degraded and its antigens loaded on major histocompatibility complex class II to activate T-cell receptor mediated adaptive immune responses (8;9). One of the main proteins involved in autophagosome formation is autophagy related 16-like 1 (ATG16L1), which is highly homologous to Atg16 in S. cerevisiae. ATG16L1 is a component of a large protein complex together with ATG5 and ATG12 that is essential for autophagosome formation.

Autophagy, and ATG16L1 in particular, has recently been implicated in the pathogenesis of Crohn's disease (CD). A non-synonymous polymorphism in *ATG16L1*, Thr300Ala (c.898A>G, rs2241880), was demonstrated to be associated with higher susceptibility to CD (10;11). Functional studies have revealed that this ATG16L1 polymorphism affects the phenotype of Paneth cells (12) and impairs autophagosome formation specifically after activation of NOD2 (13;14). However, no abnormalities were observed when autophagy was activated through TLR2 or TLR4 signalling. Recently it has been demonstrated by Saitoh et al. that macrophages derived from *ATG16L1* knockout mice, which completely lack autophagy, exhibit elevated IL-1β production after stimulation with lipopolysaccharide (LPS), suggesting an inhibitory role of autophagy on the production of this cytokine (15). While the functional consequences of the *ATG16L1* Thr300Ala polymorphism have been demonstrated with regard to the capacity to induce autophagy, no studies have been performed to elucidate its effect on the production of pro-inflammatory cytokines such as IL-1β, that drive the inflammatory process in the inflamed gut mucosa of CD patients.

In the present study we demonstrate that the genetic variant of human *ATG16L1* conferring higher risk for CD is associated with elevated production of pro-inflammatory cytokines after engagement of NOD2, thereby providing an explanation for the excessive inflammatory response to the gut residing microorganisms, which is observed in CD. We have assessed production of the pro-inflammatory cytokines IL-1β, TNF and IL-6 due to their crucial role for the inflammation in CD.

MATERIALS AND METHODS

Genotyping for ATG16L1 Thr300Ala polymorphism

DNA was isolated from whole blood by using the isolation kit Puregene (Gentra Sytems, MN, USA), according to the manufacturers' protocol. Genotyping for the presence of the *ATG16L1* Thr300Ala polymorphism was performed by applying the TaqMan single nucleotide polymorphism (SNP) assay C_9095577_20 on the 7300 ABI Real-Time polymerase chain reaction system (Applied Biosystems, CA, USA). Two independent cohorts of healthy volunteers have been assessed (N=46 and N=90). We also selected five patients homozygous for the 300Thr allele, five patients homozygous for the 300Ala allele, and five heterozygous patients from a cohort of 74 Crohn's disease patients, and from these 15 patients we isolated cells for the cytokine stimulation assays. All volunteers gave written informed consent, and the study was approved by the Ethical Committee of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

PBMC stimulation assays

Venous blood was drawn from the cubital vein of healthy volunteers or Crohn's disease patients into 10 ml EDTA tubes (Monoject). In a separate experiment, cells isolated from four individuals homozygous for the NOD2 3020insC mutation were stimulated in order to assess the role of NOD2 for cytokine production. At time of blood donation, Crohn's disease patients were in a quiescent phase, defined as a prolonged period of at least three months of mild disease without relapses or exacerbations in the absence of immunomodulatory therapy. The mononuclear cell fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech, PA, USA). Cells were washed twice in saline and suspended in culture medium (RPMI, Invitrogen, CA, USA) supplemented with gentamicin 10 µg/ml, L-glutamine 10 mM and pyruvate 10 mM. Cells were counted in a Coulter counter (Coulter Electronics) and the number was adjusted to 5 x 106 cells/ml. A total of 5 x 105 mononuclear cells in a 100 µl volume was added to round-bottom 96-wells plates (Greiner) and incubated with either 100 µl of culture medium (negative control), or the various stimuli: E.coli lipopolysaccharide (LPS, 10 ng/ml, Sigma, MO, USA), Pam3Cys (10 µg/ml, EMC Microcollections, Tübingen, Germany) or muramyl dipeptide (MDP, 10 µg/ml, Sigma. MO, USA), IL-1 receptor antagonist (10 µg/ml, Amgen, Breda, The Netherlands). Autophagy was inhibited by 3-methyl adenine (3-MA, 10 mM, Sigma) or was activated by EBSS starvation medium (Invitrogen, CA, USA). Cytokine measurements of IL-1β, IL-6 and TNFα were performed in the supernatants, whereas pro-IL-1β was measured intracellularly. All cytokines were measured after 24 hours incubation, using commercial ELISA kits (R&D Systems, MN, USA (TNFα, IL-1β, pro-IL-1β) or Sanquin, Amsterdam, The Netherlands (IL-6)).

Real-Time PCR

PBMCs stimulated for 4 or 24 hours at 37°C were treated with TRIzol Reagent (Invitrogen, CA, USA) and total RNA purification was performed according to manufacturer's instructions. Isolated RNA was subsequently transcribed into complementary DNA using iScript cDNA Synthesis Kit (Bio-Rad, CA, USA) followed by quantitative PCR using the SYBR Green method (Applied Biosystems, CA, USA). The following primers were used: for IL-1β forward 5'-GCCCTAAACAGATGAAGTGCTC-3' and reverse 5'-GAACCAGCATCTTCCTCAG-3', for ATG16L1 forward 5'-ACGTACCAAACAGGCACGAG-3' and reverse 5'-CAGGTCAGAGATAGTCTGCAAAC-3'. Data were corrected for expression of the housekeeping gene β2 microglobulin, for which the primers forward 5'-ATGAGTATGCCTGCCGTGTG-3' and reverse 5'-CCAAATGCGGCATCTTCAAAC-3' were used.

Western blot

For Western blotting, 5 × 106 cells were lysed in 100 ml of lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 40 mM a-glycerophosphate, 50 mM sodium fluoride, 200 mM sodium vanadate, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 1 mM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride). The homogenate was frozen, then thawed and centrifuged at 4°C for 10 min at 15,000 × g, and the supernatant was taken for Western blot analysis. Equal amounts of protein were subjected to SDS-PAGE using 10%, 12% and 15% polyacrylamide gels at a constant voltage of 100 V. After SDS-PAGE, proteins were transferred to nitrocellulose membrane (0.2 mm). The membrane was blocked with 5% (wt/vol) milk powder in TBS/Tween 20 for 1 hour at room temperature, followed by incubation overnight at 4°C with a caspase-1 p10 antibody (SC-515; Santa Cruz Biotechnology, CA, USA) in 5% BSA/TBS/Tween 20 or with an ATG16L1 antibody (NB110-60928, Novus Biologicals, CO, USA) in 5% milk powder in TBS/Tween 20. After overnight incubation, the blots were washed three times with TBS/Tween 20 and then incubated with HRP-conjugated swine anti-rabbit antibody at a dilution of 1:5.000 in 5% (wt/vol) milk powder in TBS/Tween 20 for 1 hour at room temperature. After being washed three times with TBS/Tween 20, the blots where developed with ECL (GE Healthcare, PA, USA) according to the manufacturer's instructions. The intensity of the bands on the Western blots were assessed by Image Lab statistical software (Bio-Rad, CA, USA).

Statistical analysis

Differences in cytokine production capacity between groups were analyzed using the Mann-Whitney U test. Data on mRNA expression levels and immunoblot intensity values were statistically tested with Student's t-tests. Differences were considered statistically significant at $P < 0.05$.

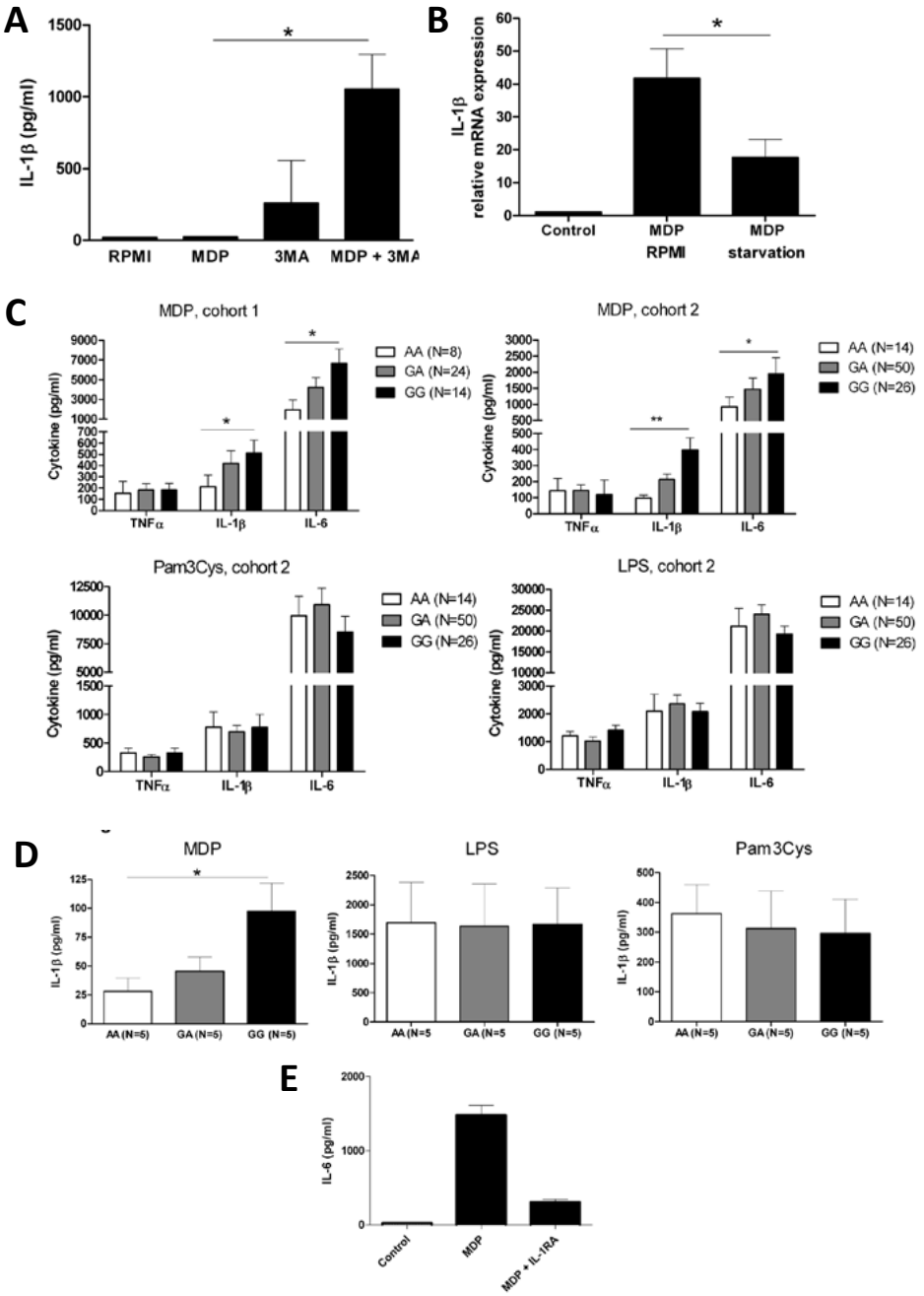

FIGURE 1. Autophagy, *ATG16L1* genotype and cytokines.

A: IL-1β production by PBMCs after stimulation for 24 hours at 37°C with the NOD2 ligand MDP, in the absence or presence of 3-methyl adenine (3-MA), an inhibitor of autophagy (N=4). Values are mean ± SD, *P < 0.05. B: IL-1β

mRNA expression after stimulation of PBMCs unstimulated or stimulated with MDP, cultured either in nutrient rich medium (RPMI) or in EBSS starvation medium (N=4). Values are means ± SD, *P<0.05. IL-6 production. PBMCs were stimulated with MDP with or without IL-1 receptor antagonist for 24 hours (N=4). C: Cytokine production capacity of TNFα, IL-1β and IL-6 by PBMCs obtained from healthy volunteers after stimulation for 24 hours with MDP, Pam3Cys or LPS, stratified for *ATG16L1* Thr300Ala genotype (A allele equals 300Thr allele, G allele equals 300Ala allele). For MDP stimulation PBMCs from two independent cohorts of healthy volunteers are shown, for LPS and Pam3Cys stimulation only the largest cohort. Values are means ± SEM, *P<0.05, **P<0.005. D: Cytokine production capacity of IL-1β by PBMCs obtained from Crohn's disease patients with functional NOD2 after stimulation for 24 hours with MDP, Pam3Cys or LPS, stratified for *ATG16L1* Thr300Ala genotype (A allele equals 300Thr allele, G allele equals 300Ala allele). Values are means ± SEM, *P<0.05. E: IL-1β dependent IL-6 production. PBMCs were stimulated with MDP with or without IL-1 receptor antagonist for 24 h (N¼4). EBSS, Earle's Balanced Salt Solution; IL, interleukin; LPS, lipopolysaccharide; 3-MA, 3-methyl adenine; MDP, muramyl dipeptide; NOD2, nucleotide-binding, oligomerisation domain 2; PBMCs, peripheral blood mononuclear cells; TNF, tumour necrosis factor.

RESULTS

Autophagy blockade and activation modulates MDP induced IL-1β

To assess the role of autophagy in the induction of IL-1β by NOD2 signalling, peripheral blood mononuclear cells (PBMCs) obtained from healthy volunteers were stimulated with the NOD2 ligand MDP alone or in combination with the autophagy inhibitor 3-MA for 24 hours (Figure 1A). The production of IL-1β was higher when cells were stimulated with both MDP and 3-MA compared to MDP alone. In contrast, when autophagy was induced in cells by culturing in starvation medium (EBSS), the MDP induced IL-1β response was decreased compared to MDP stimulation of cells in nutrient rich medium (Figure 1B).

ATG16L1 Thr300Ala genotype and MDP induced cytokine responses

The effect observed by blocking autophagy on MDP-induced IL-1β responses led to the hypothesis that the *ATG16L1* Thr300Ala genotype may influence these responses in a similar manner. Thus, PBMCs obtained from healthy volunteers with different *ATG16L1* Thr300Ala genotypes were stimulated with LPS, Pam3Cys or MDP for 24 hours (Figure 1C). Significantly higher production of IL-1β and IL-6, but not TNFα, was observed in the individuals homozygous for the 300Ala allele compared to individuals homozygous or heterozygous for the 300Thr allele, after stimulation of the cells with MDP. However, no differences were observed after stimulation with either Pam3Cys or LPS. These effects were consistent and reproducible in two independent cohorts of volunteers (Figure 1C) and in a group of Crohn's disease patients (Figure 1D). Of note, none of these individuals were bearing CD associated NOD2 variants (including R702W, G908R and 1007fsinsC). To study the role of IL-1β in the observed increased production of IL-6, PBMCs were stimulated with MDP with or without IL-1Ra for 24 hours. IL-1Ra significantly decreased the MDP-induced production of IL-6 (Figure 1E).

ATG16L1 Thr300Ala genotype and MDP stimulation in relation to 3-MA induced IL-1β

In order to examine whether the effect of the ATG16L1 polymorphism on IL-1β responses is mediated through autophagy, PBMCs collected from individuals with different ATG16L1 genotypes were stimulated for 24 hours with either MDP, 3-MA or the combination of these two stimuli. Indeed, when autophagy was completely blocked, the IL-1β response was similar between the genotypes. Notably, as also shown in Figure 1A, 3-MA also induces secretion of IL-1β (Figure 2A). To confirm that NOD2 mutations lead to a completely defective MDP induced IL-1β production, cells from Crohn's disease patients and healthy volunteers were stimulated with MDP for 24 hours and IL-1β was measured in the supernatants. MDP-induced production of IL-1β was completely dependent on recognition of MDP by NOD2 (Figure 2B).

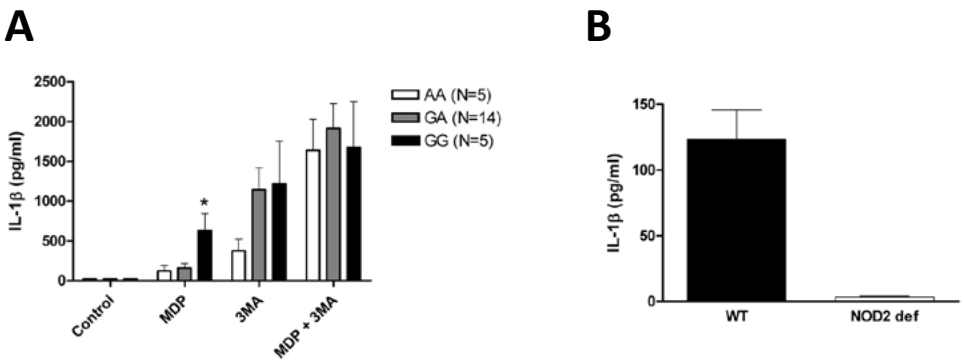

FIGURE 2 A: Production capacity of IL-1β by PBMCs after stimulation for 24 hours with MDP, 3-MA or the combination, stratified for ATG16L1 Thr300Ala genotype (A allele equals 300Thr, G allele equals 300Ala). Data are mean ± SEM, *P<0.05. B: Production capacity of IL-1β by PBMCs from healthy volunteers and NOD2 deficient Crohn's disease patients stimulated with MDP for 24 hours (N=4).

IL-1β mRNA expression, pro-IL-1β measurements and caspase-1 activation

To further dissect the mechanism underlying the effect of the *ATG16L1* Thr300Ala genotype on NOD2 induced IL-1β responses, we assessed transcriptional and post-transcriptional mechanisms of IL-1β regulation. Firstly, IL-1β mRNA expression was measured after stimulation of PBMCs with MDP for 24 hours. An increased mRNA expression of IL-1β was observed in the cells isolated from individuals homozygous for the *ATG16L1* 300Ala allele, as compared to cells from individuals heterozygous or homozygous for the 300Thr allele (Figure 3A). Similarly, an increased concentration of intracellular pro-IL-1β was observed in cells homozygous for the 300Ala allele (Figure 3B). In contrast, no difference could be observed between the *ATG16L1* genotypes regarding caspase-1 activation, either in the unstimulated condition or after stimulation with MDP. The active p35 subunit of caspase-1

could be detected in both the unstimulated and MDP stimulated condition, and was not influenced by the *ATG16L1* genotype (Figure 3C).

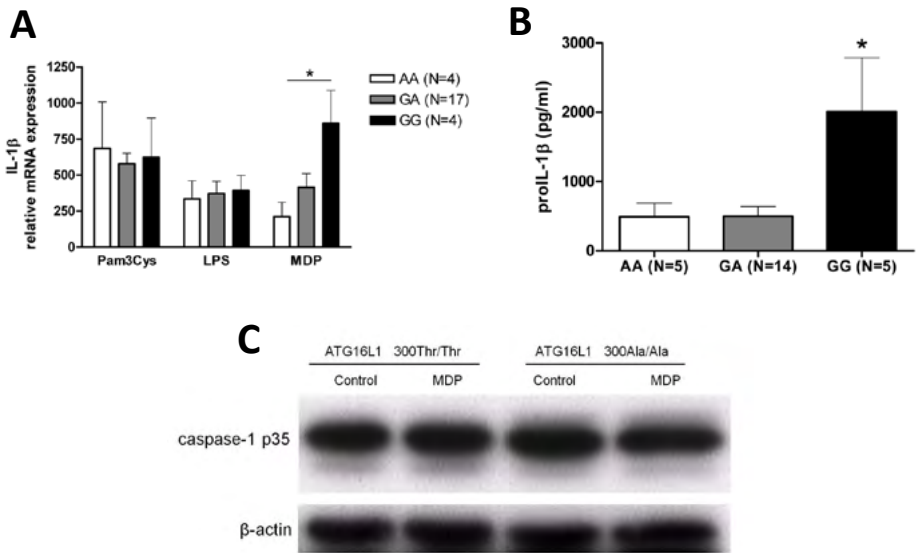

FIGURE 3. IL-1β mRNA expression, pro-IL-1β measurements and caspase-1 activation in relation to the *ATG16L1* Thr300Ala genotype

(A allele equals 300Thr, G allele equals 300Ala). A: IL-1β mRNA expression in cells stimulated for 24 hours with the indicated stimuli and stratified for *ATG16L1* Thr300Ala genotype. Data are mean ± SEM, *P<0.05. B: Intracellular pro-IL-1β in cells stimulated for 24 hours with MDP, stratified for *ATG16L1* Thr300Ala genotype. Data are mean ± SEM, *P<0.05. C: Western blot of the active p35 subunit of caspase-1 and β-actin as loading control after incubation of PBMCs for 4 hours, either unstimulated or stimulated with MDP, stratified for the ATG16L1 Thr300Ala genotype.

ATG16L1 mRNA and protein expression

Expression levels of ATG16L1 at the mRNA level were assessed between cells with different *ATG16L1* genotypes that were left unstimulated or were stimulated with either Pam3Cys, LPS or MDP for 24 hours. No differences were observed that would explain the effect of the *ATG16L1* Thr300Ala polymorphism on NOD2 induced IL-1β (Figure 4A). To study ATG16L1 protein expression, Western blot was performed with cell lysates obtained from PBMCs, either homozygous for the 300Thr allele or homozygous for the 300Ala allele, that were left unstimulated or stimulated with MDP, Pam3Cys or LPS for 3 hours (Figure 4B). It can be clearly observed that there are large differences in the amount of ATG16L1 between the genotypes comparing the unstimulated and stimulated conditions. In fact, in cells homozygous for the 300Thr allele, ATG16L1 protein expression is enhanced after MDP, Pam3Cys or LPS stimulation, whereas in cells homozygous for the 300Ala allele the amount of ATG16L1 protein remains unchanged. In addition, the absolute amount of protein after

stimulation is higher in the 300Thr genotype compared to the 300Ala genotype. These differences in ATG16L1 protein expression are quantified and shown in figure 4C.

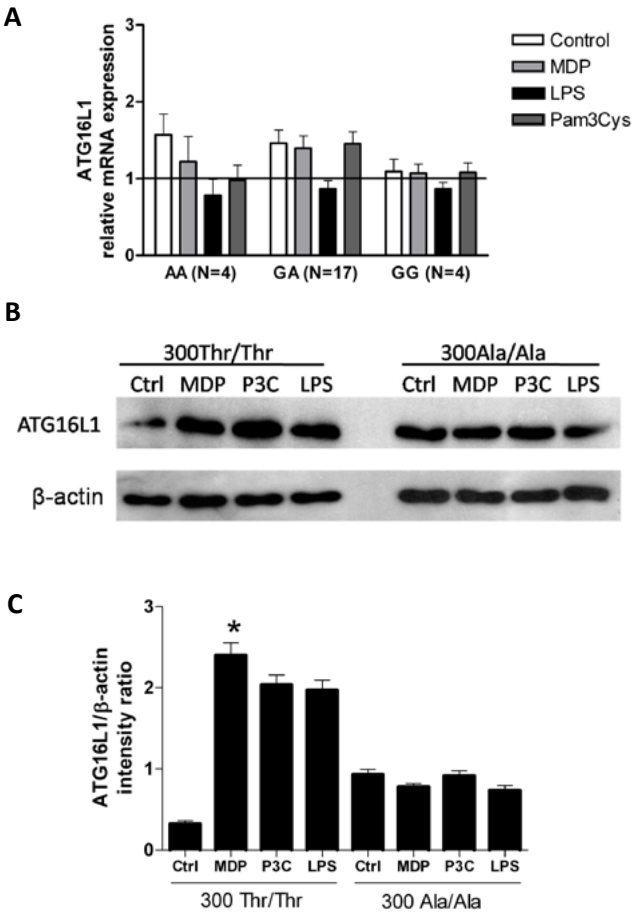

FIGURE 4. ATG16L1 mRNA and protein expression.

A: ATG16L1 mRNA expression after incubation of PBMCs for 24 hours, either unstimulated or stimulated with MDP, LPS or Pam3Cys, stratified for ATG16L1 Thr300Ala genotype (A allele equals 300Thr, G allele equals 300Ala). Data are mean ± SEM. B: Western blot of ATG16L1 protein and β-actin as loading control after incubation of PBMCs for 3 hours, either unstimulated or stimulated with MDP, P3C (Pam3Cys) or LPS, stratified for the *ATG16L1* Thr300Ala genotype. Picture is representative of three independent experiments with different donors. C: Intensity of ATG16L1 bands on the Western blot corrected for the intensity of the corresponding β-actin bands. Data are based on three experiments with different donors. Data are mean ± SD, *P<0.05.

DISCUSSION

Variation in the autophagy gene *ATG16L1* has emerged as a major genetic susceptibility factor for Crohn's disease (CD). The present study provides functional data that complement this genetic association, by demonstrating that immune cells that bear the risk variant of *ATG16L1* display enhanced production of pro-inflammatory cytokines after activation of NOD2.

Autophagy, a basic machinery in eukaryotic cells responsible for bulk degradation of cellular constituents, is also indispensable in host defence against intracellular bacteria. In this respect, autophagosomes are formed that encapsulate bacteria upon their entry into innate immune cells. Next, lysosomes fuse with the assembled autophagosomes, after which its content is degraded and made available for antigen presentation. One of the crucial proteins involved in this process is ATG16L1, of which a genetic variant is linked to CD.

Recent studies on the functional consequences of the risk variant of ATG16L1 have focussed on the capacity to form autophagosomes after microbial stimulation. Indeed, in these studies it could be demonstrated that the risk variant of ATG16L1 is associated with an impaired induction of autophagy specifically after NOD2 engagement (13;14), which could favour bacterial persistence and secondary inflammation. Interestingly, studies with cells derived from *ATG16L1* knockout mice have demonstrated a pivotal role of this protein for the inhibition of IL-1β production after endotoxin treatment in a TRIF and caspase-1 dependent manner (15). This initial finding in mice that autophagy can modulate cytokine responses upon microbial triggering of immune cells, led us to hypothesize that genetic variation in human *ATG16L1* could modulate cytokine responses, especially IL-1β.

An initial argument for a role of autophagy in NOD2-induced IL-1β responses was that inhibition of autophagy by 3-MA led to a robust increase in secretion of IL-1β, while activation of autophagy by culturing the cells in starvation medium decreased the IL-1β response after stimulation of PBMCs with a pure NOD2 ligand, MDP. To examine whether the *ATG16L1* Thr300Ala polymorphism exerts a similar effect on cytokine responses, PBMCs obtained from a large group of healthy volunteers were stimulated with ligands for TLR2, TLR4 and NOD2. Strongly increased IL-1β and IL-6 responses were observed when cells from individuals bearing the *ATG16L1* genotypes that confer an increased susceptibility to CD were stimulated with MDP, whereas TNFα responses were not different. Similarly, no differences were observed after stimulation of PBMCs with either LPS or Pam3Cys. As a confirmation, these findings were reproduced in a second set of experiments in PBMCs from another independent cohort of healthy subjects. Furthermore, similar experiments with PBMCs obtained from a group of Crohn's disease patients also revealed the specific effect of the *ATG16L1* T300A polymorphism on NOD2-induced IL-1β. Interestingly, similar experiments with cells stimulated with MDP alone or in combination with the autophagy inhibitor 3-MA,

resulted in a loss of the difference between the *ATG16L1* genotypes, indicating that the autophagy machinery is indeed involved in the effect on cytokine production. One may hypothesize whether these effects of *ATG16L1* polymorphisms are dependent on mutations in *NOD2* that are known to increased susceptibility to CD. However, no role for NOD2 mutations in the observed differences in IL-1β production could be demonstrated, since these mutations lead to a complete deficiency of *NOD2* signalling, as reported previously (16;17).

To study the mechanism underlying the effect of the *ATG16L1* genotype on MDP induced IL-1β, several stages of transcriptional and translational regulation of IL-1β were investigated. In line with the increased secretion, higher IL-1β mRNA expression and intracellular pro-IL-1β were observed. However, no difference in caspase-1 activation was apparent between cells with different *ATG16L1* genotypes, arguing for an effect on the transcription level of IL-1β. Interestingly, caspase-1 was demonstrated to be constitutively active when cells were left unstimulated. This constitutive active state of caspase-1, expressed in the monocyte fraction of PBMCs, is in line with a previous study that demonstrated that the regulatory step of IL-1β production in human monocytes is represented by transcription, and not inflammasome activation (18).

The increased production of IL-6 concomitantly with IL-1β is most probably a consequence of increased IL-1β production, since it is demonstrated that signalling through the IL-1 receptor is a major inducer of IL-6 (19;20), as we have confirmed in the present study. Interestingly, transcription of TNFα was not affected by the *ATG16L1* genotype, which argues for different pathways of gene transcription induction for IL-1β and TNFα. Indeed, recent studies suggest that TNFα transcription is mainly induced by p38 MAPK and NF-κB, whereas IL-1β transcription also involves Erk/NF-κB pathways (21;22).

Measurements of ATG16L1 mRNA and protein demonstrated that ATG16L1 expression is not influenced by the *ATG16L1* genotype on the transcriptional level. However, an evident effect of the polymorphism on ATG16L1 protein expression could be observed. Stimulation with either MDP, Pam3Cys or LPS led to either an increase in or an unchanged amount of ATG16L1 protein compared to unstimulated cells, depending on the *ATG16L1* genotype. Also, a higher total amount of ATG16L1 protein was observed after stimulation in cells bearing the 300Thr genotype compared to cells with the 300Ala genotype. Together with the recent finding that NOD2 specifically interacts with ATG16L1 in a direct fashion (13), these observations could provide an explanation for the specific effect of the polymorphism on NOD2 induced cytokine responses.

The exact mechanism underlying enhanced production of IL-1β upon MDP stimulation by cells bearing the ATG16L1 300Ala allele remains to be examined in further detail. Previously it has been shown that the polymorphism affects ATG16L1 protein expression (23). Hence, one possible explanation could be that the extent of NF-κB activation by NOD2 is influenced

by this lower availability of the ATG16L1 300Ala variant. Importantly, our findings also pinpoint to an effect of the polymorphism on protein expression. Consequently, the balance between the two effects exerted by NOD2, i.e. binding to ATG16L1 to induce autophagy on the one hand and activating NF-κB on the other hand, could be skewed in favour of Erk/NF-κB activation and IL-1β production in the case of autophagy defects in individuals bearing the *ATG16L1* polymorphism. This would implicate this polymorphism to be a modulator of the balance between NOD2 induced autophagy versus cytokine production.

Some important differences in the effect on regulation of IL-1β responses are apparent between mouse *ATG16L1* knockout cells and the human genetic variants of *ATG16L1*. Studies on *ATG16L1* knockout mouse cells revealed an inhibitory role of the protein on caspase-1 activation, whereas the human genetic variants of *ATG16L1* exert an effect on IL-1β mRNA expression. These discrepancies emphasize that important differences exist between mice and humans, but also between a complete knockout and a non-synonymous polymorphism leading to an amino acid substitution.

Innate immune responses mediated by *NOD2*, including cytokine production and induction of autophagy, have appeared to play a central role in Crohn's disease pathogenesis. At first, this was demonstrated by genetic association studies that revealed NOD2 mutations as a risk factor for developing Crohn's disease (24;25). Secondly, the role of innate host responses for the pathogenesis of Crohn's disease is further emphasized by the association of genetic variants of *ATG16L1* and *IRGM* with Crohn's disease, and the influence of these variants specifically on NOD2-induced immune responses. This has already been shown concerning the induction of autophagy by NOD2 signalling, which is affected by the ATG16L1 genotype (13;14). In addition to these findings, the present study demonstrates the association of ATG16L1 genotype with increased NOD2 induced IL-1β responses, providing a direct link of the CD risk-associated ATG16L1 300Ala allele with excessive inflammation that is pathognomonic of CD.

Acknowledgements

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ENHANCED INTERLEUKIN-1β PRODUCTION OF PBMCS FROM PATIENTS WITH GOUT AFTER STIMULATION WITH TOLL-LIKE RECEPTOR-2 LIGANDS AND URATE CRYSTALS

Mylona EE
Mouktaroudi M
Crisan TO
Makri S
Pistiki A
Georgitsi M
Savva A
Netea MG
van der Meer JW
Giamarellos-Bourboulis EJ
Joosten LA

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SUMMARY

Introduction

Monosodium urate monohydrate (MSU) crystals synergize with various TLR ligands to induce cytokine production via the activation of NLPR3 inflammasome. This has been demonstrated in vitro using human cell lines or monocytes of healthy volunteers. In the present study, we have investigated the effect of MSU crystals and of their combination with TLR ligands in peripheral blood mononuclear cells (PBMC) of patients with gout.

Methods

PBMCs from 18 patients with primary gout and 12 healthy donors were exposed to MSU crystals in the presence or absence of saturated fatty acid C18:0 (free fatty acid, TLR2 ligand), Pam3Cys (TLR1/2 ligand) and FSL-1 (Fibroblast Stimulating Factor-1, TLR 2/6 ligand). Production of IL-1β, IL-6, IL-17 and TNFα was determined by ELISA. mRNA transcripts of IL-1β were measured by real-time PCR.

Results

MSU crystals alone failed to induce IL-1β, IL-6 and TNFα in both patients and control groups, but a stronger synergy between MSU/Pam3Cys and MSU/C18:0 for the induction of IL-1β was found in gout patients compared to healthy controls. IL-6 followed the kinetics of IL-1β. No production of the neutrophil-recruiting IL-17 was detectable after stimulation of patients PBMCs with MSU in both the presence or absence of TLR ligands. No change of gene transcripts of IL-1β after stimulation with MSU and Pam3Cys or with MSU and C18:0 was found. A positive correlation was found between synergy in IL-1β production from PBMCs of patients between C18:0 and MSU crystals and the annual number of attacks of acute gouty arthritis (rs: +0.649, p: 0.022).

Conclusion

The synergy between MSU crystals and TLR-2 ligands is more prominent in patients with gout than in controls. This is likely mediated by the enhanced maturation of pro-IL-1β into IL-1β.

Key-words

gout; interleukin-1; urate; inflammasome

INTRODUCTION

Gout is a crystal-induced inflammatory disease induced by the deposition of crystals of monosodium urate monohydrate (MSU) in the joints and in the synovial membranes [1]. The arthritis is mediated by pro-inflammatory cytokines, produced by activated innate immune cells [2]. Among these cytokines, IL-1β seems to play a pivotal role [3]. This was proven in clinical studies, in which selective blockade of IL-1β effectively suppressed pain and inflammation in patients with gout that was refractory to other treatments [4-6].

Synthesis of bioactive IL-1β is induced in two steps: in the first step, gene expression leads to synthesis of inactive pro-IL-1β; in the second step, pro-IL-1β is cleaved by the protease caspase-1 to yield mature IL-1β [7]. For activation of caspase-1, intracellular molecular platforms called inflammasomes have to be assembled [8]. MSU crystals have been reported to activate the NLPR3 inflammasome and induce IL-1β production via caspase-1-dependent processing [9, 10]. However, purified MSU crystals induce moderate amounts of IL-1β by themselves [11, 12]; they require co-stimulation with TLR ligands such as LPS [11] or free fatty acids (FFA) [12].

This necessity of co-stimulation is in agreement with the clinical wisdom that in the context of chronically elevated uric acid concentrations, attacks of gout are preceded by infections or excess intake of alcohol or dietary fat.

The in-vitro studies performed until now have either studied human monocyte cell lines [9] or primary mononuclear cells of healthy volunteers [9, 11, 12] but, the IL-1β production capacity of primary cells of patients with gout has not been investigated. In this paper, we investigated whether primary cells (PBMCs) of patients with gout also need this dual stimulation for cytokine production. In order to assign the pathophysiological phenomena of attacks of acute gouty arthritis to IL-1β stimulation of other cytokines mediating chemotaxis of neutrophils, namely IL-8 and IL-17, at the inflamed synovium was studied.

MATERIAL AND METHODS

Patients

Eighteen patients with gout (13 men, five women, aged 63.5 +/- 13.6 years, Table 1) and 12 healthy volunteers (6 men, 6 women, aged 41.17 +/- 14.41) were asked to donate blood. The study protocol received permit form the Ethics Committee of ATTIKON University Hospital. Patients were enrolled after written informed consent. Inclusion criteria were: a) diagnosis of primary gout; b) history of at least two acute gouty attacks; and c) blood sampling in interictal periods. Patients under any anti-inflammatory medication the last 15 days were excluded from the study. Primary gout and acute attack of gout were defined according to the criteria outlined by the American Rheumatism Association [13]. After informed consent, 30ml of venous blood was collected under sterile conditions.

TABLE 1. Clinical characteristics of 18 patients with gout enrolled in the study

No	Age (years)	Co-morbidities	Number of affected joints	Number of annual attacks	Therapy of attacks
1	75	DM2, VH, COPD	2	2	Colchicine
2	80	DM2, VH, CHD	4	5	Colchicine
3	62	DM2, VH, obesity	1	1	Colchicine
4	80	DM2, COPD, CHD	1	1	Colchicine
5	45	VH, obesity	1	1	Colchicine
6	70	VH	1	1	Colchicine
7	67	DM2, VH, CHD	7	5	NSAIDs
8	65	VH, obesity	1	1	Colchicine
9	62	VH	1	1	Colchicine
10	55	VH, obesity	2	1	Colchicine
11	80	DM2, VH	2	2	NSAIDs
12	32	Obesity	4	6	NDAIDs
13	70	VH	1	1	Colchicine
14	70	DM2, VH	3	2	Colchicine
15	68	Obesity	1	3	Colchicine
16	52	DM2, CRD	1	10	Colchicine
17	69	CRD	5	4	Colchicine
18	46	CRD	1	5	Colchicine

Abbreviations: CHD: coronary heart disease; COPD: chronic obstructive pulmonary disease; CRD: chronic renal disease; DM2: diabetes mellitus type 2; VH: vascular hypertension

FIGURE 1. Polarized microscopy of the prepared crystals of monosodium urate

A 200mM stock solution of ultrapure C18:0 fatty acids (Sigma Co) at a volume of 5ml of ethanol 100% was prepared. The stock was warmed at 370C in a water bath and pre-diluted to 20mM with ethanol 100%, before usage. A concentration of 200μM C18:0 was used to stimulate PBMCs and the final concentration of ethanol in the wells was 0.55 %. Although this concentration appears high, it is consistent with the concentrations that exist in the joint as reflected indirectly by the circulating concentrations of free fatty acids of patients before and after total hip arthroplasty [15].

Preparation of monosodium urate monohydrate (MSU) crystals and of ultrapure C18:0

MSU crystals were prepared according to the method described by Seegmiller et al [14]. Briefly, a solution of 0.03 M of MSU at a volume of 200 ml was prepared after diluting 1.0 g of uric acid (Sigma Co, St Louis, USA) in 200 ml of sterile water containing 24 g of NaOH. The pH was adjusted to 7.2 after addition of HCl and the solution became pyrogen-free after incubation for six hours at 1200C. Concentrations of lipopolysaccharides were below 0.01 ng/ml as measured by the kinetic LAL QCL-1000 assay (BioWhitaker) The solution was left to cool at room temperature and stored at 40C. Produced crystals were 5-25μm long (Figure 1). On each day of experiment, a small amount of crystals was weighted under sterile conditions for application.

Stimulation of cytokine production

PBMCs were isolated after gradient centrifugation of heparinized whole blood over Ficol Hypaque (Biochrom, Berlin, Germany). After three consecutive washings in ice-cold phosphate buffered saline pH 7.2 (Biochrom), PBMCs were counted in a Neubauer chamber after trypan blue exclusion of dead cells. They were then distributed into wells of a 96-well plate at a final concentration of 2x106/ml in RPMI 1640 (Biochrom) enriched with 2mM glutamine (Biochrom), 10 μg/ml of gentamicin and 100 U/ml of penicillin G. PBMCs were stimulated with 200μg/ml of highly purified MSU; with 200μM of ultrapure fatty acid [C18:0] which is a TLR2 ligand; with 10μg/ml of the TLR1/2 ligand palmitoyl-3-cystein [Pam3Cys- SKKKK] (EMC Microcollections GmbH) and with 1μg/ml of the TLR2/6 ligand fibroblast stimulating ligand-1 [FSL-1] (EMC Microcollections GmbH). Pam3Cys and FSL-1 stock solutions in distilled water were used. All experiments were run in duplicate with cells of at least one healthy donor per day of experiment. After 24h of incubation, plates were centrifuged and supernatants were collected and stored at -800C until the cytokine assays were performed. For assessment of IL-17 production, the culture period was 5 days with fetal bovine serum (FBS) added in the culture medium at a final concentration of 10%.

PBMCs used for this analysis consists by lymphocytes and monocytes at an almost 4:1 ratio. Stimulation of PBMCs for 24 hours provides cytokines coming from the monocyte fraction. As such, they reflect with accuracy the function of circulating monocytes whereas ex vivo stimulation by the experimental procedure itself in the attempt to purify monocytes is avoided.

Cytokine measurement

Human TNFα, IL-1β, IL-6, IL-8 and IL-17 in supernatants were measured by an enzyme-linked immunosorbent assay (R&D systems, Minneapolis) according to the instructions of the manufacturer. The lowest limits of detection were 40 pg/ml for TNFα; 20 pg/ml for IL-1β; 16 pg/ml for IL-6; 165 pg/ml for IL-8; and 80 pg/ml for IL-17.

Quantitative PCR for mRNA expression of IL-1β

PBMCs were cultured with the stimuli as mentioned above and after 4 hours of incubation at 370C in 5% CO2 the plates were centrifuged, the cell pellets were lysed with 400 μl of Trizol (AppliChem GmbH) and kept at -800C until extraction of RNA.
RNA was extracted with chloroform and gradient centrifugation for 15 minutes at 40C and 12,000 g followed by treatment for 30 minutes at 370C with 0.04 U/μl of DNAase (New England BioLabs, Ipswich, MA). RNA was recognized after 3% agarose gel electrophoresis and ethidium bromide staining. 1.5 μg of RNA (Pharmacia Biotech photometer) was applied for the production of cDNA using 0.4 mM of dNTPs (New England BioLabs), 1 U of RNA-sin (New England BioLabs), 10 mM DTT (AppliChem GmbH) and 5x of the reverse transcriptase buffer in a Sensoquest thermal cycler LabCycler Gradient using appropriate blanks (Eppedorf). After an initial incubation step of 10 minutes at 600C, 1 μU of reverse transcriptase (New England BioLabs) was added followed by three steps: 10 minutes at 250C, 50 minutes at 420C and 15 minutes at 700C. cDNA was kept at -800C until assayed
Expression of mRNA was tested by the iCycler system (BioRad, Philadelphia, PA, USA) using per reaction tube 1 μl of cDNA, 0.1 mg/ml of sense and antisense primers, 3mM of MgCl2 (New England BioLabs), 0.25 mM of dNTPs (New England BioLabs), 10x buffer and 1 mM of Taq polymerase with SYBR-Gr as a fluorochrome. Primer sequences were: for IL-1β sense 5'- CAG CTA CGA ATC TCC GAC CAC-3' and antisense 5'- GGC AGG GAA CCA GCA TCT TC-3', and for β2-microglobulin sense 5'-ATG AGT ATG CCT GCC GTG TG-3' and antisense 5'-CCA AAT GCG GCA TCT TCA AAC-3'. After an initial denaturation step for 10 minutes at 950C, 34 cycles were performed. Each cycle consisted of three steps: denaturation for 30 seconds at 950C, annealing for 30 seconds at 720C and elongation for 30 seconds at 950C. Amplification was followed by a melting curve, appropriate blanks sere applied. The PCR product was recognized after 3% agarose gel electrophoresis and ethidium bromide staining. Quantitative results were expressed as defined by the PFAFFL equation [16], using the efficiency of a standard curve created with known cDNA.

Statistical analysis

Data of cytokines are expressed as medians +/- 95% confidence intervals (CI); those of gene transcripts by their mean +/- SE. Comparisons between groups were done by the Mann-Whitney U test. Percentages of change of cytokine production after treatment with a combination of stimuli compared with the most active single stimulus were calculated; comparisons between patients and healthy volunteers were done by the Mann-Whitney U test. Any more than 30% increase was considered as significant synergy. Comparison between groups was done by the Fischer's exact test. Correlations between folds of changes of cytokine production and of the annual number of attacks of acute gouty arthritis of patients were done according to Spearman. P values less than 0.05 were considered significant.

RESULTS

Cytokine production after exposure to MSU and TLR ligands

We stimulated PBMCs of patients with gout and of healthy donors with either TLR2/6 ligand FSL-1 or TLR1/2 ligand Pam3Cys (Figure 2). We found that production of IL-1β did not significantly differ between patients and controls, although IL-1β production in cells of gout patients showed a wide variation and a tendency to higher production when exposed to Pam3Cys (Figure 2A).

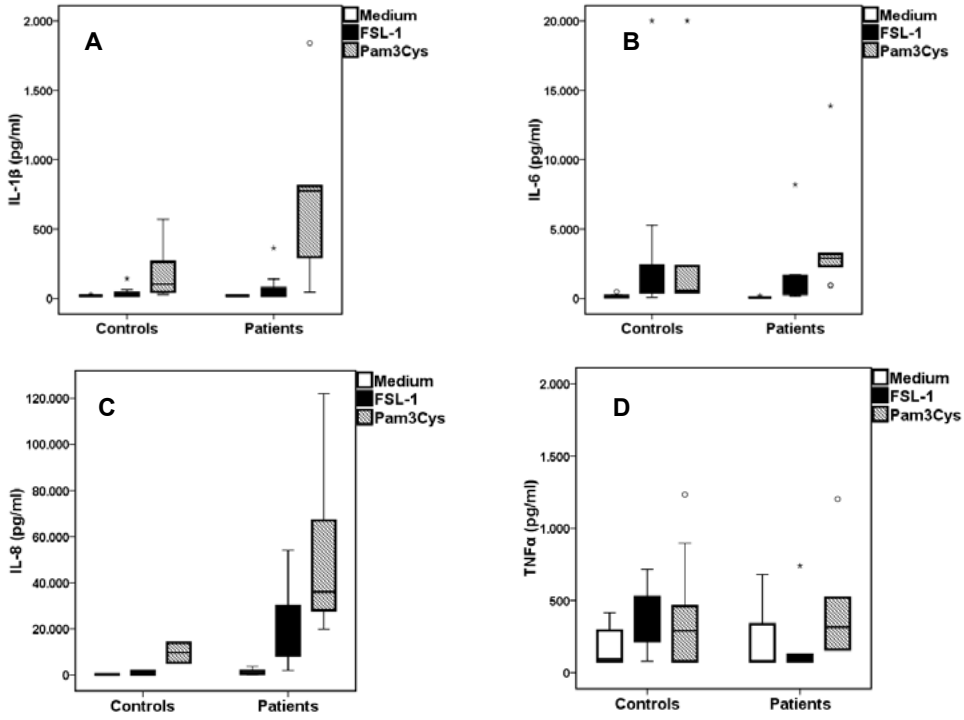

FIGURE 2. PBMCs from gout patients produce more IL-1β and IL-6 compared to healthy controls when exposed to TLR ligands.

Release of IL-1β (A), IL-6 (B), IL-8 (C) and TNFα (D) by PBMCs of patients with gout and healthy donors after their stimulation with growth medium (RPMI), palmitoyl-3-cystein [Pam3Cys] (TLR2/1 agonist) or fibroblast stimulation ligand-1 [FLS-1] (TLR2/6 ligand). Asterisks denote outliers and circles denote extremes. Gout patients n=18 and healthy controls n=12.

Exposure of PBMCs to ultrapure MSU crystals showed that the latter did not induce IL-1β production in either of the two groups (Figure 3A). Co-incubation of MSU with FSL-1 did not change the release of cytokines achieved by single FSL-1 (Figure 3). When PBMCs of

gout patients were incubated with MSU crystals together with Pam3Cys, IL-1β production in cells of gout patients was almost 10-fold higher when exposed to the combination of Pam3Cys and MSU crystals than to Pam3Cys alone. IL-6, IL-8 or TNFα released by PBMCs was not significantly different between gout patients and controls for any of the stimuli, single or in combination (Figures 2, 3 and 4).

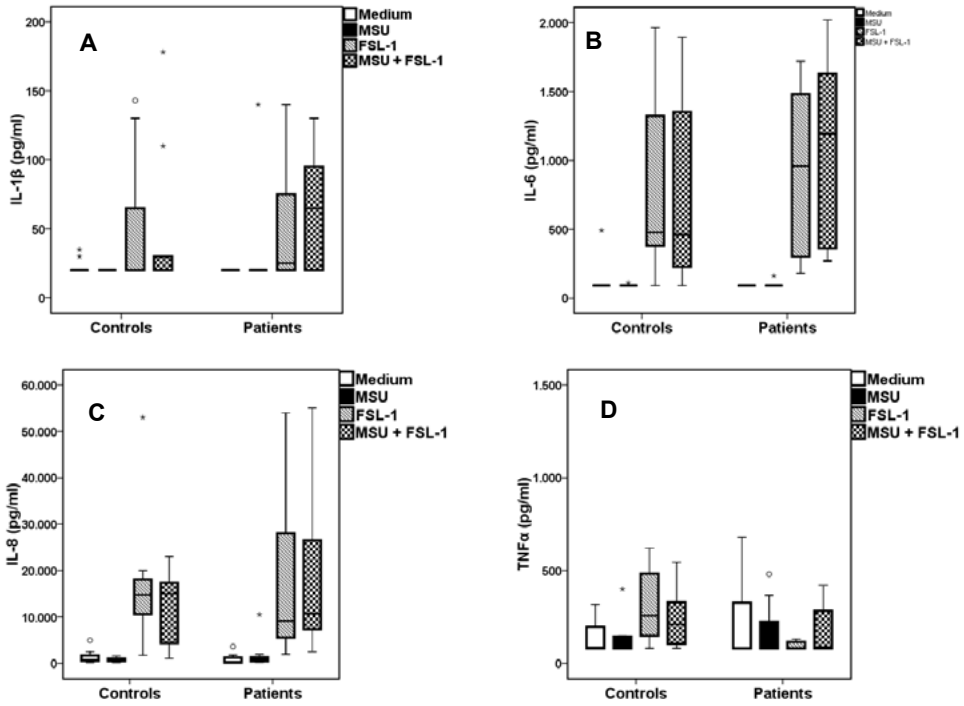

FIGURE 3. Lack of synergy between MSU and FSL-1 in the induction of IL-1β.

Release of IL-1β (A), IL-6 (B) IL-8 (C) and TNFα (D) by peripheral blood mononuclear cells of patients with gout and healthy donors after having been left untreated (RPMI) or stimulated by monosodium urate monohydrate (MSU) crystals alone or in combination with fibroblast stimulation ligand-1 [FLS-1]. Asterisks denote outliers and circles denote extremes. P values indicate significant differences between respective values of patients and healthy controls by Mann-Whitney U-test. Gout patients n=18 and healthy controls n=12.

Cytokine induction by MSU and saturated fatty acids

Recently, we demonstrated that the addition of C18:0 fatty acid augments IL-1β production of PBMCs exposed to MSU [12]. In the present study, we show that PBMCs from gout patients produce more IL-1β than controls when incubated with C18:0 and MSU (Figure 5A). However, we noted that exposure of PBMCs to C18.0 alone also stimulated IL-1β production, most likely due to a different preparation of C18.0 stimuli than in previous studies (containing a low final concentration of 0.55% ethanol). The production of IL-6 by

MSU/C18:0 was in line with IL-1β production in cells isolated from gout patients, with a trend towards higher cytokine production in gout patients than in healthy controls. Release of TNFα and of IL-8 after co-stimulation with C18:0 and MSU did not differ between patients and healthy controls.

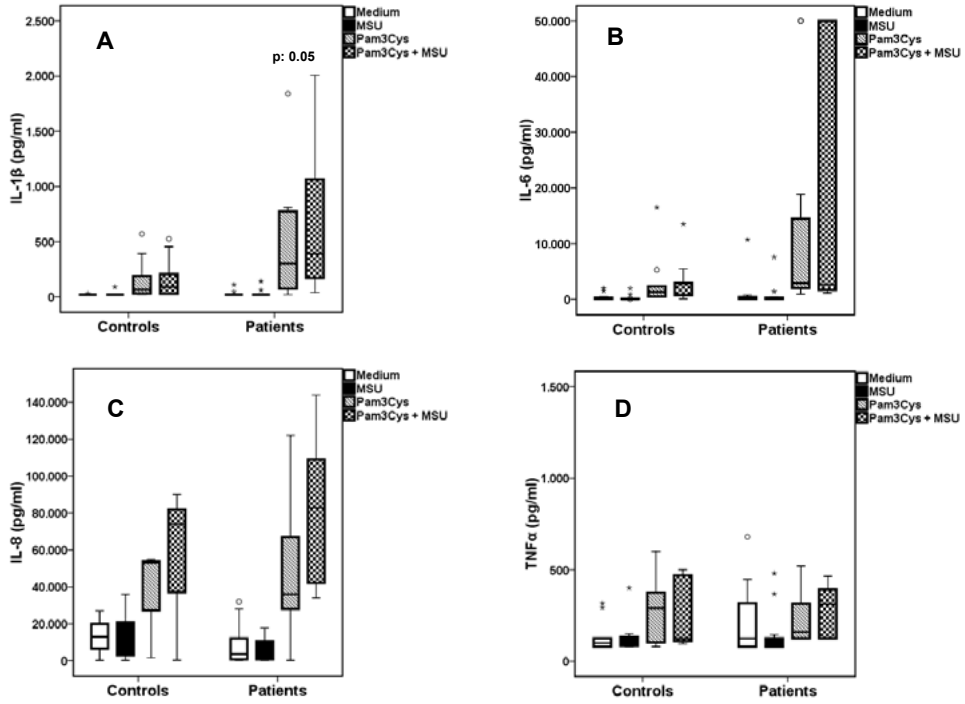

FIGURE 4. Synergy between MSU and Pam3Cys in the induction of IL-1β was greater in patients than controls.

Release of IL-1β (A), IL-6 (B), IL-8 (C) and TNFα (D) by peripheral blood mononuclear cells of patients with gout and healthy donors after having been left untreated (RPMI) or stimulated by monosodium urate monohydrate (MSU) crystals alone or in combination with palmitoyl-3-cystein [Pam3Cys]. Asterisks denote outliers and circles denote extremes. P values indicate significant differences between respective values of patients and healthy controls by Mann-Whitney U-test. Gout patients n=18 and healthy controls n=12.

Undoubtedly, the wide variation in cytokine production raises questions about the robustness of the finding. In order to make the result robust four types of analysis were performed: a) statistical comparisons reported in Figures 5A and 5B were performed using non-parametric tests; b) qualitative analysis disclosed that synergy of FFA C18:0 with MSU crystals for the production of IL-1β and IL-6 was found in 15 out of 18 patients versus 2 out of 12 controls, p= 0.001); c) fold changes of IL-1β and of IL-6 production after stimulation of PBMCs of patients with the combination of C18:0 and MSU crystals compared to C18:0

alone were significantly greater compared to respective changes of healthy controls (Figure 6); and d) a positive correlation was found between the change of IL-1β production after stimulation of PBMCs of patients with the combination of C18:0 and MSU crystals compared to C18:0 alone and the annual number of attacks of acute gouty arthritis of these patients (p: 0.022, Figure 7). Significant correlations were not found between changes of IL-1β by the combination of Pam3Cys and MSU compared to Pam3Cys alone and the annual number of attacks of acute gouty arthritis (data not shown, rs: +0.596, p: 0289).

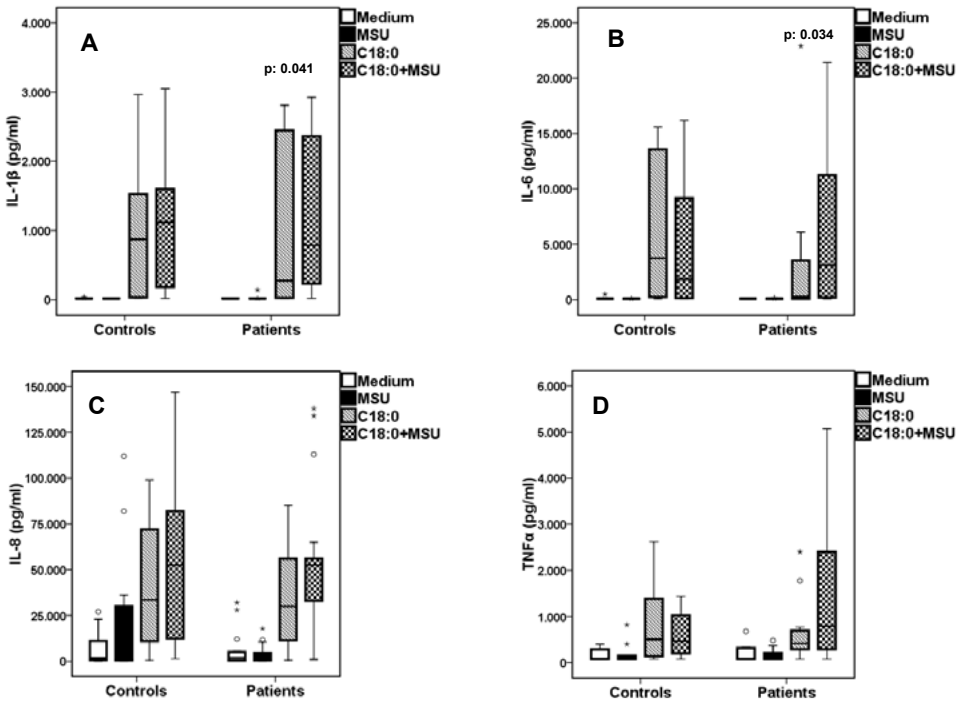

FIGURE 5. Patients displayed greater synergy between MSU and C18:0 in the induction of IL-1β than healthy donors.

Release of IL-1β (A), IL-6 (B), IL-8 (C) and TNFα (D) by peripheral blood mononuclear cells of patients with gout and healthy volunteers after stimulation with medium alone (RPMI), monosodium urate monohydrate (MSU) crystals and saturated fatty acids C18:0 alone or in combination with MSU. Asterisks denote outliers and circles denote extremes. P values indicate significant differences between respective values of patients and healthy controls by Mann-Whitney U test. Gout patients n=18 and healthy controls n=12.

To exclude the possibility that part of the explanation for the found synergy between C18:0 and MSU is due to the younger age of healthy controls, correlation analysis was done between the age of both healthy donors and patients and the fold change of IL-1β production by the combination compared with C18:0 alone. No statistically significant results were found (rs: +0.170, p: 0.428).

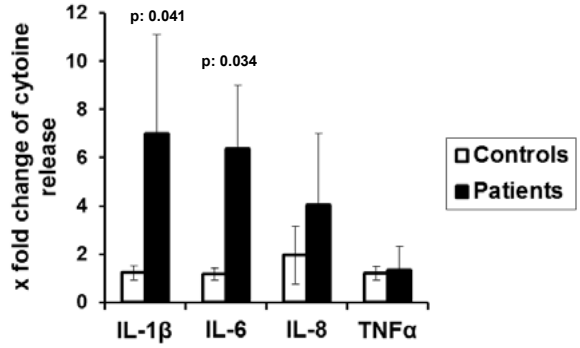

FIGURE 6. Changes of production of cytokines from PBMCs of gout patients and of healthy volunteers after stimulation with FFA C18:0 and MSU.

x fold refers to changes compared with the most active single stimulus. P values indicate significant differences between respective values of patients and healthy controls by Mann-Whitney U-test. Gout patients n=18 and healthy controls n=12.

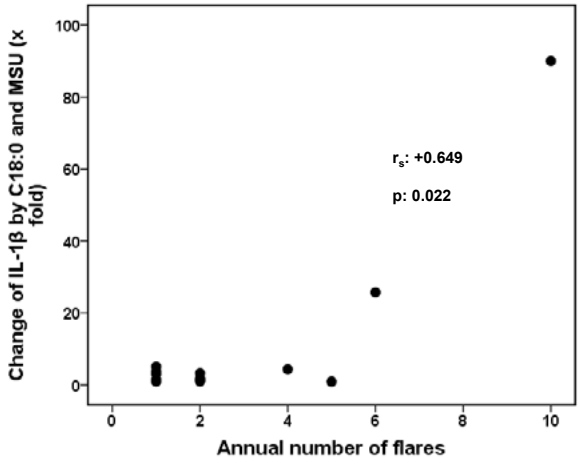

FIGURE 7. Correlation between synergy for IL-1β production and the annual number of attacks of acute gouty arthritis.

The x fold change of IL-1β produced by the combination of free fatty acids C18:0 and monosodium urate (MSU) compared with C18:0 alone is shown

Lack of IL-17 production by PBMCs after MSU and TLR2 ligands

A hallmark of acute gout is the influx of neutrophils into the synovium and joint fluid [17]. In order to define over-production in IL-1β release as the major culprit for attacks of gout, production of other cytokines mediating chemotaxis of neutrophils, notably IL-8 and IL-17, should not be affected by the co-incubation with C18:0 and MSU. The cytokine IL-17 induces neutrophil-attracting chemokines and granulopoietic cytokines, and recruits neutrophils to

the site of inflammation [18]. PBMCs of both gout patients and healthy controls exhibited no detectable production of IL-17 after stimulation with MSU crystals for 5 days, either in the presence or absence of FSL-1, Pam3Cys or C18:0 fatty acids (data not shown).

Effect on gene transcription

The synergy between TLR-2 ligands and MSU crystals might lead either to enhanced expression of the IL-1β gene or to increased cleavage of pro-IL-1β by caspase-1. mRNA transcripts of IL-1β in the PBMCs of gout patients did nor have any difference after addition of MSU crystals to FFA C18:0, compared with healthy donors (Figure 8A). Similar findings were observed after stimulation with MSU and Pam3Cys (Figure 8B).

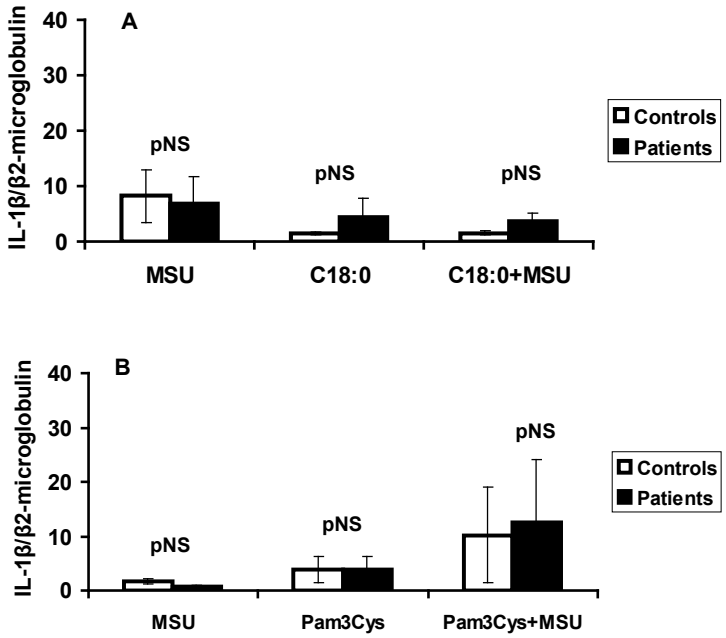

FIGURE 8. Gene expression of IL-1β in PBMCs from gout patients stimulated with MSU and Pam3Cys compared with healthy controls.

Measurement of mRNA transcripts of IL-1β after stimulation of PBMCs of patients with gout and controls with FFA C18:0 (A) or Pam3Cys (B) alone or in combination with monosodium urate (MSU) crystals. Results are shown as ratios with the transcripts of β2- microglobulin. Gout patients n=8 and healthy controls n=6.

DISCUSSION

In the present paper, we report that PBMCs of gout patients that have experienced attacks of arthritis produce more IL-1β than PBMCs of healthy volunteers, when the cells are exposed to a combination of TLR2 ligands and MSU crystals. In addition, the IL-1β response in the presence of C18:0 fatty acids together with MSU is augmented in the majority of the gout patients.

The responses in the gout patients show a much wider variation than that in healthy volunteers. These variations may be partly explained by the variations of the annual attacks of acute gouty arthritis in the patient group since synergy for IL-1β production is pronounced for patients with frequent attacks of acute gouty arthritis. We found that the synergism between MSU and FFA C18:0 leading to production of IL-1β is not due to enhanced gene expression. The most likely explanation is increased cleavage of pro-IL-1β to IL-1β in patients with gout. Enhanced cleavage may be mediated through the NLRP3 inflammasome, since MSU which is one agonist for NLRP3 inflammasome was used for stimulation. Our finding that this synergy was also present for IL-6 (which is at least in part induced by endogenous bioactive IL-1) underscored the activation of the inflammasome. However, it should not be neglected that other proteinases, namely neutrophil elastase and cathepsin G, may mediate cleavage of pro-IL-1β to IL-1β [19] but this is unlikely for the present study since they are present at very low levels in the PBMC preparations.

Our findings suggest that the NLRP3 inflammasome may be activated readily in patients with gout than in healthy controls. The synergistic effects for the production of IL-1β were mainly found between MSU and Pam3Cys and between MSU and FFA C18:0, but not between MSU and FSL-1, providing evidence that MSU synergize with TLR2/1 agonists and not with TLR2/6.

Several groups showed that MSU crystals function as inflammasome activators [9-11]. Initial reports suggested that urate crystals were able to induce IL-1β on their own. When we reported that MSU crystals need the presence of a second stimulus such as LPS [11] or FFA C18:0 [12], it turned out that others also used LPS as an inducer of pro-IL-1β transcripts [13]. This would fit with everyday clinical practice: while hyperuricemia is a prerequisite for gout, its presence does not always lead to disease and a second stimulus such as infection, excessive alcohol intake or fatty meal is needed for an attack to occur. The data in the literature on IL-1β production in gout was obtained by the use of either primary mononuclear cells of healthy volunteers [9, 11, 12] or human monocytic cell lines (THP-1) cells [10] and this is the first study using cells of gout patients.

The infiltration of the synovium by neutrophils is considered to be a feature of the acute inflammation in gout [1]. The recent literature indicates that IL-17 is a potent mediator of neutrophil influx, by expansion of the neutrophil lineage through induction of G-CSF as well as by recruitment of neutrophils through induction of chemokine [18]. IL-17 is mainly

released by a distinct subset of T helper cells, called Th17, which derive from the naïve CD4+ T-cells [20]. We measured IL-17 in order to find out whether acute gouty inflammation is under control of IL-17. The lack of production of IL-17, observed in the present study, supports the view that neutrophil influx in gouty inflammation is probably due to the release of other neutrophil-recruiting chemokines by IL-1β. In fact, Th17 cells are generated after days and this is not compatible with the rapid occurrence of inflammation in gout.
Despite our inability to find a direct relationship between uric acid concentrations in serum and the degree to which the inflammasome is activated, hyperuricemia still may be the explanation for the increased activation of the inflammasome in patients with gout. At the molecular level uric acid, in its soluble form, has been reported to have a role in regulating redox homeostasis by forming radicals in reaction with other oxidants [21]. Moreover, it has been suggested that reactive oxygen species (ROS) may activate NLRP3 inflammasome [22]. However, based on the findings of a previous study by our group, ROS are not involved in inflammasome activation, since PBMCs from ROS deficient patients produce significantly more IL-1β after exposure to MSU crystals than the cells from healthy individuals [23].
Our findings were generated with circulating PBMCs of patients. As such, it may be speculated that the over-inflammatory state produced in gout is systematic and not limited to joints. This speculation is consistent with the wide variation of co-morbidities recognized in these patients [1].

CONCLUSIONS

The present study provides evidence that the PBMCs of patients with gout display a more prominent synergistic production of IL-1β after stimulation with MSU crystals and TLR-2/1 ligands, compared to healthy controls. This synergy may be mediated by enhanced maturation of pro-IL-1β into IL-1β.

Abbreviations

CHD: coronary heart disease; COPD: chronic obstructive pulmonary disease; CRD: chronic renal disease; DCs: dendritic cells; DM2: diabetes mellitus type 2; FFA: free fatty acids; FSL-1: fibroblast stimulating ligand-1; IL interleukin; LPS: lipopolysaccharide; MSU: Monosodium urate monohydrate Pam3Cys: palmitoyl-3-cystein; PBMCs: peripheral blood mononuclear cells; PCR: polymerase chain reaction; TLR: toll-like receptors; TNFα: tumor necrosis factor alpha; ROS: reactive oxygen species; VH: vascular hypertension

Competing Interest

The authors declare that they have no competing interests

Authors' contributions

EM and MM participated in the acquisition of data, analysis and interpretation and the preparation of the manuscript. TOC, SM, AP, MG and AS contributed to the acquisition of data. EG-B, MN, JvdM and LJ participated in study design, analysis and interpretation of data and MS preparation. All authors approved the final version of the article.

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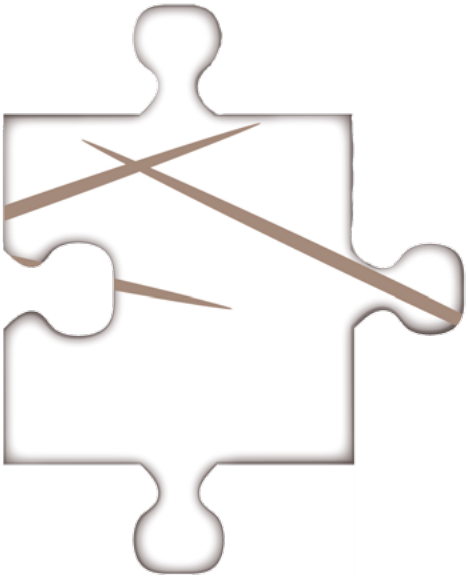

SOLUBLE URIC ACID PRIMES TLR-INDUCED PROINFLAMMATORY CYTOKINE PRODUCTION BY HUMAN PRIMARY CELLS VIA INHIBITION OF IL-1RA

Crișan TO
Cleophas MC
Oosting M
Lemmers H
Toenhake-Dijkstra H
Netea MG
Jansen TL
Joosten LA

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SUMMARY

Objectives

The study of the pro-inflammatory role of uric acid has focused on the effects of its crystals of monosodium urate (MSU). However, little is known whether uric acid itself can directly have pro-inflammatory effects. In this study we investigate the priming effects of uric acid exposure on the cytokine production of primary human cells upon stimulation with gout-related stimuli.

Methods

Peripheral blood mononuclear cells (PBMCs) were harvested from gout patients and healthy volunteers. Cells were pre-treated with or without uric acid in soluble form for 24 hours and then stimulated for 24 hours with TLR2 or TLR4 ligands in the presence or absence of MSU crystals. Cytokine production was measured by ELISA, mRNA levels were assessed using qPCR.

Results

The production of interleukin (IL)-1β and IL-6 was higher in patients compared to controls and this correlated with serum urate levels. Pro-inflammatory cytokine production was significantly potentiated when cells from healthy subjects were pre-treated with uric acid. Surprisingly, this was associated with a significant downregulation of the anti-inflammatory cytokine IL 1 receptor antagonist (IL-1Ra). This effect was specific to stimulation by uric acid and was exerted at the level of gene transcription. Epigenetic reprogramming at the level of histone methylation by uric acid was involved in this effect.

Conclusions

In this study we demonstrate a mechanism through which high soluble uric acid (up to 50 mg/dl) influences inflammatory responses by facilitating IL-1β production in PBMCs. We show that a mechanism for the amplification of IL-1β consists in the downregulation of IL-1Ra and that this effect could be exerted via epigenetic mechanisms such as histone methylation. Hyperuricaemia causes a shift in the IL-1β/IL-1Ra balance produced by PBMCs after exposure to MSU crystals and TLR-mediated stimuli and this phenomenon is likely to reinforce the enhanced state of chronic inflammation.

INTRODUCTION

Gout is one of the oldest described rheumatic diseases which affects approximately 1% of the world's population,[1] and reaches 2.5-3.9% prevalence in developed countries,[2 3]. Gout is characterized by painful, recurrent and initially self-limited attacks of acute inflammation with long-term progression towards chronic tophaceous gout in some patients,[4]. The biological culprit of gout is represented by monosodium urate (MSU) crystals,[5] that are formed during hyperuricaemia and elicit inflammatory events,[4]. The main proinflammatory cytokine that has been proven to strongly mediate acute gouty inflammation is IL-1β,[6-10]. MSU crystals have been associated with the activation of the NLRP3 inflammasome,[7] and proven to exert this effect via microtubule-mediated co-localisation of ASC to the site of inflammasome formation,[11]. Nevertheless, second signals are equally required to induce pro-IL-1β and synergize with MSU crystals,[9 10], in line with the clinical situation where gouty flares are precipitated in specific environmental situations, despite continuous deposition of MSU crystals in the joint,[12].

The single factor that is significantly associated with gout susceptibility and represents a necessary cause in gout development is hyperuricaemia,[13]. This is the elevation of serum uric acid levels above the threshold of 0.36 mM,[14] when uric acid crystallization ensues. Uric acid is the end-product of purine metabolism in humans and higher primates due to the evolutionary loss of uricase activity, an enzyme that metabolizes uric acid to the more soluble product allantoin,[15]. Recently, it has been shown that the loss of uricase activity is gradually lost during evolution, allowing adaptation to less enzymatic activity and slow rise of uric acid levels in the blood,[16]. Additionally, several uric acid transporters are involved in the reabsorption of approximately 90% of the total urate that has been filtered via the glomeruli,[17]. On the one hand, these two mechanisms have led to several hypotheses of evolutionary advantage that might be conferred by higher serum urate levels in primates,[18 19]. On the other hand, they make humans more prone to develop hyperuricaemia and gout. Consistently with uric acid being the major risk factor for gout, the most clear genetic associations with gout susceptibility were obtained for genetic variations in genes encoding urate transporters,[20 21].

Up to now, major lines of research investigating the pro-inflammatory effects of uric acid have focused on MSU crystal-induced processes,[22]. However, emerging data suggest that uric acid in soluble form might also have pro-inflammatory effects. It has been observed that hyperuricaemic mice have a higher cytokine production upon LPS challenge compared to control animals,[23] and different studies have shown NF-kB activation in renal and pancreatic tissue of mice receiving intraperitoneal injections with uric acid,[24 25].

In this study we hypothesize that uric acid might exert pro-inflammatory properties not only by predisposing to MSU crystallization, tissue deposition and acute inflammation, but also through a direct effect on human primary PBMCs. Here, we show that cells originating

from gout patients differ in their cytokine production capacity from control volunteers and that these differences correlate with serum uric acid levels. Furthermore, we describe enhanced pro-inflammatory cytokine production by primary cells exposed to high uric acid concentrations in-vitro and show that this effect is due to downregulation of IL-1Ra, the natural antagonist of the IL-1 receptor type I (IL1RI).

MATERIALS AND METHODS*

*A detailed Materials and methods version is provided in the Supplementary information of the manuscript

PBMC isolation and stimulation

Peripheral blood was drawn from healthy volunteers or gout patients, after informed consent. PBMCs were separated using Ficoll-Paque and suspended in culture medium RPMI (Roswell Park Memorial Institute).Cells were stimulated for 24 hours with Pam3Cys or C16:0 with or without MSU crystals. Separately, cells were incubated for 24 hours with culture medium, uric acid or allantoin (priming). After priming, culture medium was removed and remaining adherent cells were re-stimulated with medium, Pam3Cys, LPS with or without MSU crystals.

Flow cytometry

Cells primed for 24 hours uric acid were stained with annexin V-FITC (BioVision) and propidium iodide (Invitrogen) for assessment of cell death. Fluorescence was measured using Cytomics FC500 (Beckman Coulter).

Cytokine measurements

Cytokine concentrations were determined using ELISA kits for IL-1β, TNFα, IL-1α, IL-1Ra (R&D Systems), IL-6 and IL-10 (Sanquin).

Quantitative-PCR

Samples stimulated for 4 hours were treated with TRIzol Reagent (Invitrogen) for total RNA purification. Isolated RNA was transcribed into complementary DNA using iScript (Bio-Rad) followed by quantitative-PCR using Sybr Green.

Statistical analysis

Data was analysed using Kruskal-Wallis, Mann-Whitney or Wilcoxon signed rank test according to the number of datasets and experimental design, $\alpha<0.05$.

RESULTS

Enhanced cytokine production in PBMCs of gout patients compared to healthy volunteers

To assess the cytokine production of PBMCs of gout patients compared to healthy controls, 24 hours stimulations were performed using MSU crystals alone or in combination with TLR2 ligands Pam3Cys and saturated fatty acid palmitate (C16:0). MSU crystals alone did not induce IL-1β or IL-6 on their own, but significantly increased the effects of Pam3Cys or C16:0 in both patients and controls (Fig.1 A, B). Nevertheless, the overall cytokine levels observed in PBMCs originating from gout patients were higher compared to controls (Fig.1 A, B). Of high interest, a positive relation was observed between serum uric acid levels and ex-vivo IL-1β secretion in gout patients-derived cells, with hyperuricaemic patients (>0.36 mM) having a higher response than normouricaemic patients (<0.3 mM) (Fig.1 C, D, Fig.S1).

Uric acid pre-treatment enhances pro-inflammatory cytokine production in primary PBMCs of healthy volunteers

In order to mimic in-vitro the effects of hyperuricaemia, cells were exposed to soluble uric acid or were left untreated for 24 hours. Subsequently, they were stimulated with MSU crystals in the presence or absence of TLR2 ligand Pam3Cys or TLR4 ligand LPS. Cells and media were microscopically assessed at the end of the first 24 hours incubation for the presence of MSU crystal precipitates. No MSU crystallization was observed indicating that uric acid was stable for the time and conditions used. Uric acid alone after 24 hours priming did not induce detectable IL-1β or TNF concentrations but slightly increased IL-6 (Fig.2 A-C). Nevertheless, after stimulation, significantly increased cytokine levels were measured in uric acid pre-treated cells compared to RPMI pre-treated, effects that occurred in a dose-dependent manner (Fig.2 A-E). This effect was also seen in a time-dependent manner (Fig. S2 A). To assess mRNA modifications, after the first 24 hours priming with uric acid, cells were re-stimulated for another 4 hours. Uric acid pre-treated cells exhibited higher relative IL-1β mRNA compared to control conditions (Fig.2 F) and this effect was also observed in a dose-dependent manner (Fig.S2 B). TNF and IL-6 mRNA levels were also enhanced (Fig. S2 C, D). This effect of uric acid was most prominent at the highest concentration (50 mg/dl). Nevertheless, lower uric acid levels show the same tendency towards increased IL-1β production (Fig.S3 A). The effect on IL-1β was replicated in purified monocyte cell suspensions isolated from PBMCs (Fig.S4).

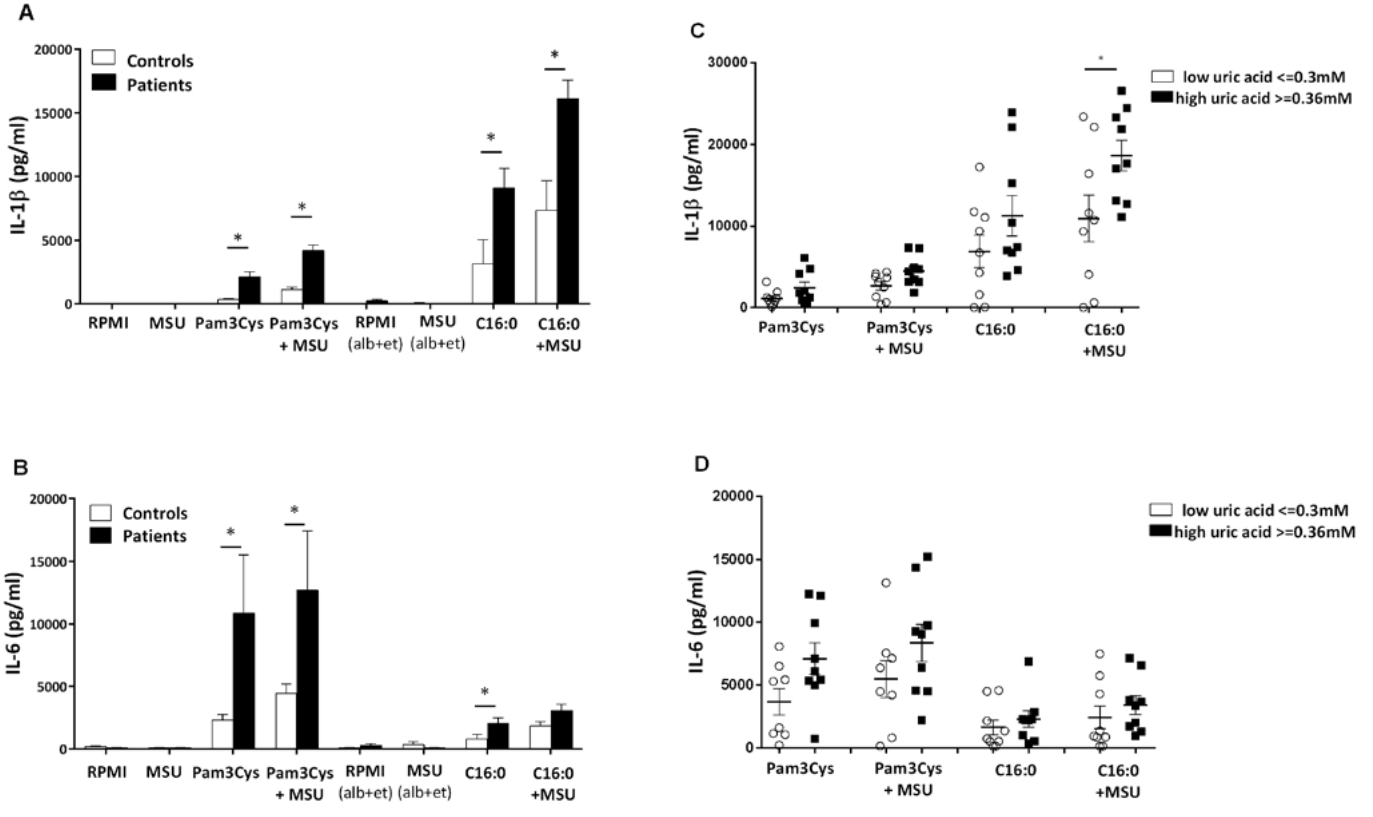

FIGURE 1. Enhanced cytokine production in cells from gout patients compared to healthy volunteers.

Freshly isolated PBMCs from gout patients (n=19) and healthy controls (n=7) were stimulated for 24h with Pam3Cys (10 µg/ml) or C16:0 (200 µM) in the presence or absence of MSU crystals (300 µg/ml). C16:0 vehicle (albumin and ethanol) was added to controls. Cytokine measurements were performed by ELISA for IL-1β (A) and IL-6 (B). Cytokine response differences between low (n=9) and high (n=9) uric acid gout patients are shown for IL-1β (C) and IL-6 (D). Data are shown as means ± SEM, Mann-Whitney test *p<0.05.

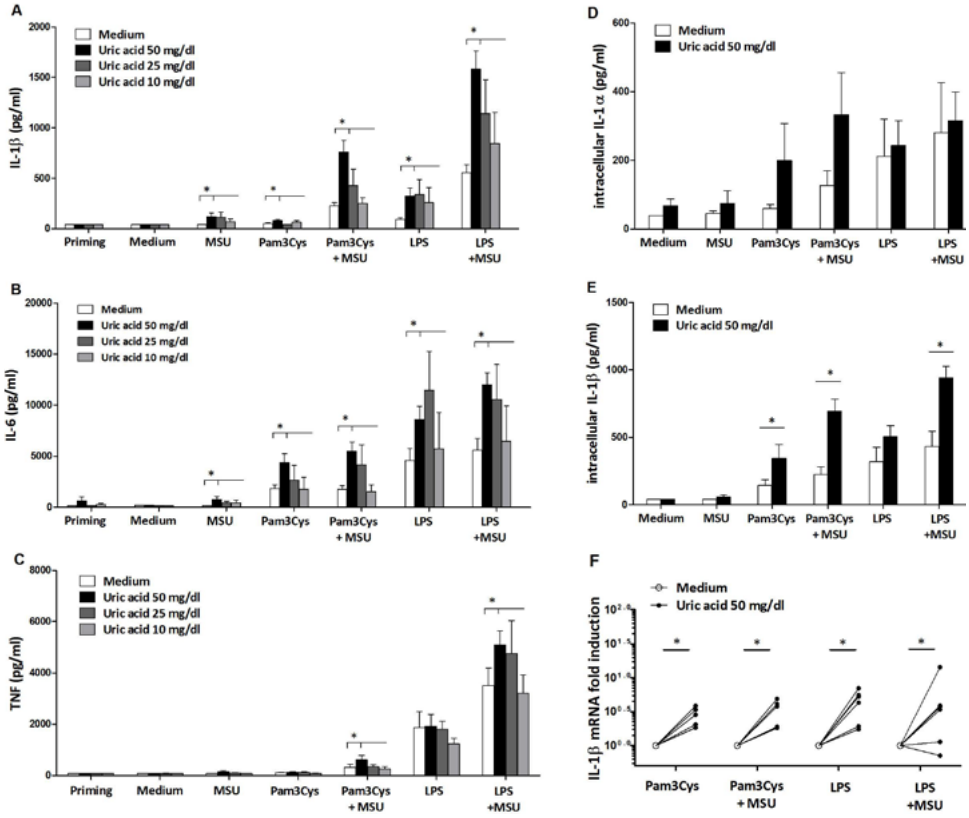

FIGURE 2. Uric acid pre-treatment increases pro-inflammatory cytokines.

PBMCs of healty volunteers were treated for 24h with culture medium (RPMI with 10% human serum) or with increasing concentrations of uric acid, followed by removal of medium and re-stimulation with RPMI, Pam3Cys (10 µg/ml) or LPS (10 ng/ml) in the presence or absence of MSU crystals (300 µg/ml). Cytokine production is shown in supernatants after the first 24h of pre-treatment (priming) and after the second 24h stimulation for secreted IL-1β (A), IL-6 (B) and TNF (C). Cells were lysed after stimulation by three sequential freeze-thaw cycles and intracellular IL-1β (D) and IL-1α (E) were measured. Data represents means±SEM of values observed in at least 5 up to 37 volunteers, from at least 3 independent experiments. Cells exposed to control medium or uric acid (50 mg/dl) for 24h were re-stimulated for 4h followed by mRNA isolation and qRT-PCR for IL-1β mRNA assessment. Data is shown as relative fold induction of IL-1β mRNA levels by uric acid in 6 volunteers from 3 independent experiments (F). Kruskall Wallis (A-C) or Wilcoxon (D-F) *p<0.05

Uric acid specifically down regulates the production of the anti-inflammatory cytokine IL-1Ra

To further decipher the mechanism of uric acid induction of pro-inflammatory cytokines, the effect on anti-inflammatory cytokines IL-1 receptor antagonist (IL-1Ra) and IL-10 was investigated. Of interest, the IL-1Ra concentrations were found to be significantly decreased in PBMCs treated with uric acid compared to medium pre-treated cells (Fig.3 A), whereas IL-10 levels were not affected (Fig.3 B). This surprising effect of uric acid on IL-1Ra production

was observed at the level of transcription after 4 hours uric acid exposure (Fig.3 C) and after 24 hours priming followed by 4 hours stimulation (Fig.3 D). This implies a specific modulation of uric acid on IL-1 natural inhibition. IL-1Ra downregulation was also obvious at the lower uric acid doses situated in the range of clinical hyperuricaemia (Fig.S3 B) and was present in selected monocytes (Fig.S4). Addition of exogenous IL-1Ra (recombinant protein) dose-dependently reversed the effects of uric acid (Fig.S5).

Modulation of immune responses by uric acid is independent of allantoin, myeloperoxidase (MPO) inhibition or cell death

To determine whether the effect of uric acid is specific or whether it can also be induced by related metabolites, allantoin was used as control in the same experimental conditions as uric acid. Uric acid induced significantly higher IL-1β production and strongly supressed IL-1Ra, which was not seen in allantoin treated PBMCs (Fig.4 A, Fig.S6 A,B). MPO inhibitor, 4-ABH, was used to assess whether oxidation products resulting after MPO-mediated oxidation of uric acid might play a role in the effects observed. However, the effect of uric acid on cytokine production was not modified by MPO inhibition (Fig.4 B, Fig.S6 C,D). Moreover, annexinV/PI staining after uric acid treatment did not show differences in cell death due to uric acid exposure (Fig.4 C).

PBMCs of gout patients reveal to be less responsive to uric acid priming for IL-1β induction when compared to PBMCs from healthy controls

Furthermore, we investigated whether uric acid priming is a possible mechanism explaining the higher cytokine production observed in cells of gout patients (as described in Fig.1). For testing this hypothesis, the experimental setup of 24 hours uric acid priming and 24 hours stimulation of PBMCs was replicated in a larger cohort of healthy controls and gout patients aiming at overruling bias due to cytokine variation. Indeed, in consistency with previous data shown in Figure 1, the absolute cytokine concentrations determined in cells from gout patients were significantly higher than in controls (white bars, Fig.5 A). The enhancement of IL-1β (Fig.5 A) and suppression of IL-1Ra (Fig.5 B) due to uric acid pre-treatment was observed in both groups. However, the degree of IL-1β induction by uric acid was significantly lower in gout patients despite identical dose of uric acid used, as observed by relative induction of IL-1β by uric acid (Fig.5 C). CD14+ monocytes of gout patients also exhibited higher steady-state IL-1β mRNA levels than healthy controls in unstimulated conditions (Fig.5 D), suggesting that cells of gout patients might have encountered factors in-vivo inducing a facilitated state for IL-1β production. IL-1Ra production was significantly decreased upon uric acid priming, with a lower basal level of IL-1Ra in unstimulated cells from gout patients compared to controls (Fig.5 B). However, the level of IL-1Ra inhibition by uric acid in gout patients does not significantly differ from controls (Fig.5 C).

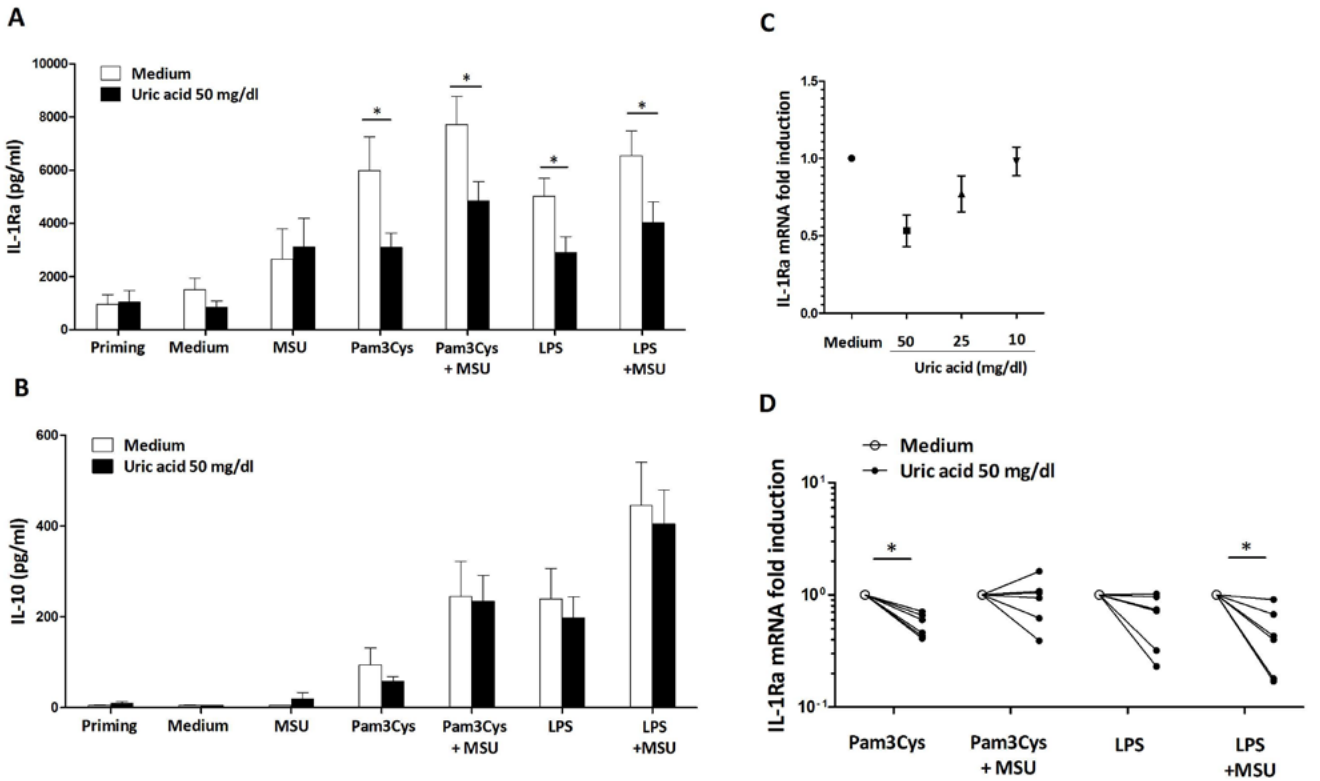

FIGURE 3. Specific downregulation of IL-1Ra due to uric acid exposure.

PBMCs of healty volunteers were treated for 24h with culture medium (RPMI with 10% human serum) or with uric acid 50 mg/dl, followed by removal of medium and re-stimulation with RPMI, Pam3Cys (10 µg/ml), LPS (10 ng/ml) in the presence or absence of MSU crystals (300 µg/ml). Cytokine production is shown in supernatants after the first 24h of pre-treatment (priming) and after the second 24 h stimulation for secreted IL-1Ra (n=13) (A) and IL-10 (n=5) (B). Data is shown as mean ± SEM, Wilcoxon *p<0.05. PBMCs were treated with medium or uric acid for either 4h (C) or for 24h followed by 4h restimulation (D) and were subjected to mRNA isolation and qRT-PCR for IL-1Ra transcription assessment. Data represents relative fold induction of IL-1Ra mRNA in 4 (C) or 6 (D) volunteers, Wilcoxon *p<0.05.

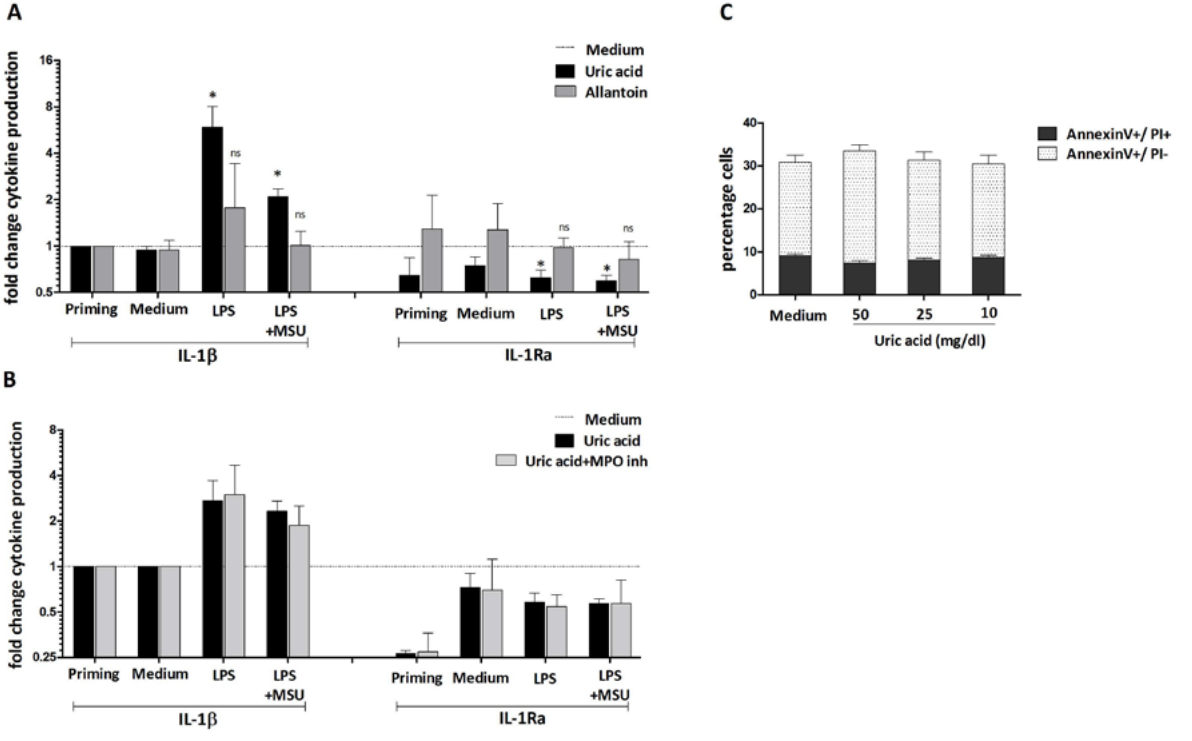

FIGURE 4. Uric acid effects are independent of similar or secondary metabolites or cell death.

PBMCs of healty volunteers were treated for 24h with culture medium, uric acid 50 mg/dl or equivalent concentrations of allantoin, followed by removal of medium and re-stimulation with RPMI, Pam3Cys (10 µg/ml), LPS (10 ng/ml) in the presence or absence of MSU crystals (300 µg/ml). Relative fold induction of IL-1β and IL-1Ra cytokines by uric acid or allantoin compared to medium control is shown (A). Data represents mean ± SEM of data obtained in 7 volunteers from 4 independent experiments, Wilcoxon *p<0.05. Priming with uric acid was performed in the presence or absence of myeloperoxidase (MPO) inhibitor, 4-aminobenzoic hydrazide (100 uM), and fold IL-1β and IL-1Ra induction of cytokines by uric acid compared to medium control is shown (n=4) (B). Cells were treated for 24h with medium or increasing concentrations of uric acid and cell death was assessed by flowcytometry after Annexin V/Propidium Iodide (PI) staining. Percentages of early apoptotic (Annexin V+/PI-) and late apoptotic (Annexin V+/PI+) cells are shown in samples derived from 3 volunteers (C).

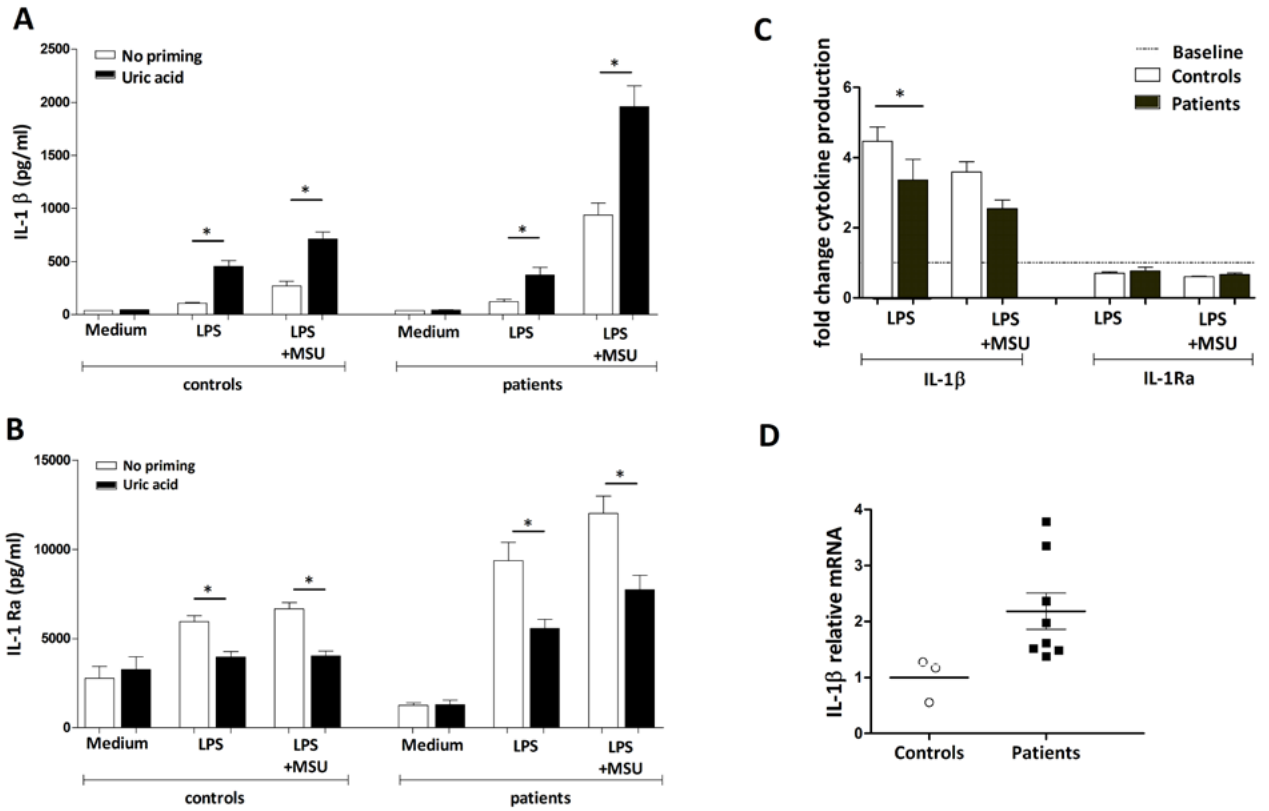

FIGURE 5. Reduced priming effects in cells from gout patients compared to healthy controls.

Freshly isolated human PBMCs from 114 healthy volunteers and 42 gout patients were primed with medium or uric acid 50 mg/dl for 24h, followed by stimulation with medium, LPS (10 ng/ml) or LPS+MSU(300 µg/ml) for another 24h. Absolute values and relative fold of change due to uric acid priming were assessed for IL1β (A and C) and IL-1Ra (B and C). CD14+ cells were positively selected using magnetic beads from 3 healthy volunteers and 8 gout patients and basal IL-1β mRNA levels were assessed in unstimulated CD14+ cells (E). Data is shown as means±SEM, Mann-Whitney *p<0.05.

Pharmacological inhibition of histone modifying enzymes reverse the priming effect of uric acid

Uric acid primes PBMCs for enhanced cytokine production at the transcription level, and we hypothesized that epigenetic modifications known to influence cytokine production, such as histone methylation and acetylation, are involved. In order to investigate the role of epigenetic histone modifications on uric acid induced priming that results in aggravated IL-1β, histone methyltransferase inhibitor, MTA, and histone acetyltransferase inhibitor, EGCG, were co-incubated with uric acid. Of high interest, upon restimulation, the enhancement of IL-1β by uric acid was reversed by MTA (Fig.6 A) while staying unmodified by acetyltransferase inhibitor EGCG (Fig.6 B).

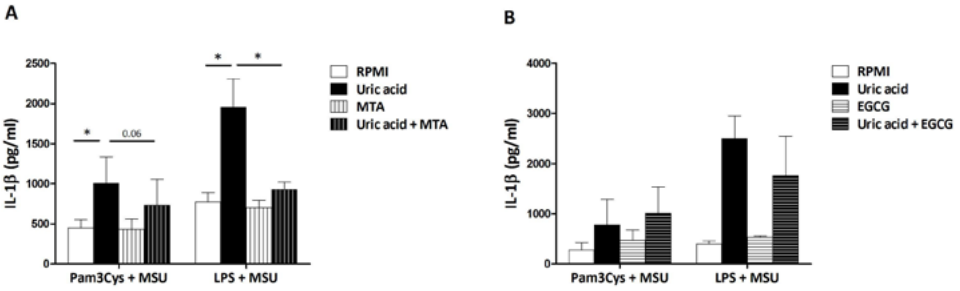

FIGURE 6. Reversal of uric acid effects by pharmacological inhibition of histone methyl transferases.

PBMCs of healty volunteers were primed with uric acid 50 mg/dl in the presence or absence of broad spectrum histone methyltransferase inhibitor MTA (5'-deoxy-5'-methylthio-adenosine), 1 mM (A) or histone acetyltransferase inhibitor EGCG (epigallocatechin-3-gallate), 30 μM (B). IL-1β levels were measured in the supernatants after 24h priming followed by 24h re-stimulation. Data represents mean ± SEM of data obtained in 6 volunteers from 3 independent experiments, Wilcoxon *p<0.05.

DISCUSSION

In this study, we have re-visited the hypothesis of uric acid acting as a possible pro-inflammatory agent, independently of its precipitated form in MSU crystals. As previously reported,[26], here we confirm the finding that ex-vivo stimulated PBMCs from gout patients reveal enhanced inflammatory cytokine production and we show that this is linked with serum uric acid levels of the donors.

Using primary human PBMCs as well as purified monocytes, we have performed an in-vitro stimulation protocol mimicking hyperuricaemia for 24 hours of initial treatment of the cells (priming) followed by re-stimulation with TLR ligands and MSU. These experiments revealed that significantly increased levels of pro-inflammatory cytokines (up to 3 fold more IL-1β) were produced by uric acid primed cells.

We next asked the molecular level at which uric acid exerts its effect: remarkably, we show that IL-1β production is enhanced at both intracellular and extracellular compartments, suggesting that enhanced secretion is probably not the explanation of these effects, but transcriptional upregulation of cytokines is the likely mechanism. Together with IL-1β, other pro-inflammatory cytokines like IL-6 and TNF were also increased, probably at least in part secondarily to IL-1β induction. However, most interestingly, a specific inhibition of IL-1Ra production was observed after uric acid pre-exposure, both at protein and transcription level. The increased IL-1β and decreased IL-1Ra levels were similarly observed in monocytes purified from the PBMC suspension. This finding is of high importance, as IL-1Ra is the natural inhibitor of IL-1RI, and it is known to be up-regulated in parallel to IL-1β production in a feedback loop aiming to control IL-1β and IL-1α-driven inflammation,[27]. To our knowledge this is a unique finding of a signal that does not up-regulate both IL-1β and IL-1Ra in the same direction, but rather decreases IL-1Ra production. Therefore, this promotes IL-1β-induced IL-1β production without the natural antagonist counterbalancing this pathway.

Exogenous IL-1Ra addition to uric acid treated samples during the first 24h of priming re-established the IL-1Ra levels during the first 24h. This however, did not have lasting effects for the second 24h stimulation on IL-1β (Fig.S5 A,B). Adding IL-1Ra also at the moment of re-stimulation, dose-dependently restored IL-1Ra and diminished IL-1β (Fig.S5 C,D). This proves that IL-1Ra downregulation by uric acid is an important part of the mechanism of IL-1β enhancement, not only an associated phenomenon with no functional significance.

Cells isolated from gout patients were found to be less prone to potentiate IL-1β induction after uric acid pre-exposure, compared to healthy volunteers. This effect was due to an already enhanced state of IL-1β production, most likely due to the effects of hyperuricaemia already present in these patients. In line with this, steady-state IL-1β mRNA levels appear to be higher in patients with gout than in controls and basal levels of IL-1Ra in unstimulated cells are lower than those observed in healthy volunteers. These observations suggest that the priming effects of soluble uric acid, that enhance IL-1β while decreasing IL-1Ra, are biologically relevant and present in-vivo in gout patients.

In experiments using PBMCs of gout patients and healthy controls for in-vitro priming, uric acid was not present during the time of re-stimulation with TLR ligands and/or MSU crystals. This implies a long-term consequence of uric acid exposure that induces an effect reminiscent of non-specific immunological memory ('trained immunity') of monocytes observed after infections and vaccinations,[28 29]. Long term adaptive effects on innate immune cells resulting in enhanced cytokine production have been shown to be mediated by epigenetic reprogramming of cells,[28 29]. The increased long-term H3K4me1 induced by inflammatory stimuli has been reported to increase cell responsiveness, and these epigenetic marks have been termed 'latent enhancers',[30]. Because uric acid exposure

induces a long-term increase in IL-1β gene transcription (accompanied by less IL-1Ra), we hypothesized that this effect may be mediated by histone modifications. Using pharmacological inhibitors of either histone methylation or acetylation, two major histone modification marks with functional consequences on gene expression, we show that uric acid effects were abolished when histone methyl transferase inhibitor was used. This is the first indication that indeed uric acid might exert effects at the epigenetic level and that these effects could have long term consequences on the individual cytokine profile. Future studies are warranted to assess the role of hyperuricaemia on the specific epigenetic marks and complete functional signature associated with high urate exposure.

A link between soluble uric acid and induction of inflammation has also been suggested by previous reports where uric acid has been identified as a danger signal released in the context of cell death inducing inflammation,[31] or adaptive immunity,[32]. Although the initial event in these studies is also the release of uric acid and formation of a high urate environment, the main inflammatory role in this context is still in fact attributed to MSU crystals,[33]. In contrast, data linking uric acid with higher LPS responses,[23] and NF-kB activation,[24 25] were obtained in mice receiving intraperitoneal uric acid injections. In this study, despite the high uric acid concentrations used (approximately 8 times higher than the threshold for hyperuricaemia) we were unable to detect MSU crystals being formed over the 24 hours of cell culture in the presence of uric acid, arguing for the stability of the solutions used for the time and conditions of our experimental setup. Moreover, MSU crystals and soluble uric acid have totally opposite effects on IL-1Ra levels: as shown in Fig.3 A and Fig. 5 B, MSU crystals increase the levels of IL-1Ra secreted upon MSU stimulation alone, or in synergism with other ligands, while soluble urate significantly downregulates IL-1Ra. Therefore, in this study, we attribute our findings not to the crystalline form of uric acid but to high uric acid concentrations in the cellular environment.

The high concentrations of uric acid used can be a limitation of this study. Nevertheless, we provide evidence (Fig.S2 C, D) that the same cytokine profile is observed also at lower uric acid levels, in the range of clinical hyperuricaemia and within the solubility limits of uric acid. The rationale of using a high dose was to have the ability to best observe these significant effects in an in-vitro setting, allowing further experimental intervention. In Figure 5 we show data supporting that similar features of uric acid priming were also present in gout patients, sustaining the biological relevance of this finding. Further studies using in-vivo models are needed to demonstrate this in more detail.

This mechanism of uric acid induced modulation of pro-inflammatory cytokines and, more specifically, IL-1β, is of high relevance also for other metabolic diseases. There is large body of evidence that associates uric acid with incidence and risk factors for metabolic syndrome, atherosclerosis, hypertension, type 2 diabetes, chronic kidney disease,[34-38]. In this context, mechanistic data on the pro-inflammatory effects of uric acid are likely to turn

into a potential link between the diseases of modern society associated with chronic low grade inflammation.

In conclusion, in this study we propose a mechanism through which high uric acid concentrations mediate the metabolic modulation of inflammatory responses by facilitating IL-1β production in immune cells. We show that a mechanism for the amplification of IL-1β is via the unique inhibitory effect on IL-1Ra production that would normally counterbalance the effects of high IL-1 and the IL-1β auto-induction loop. This effect of uric acid is likely to be epigenetically mediated by histone marks that modify the degree of gene transcription for cytokine genes. As a consequence, patients having hyperuricaemia could be at risk of exhibiting increased reactivity of their immune cells upon encounter of acute inflammatory stimuli (infectious or sterile danger signals) and this might induce enhanced states of (auto) inflammation in the affected patient. Thus, we provide here the first evidence that uric acid represents a silent modulator of cytokine production in primary human cells. This pinpoints to the role of metabolic triggers on the inflammatory properties of the circulating cells and could represent a relevant link for understanding the pathogenesis of gout and other metabolic diseases with inflammatory components.

Acknowledgements

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Competing interests

The authors declare no competing interests.

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SUPPLEMENTARY INFORMATION

Materials and methods

Participants

Subjects to this study consisted in healthy volunteers and patients diagnosed with crystal-proven gout (TJ) being followed at the Rheumatology Department of the Radboud University Medical Center. Participants were enrolled after written informed consent and peripheral blood was collected on EDTA under sterile conditions. The patient study was approved by the Ethical Committee of the Radboud University Medical Center (registration number 2012/482). Blood from a total of 57 gout patients of Dutch nationality has been used in 24h stimulation experiments (n=19) or in 48h uric acid priming experiments (n=49). Mean age was 62.8 (range 29-85). A set of main clinical parameters is shown in Table S1. All patients were under either urate lowering therapy, anti-inflammatory therapy for control of the attacks, or both. Urate lowering therapy consisted mainly in allopurinol (or febuxostat when allopurinol was counterindicated), occasionally in combination with benzbromarone. Anti-inflammatory therapy consisted mainly in colchicine, with fewer cases on steroids or non-steroidal anti-inflammatory drugs.

Reagents

Uric acid, palmitic acid (C16:0), LPS (E. coli serotype 055:B5), allantoin, 4-aminobenzoic hydrazide (4-ABH), 5'-deoxy-5' (methylthio) adenosine (MTA) and epigallocatechin-3-gallate (EGCG) were purchased from Sigma. Synthetic Pam3Cys was purchased from EMC Microcollections. Recombinant IL-1Ra was obtained from R&D. LPS was subjected to ultrapurification before cell culture experiments. In experiments using C16:0, albumin (Albuman, human albumin 20% for intravenous use, Sanquin) was used to initially conjugate fatty acids before applying to cells. Equal amounts of albumin and ethanol were added to controls. In experiments using 4-ABH and MTA equal amounts of DMSO vehicle were added to control wells.

Preparation of MSU crystals.

A 0.03 M solution of MSU at a volume of 200 ml was prepared after diluting 1.0 gram of uric acid in 200 ml of sterile water containing 24 grams of NaOH. The pH was adjusted to 7.2 after the addition of HCl, and the solution became pyrogen-free after heating for 6 hours at 120°C. The solution was left to cool at room temperature and stored at 4°C. Crystals produced were 5–25 µm in length.

PBMC isolation and stimulation

Peripheral blood was harvested from the antecubital vein of healthy volunteers or gout patients, after obtaining informed consent. The PBMC fraction was obtained by differential centrifugation over Ficoll-Paque (Pharmacia Biotech). Cells adjusted to 5x106 or 10x106 cells/ml (depending on experiment setup) were suspended in culture medium RPMI (Roswell Park Memorial Institute) 1640, supplemented with 50 µg/ml gentamicin, 2 mM L-glutamine and 1 mM pyruvate. PBMCs were plated at 5x105 cells/well in 96-well plates (Corning). Cells were stimulated for 24h in round bottom plates with Pam3Cys (10 µg/ml) or C16:0 (200 µM) in the presence or absence of MSU crystals (300 µg/ml).
In different sets of experiments, cells were incubated in flat bottom 96-well plates for 24h with culture medium (RPMI with 10% human serum), uric acid 50 mg/dl or allantoin 50 mg/dl. In experiments investigating dose-dependent effects, lower uric acid concentrations were also used. Experiments investigating the effects of pharmacological inhibitors on uric acid priming consisted in 1 hour pre-incubation with or without inhibitor, followed by 24 hours exposure to uric acid. After this initial treatment with uric acid, culture medium was removed and the remaining adherent cells were stimulated for another 24 hours with RPMI medium, Pam3Cys (10 µg/ml), LPS (10 ng/ml) in the presence or absence of MSU crystals (300 µg/ml). Cytokine induction experiments were performed in duplicate wells and the replicates were pooled before measurement.

Monocyte isolation

Purified monocyte suspensions were obtained from PBMCs using MACS beads according to manufacturer's instructions (Miltenyi Biotec). CD14+ cells were isolated using CD14 microbeads followed by magnetic separation over selection columns. Negative selection of monocytes was performed after an initial monocyte-lymphocyte separation of PBMCs using high-density hyperosmotic Percoll solution, followed by MACS. This was carried out either by labeling CD3+, CD19+ and CD56+ cells using the corresponding microbeads and cell separation over depletion columns, or by using the pan monocyte isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Monocyte suspensions were adjusted to 1x106 cells/ml and experiments were performed using 1x105 cells/well.

Flow cytometry

Cells treated for 24 hours with or without uric acid were stained with annexin V-FITC (BioVision) and propidium iodide (PI) (Invitrogen) for assessment of cell death. In brief, annexin V-FITC was diluted 1/100 in CaCl2-enriched RPMI (6 µM), samples were stained with 100 ul annexin V staining solution and were incubated for 15 minutes on ice in the dark. This was followed by PI staining (1 mg/ml, 1.5 µl per sample) and 5 minutes incubation

before measurement. Purity of cells obtained through MACS isolation was assessed by 30 minutes staining on ice in the dark of 1x105 freshly isolated cells with CD14-FITC, CD56-PE, CD19-ECD, CD3-PC5, CD45-PC7, followed by 1 washing step with 1% BSA. Fluorescence was measured using Cytomics FC500 (Beckman Coulter).

Cytokine measurements

Cytokine concentrations were determined using specific sandwich ELISA kits for IL-1β, TNFα, IL-1α, IL-1Ra (R&D Systems), IL-6 and IL-10 (Sanquin). Secreted cytokines were measured in the supernatant collected at the end of the experiment. For intracellular cytokine levels, after removal of supernatant, 200µl medium was added to the remaining cells and was subjected to three sequential freeze-thaw cycles. These induce the disintegration of the membrane and release of intracellular cytokine content into the medium.

Quantitative-PCR

Samples stimulated for 4 hours at 37°C were treated with TRIzol Reagent (Invitrogen) and total RNA purification was performed according to manufacturer's instructions. Isolated RNA was subsequently transcribed into complementary DNA using iScript cDNA Synthesis Kit (Bio-Rad) followed by quantitative PCR using the Sybr Green method. The following primers were used in the reaction: IL-1β forward 5'-GCCCTAAACAGATGAAGTGCTC-3' and reverse 5'-GAACCAGCATCTTCCTCAG-3', IL-1Ra forward 5'-GCCTCCGCAGTCACCTAAT-3' and reverse 5'-TCCCAGATTCTGAAGGCTTG-3', β2-microglobulin forward 5'-ATGAGTATGCCTGCCGTGTG-3' and reverse 5'-CCAAATGCGGCATCTTCAAAC-3' (Biolegio). Results are shown as fold change in mRNA levels in stimulated samples compared to controls.

Statistical analysis

Data was analysed using GraphPad Prism version 5.03 and SPSS version 20. The differences were analysed using Kruskal Wallis test, Mann-Whitney test or Wilcoxon signed rank test according to the number of datasets and experimental design. Correlation analysis was performed using Spearman's correlation. Data was considered statistically significant at a p-value<0.05. Data is shown as cumulative results of levels obtained in all volunteers (means + SEM).

SUPPLEMENTARY TABLES AND FIGURES

TABLE S1. Main clinical parameters of gout patients

Gout patients		**Total**		**Men**	**Women**	
	n=	**57**		**52**	**5**	
	%	**100,0**		**91,2**	**8,8**	
Time since last attack	**Months:**	**0**	**3**	**6**	**12**	**undetermined**
	n=	**4**	**12**	**5**	**29**	**7**
	%	**7,0**	**21,1**	**8,8**	**50,9**	**12,3**
Therapy		**Urate lowering**		**Anti-inflammatory**		**Others:**
		yes	**no**	**yes**	**no**	**Statins**
	n=	**51**	**6**	**47**	**10**	**12**
	%	**89,5**	**10,5**	**82,5**	**17,5**	**21,1**

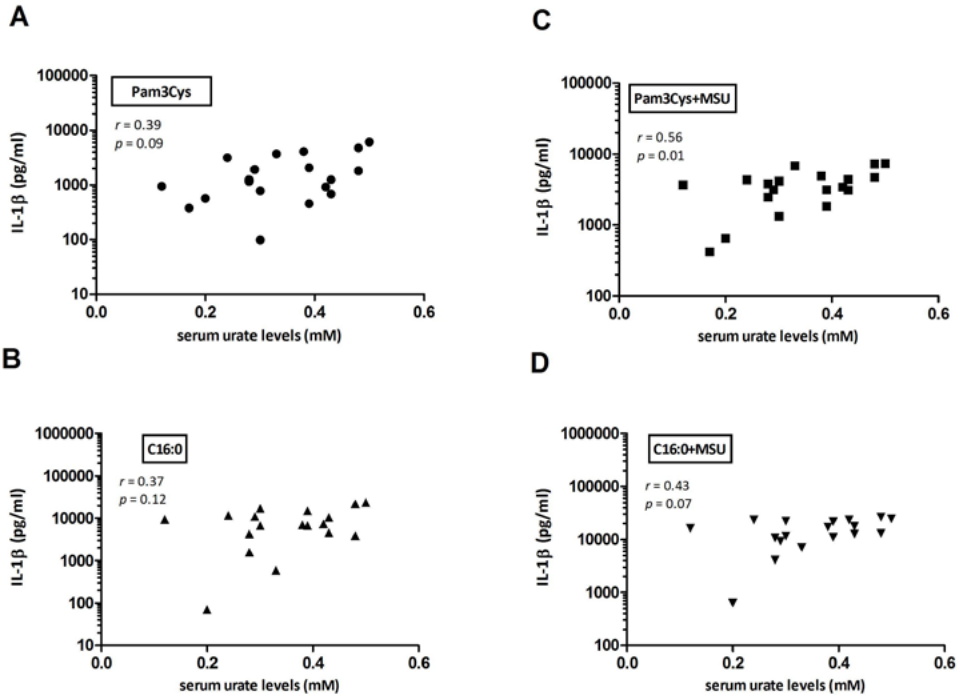


FIGURE S1. Freshly isolated PBMCs from gout patients (n=19) were stimulated for 24h with Pam3Cys (10 µg/ml) or C16:0 (200 µM) in the presence or absence of MSU crystals (300 µg/ml). C16:0 vehicle (albumin and ethanol) was added to control wells. Cytokine measurements were performed by ELISA and Spearman correlation was assessed between serum uric acid levels and cytokine production upon specific stimulations (Spearman r and p-values depicted in figure, $\alpha<0.05$).

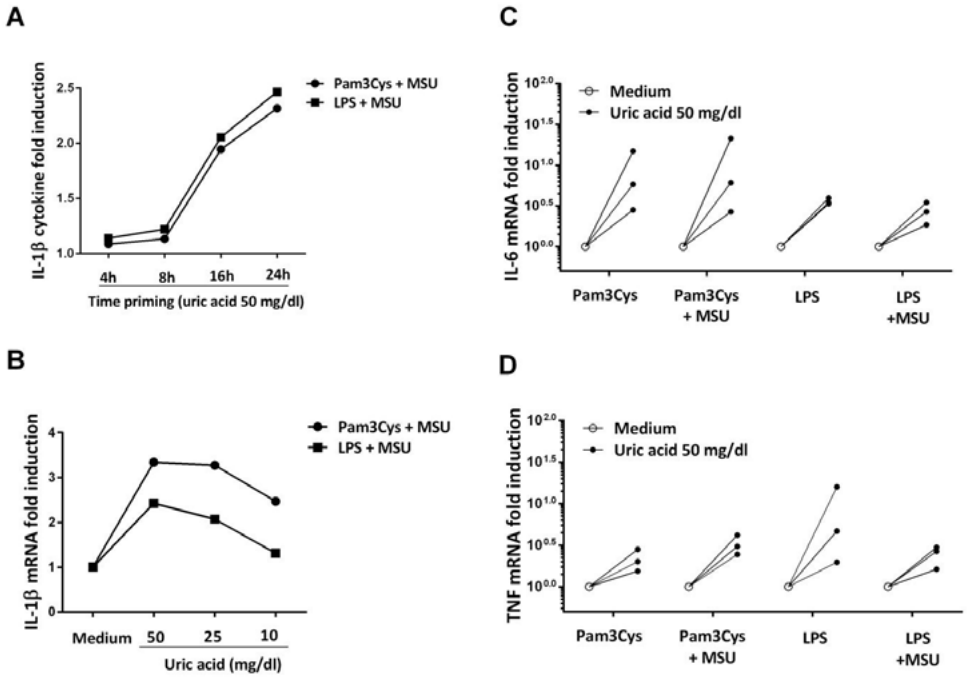


FIGURE S2. Cells were exposed to control medium or uric acid (50mg/dl) for 4, 8, 16 and 24h and cytokine production after re-stimulation was assessed. Relative fold induction of cytokines by uric acid compared to medium control in response to Pam3Cys+MSU or LPS+MSU is shown (n=2) (A). Cells were treated for 24h with culture medium (RPMI with 10% human serum) or with increasing concentrations of uric acid, followed by 4 h re-stimulation for IL-1β mRNA assessment. Data is shown as relative fold induction of IL-1β mRNA levels observed in 2 volunteers. PBMCs were treated with medium or uric acid for either for 24h followed by 4h restimulation and were subjected to mRNA isolation and qRT-PCR. Data represents relative fold induction of IL-6 (C) and TNF(D) mRNA in 3 volunteers.

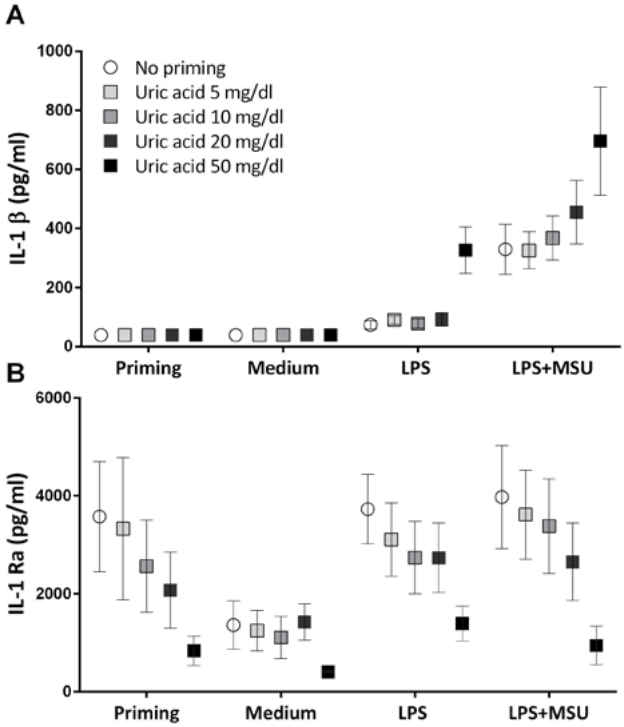


FIGURE S3. Cells were exposed to lower uric acid concentrations (5-20 mg/dl range) and compared to 50 mg/dl for 24h priming and 24h stimulation with the mentioned stimuli. Data represents cytokine levels in 4 volunteers for IL-1β (A) and IL-1Ra (B).

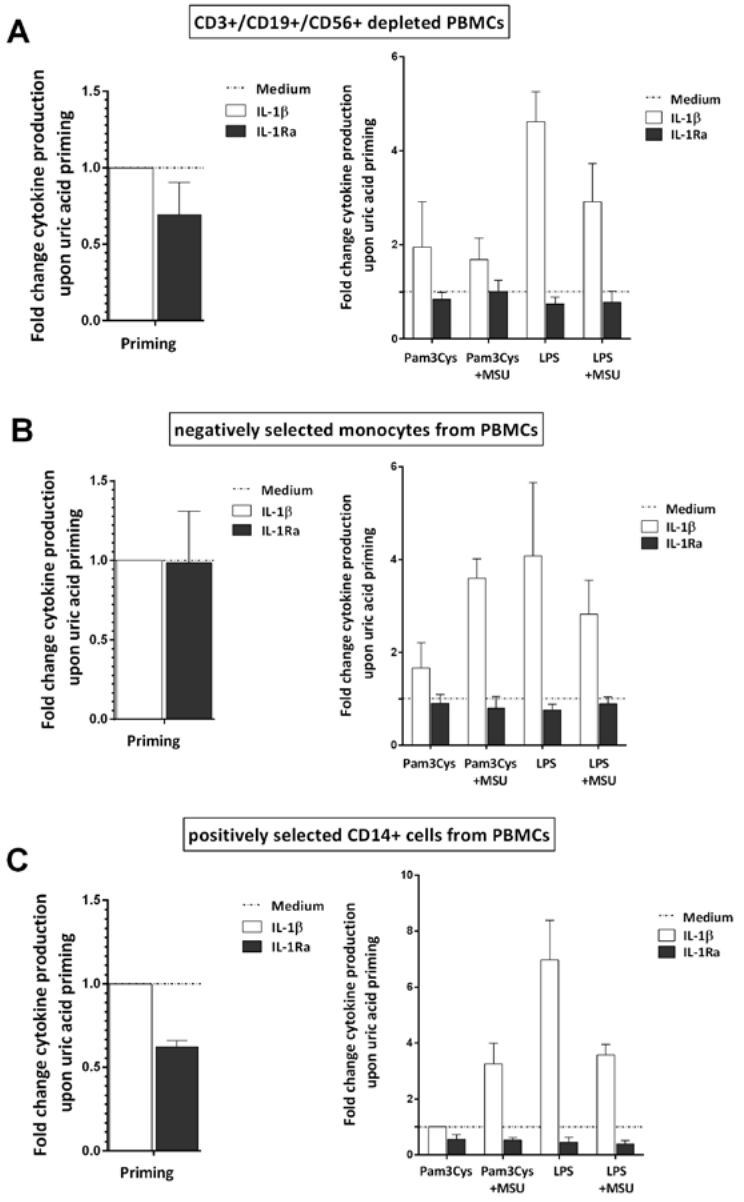


FIGURE S4. Monocytes were negatively or positively selected from the PBMC fraction using magnetic separation and were subjected to the priming protocol consisting of 24h incubation with or without uric acid 50 mg/dl followed by another 24h stimulation RPMI medium, Pam3Cys (10 µg/ml), LPS (10 ng/ml) in the presence or absence of MSU crystals (300 µg/ml). Fold induction or supression of cytokines by uric acid compared to medium control is displayed for the first 24h of priming and for the 24h restimulation in CD3+/CD19+/CD56+ depleted PBMCs (A), enriched monocytes after pan-monocyte isolation (B) or positively selected CD14+ cells (C). Data represents means ± SEM of at least

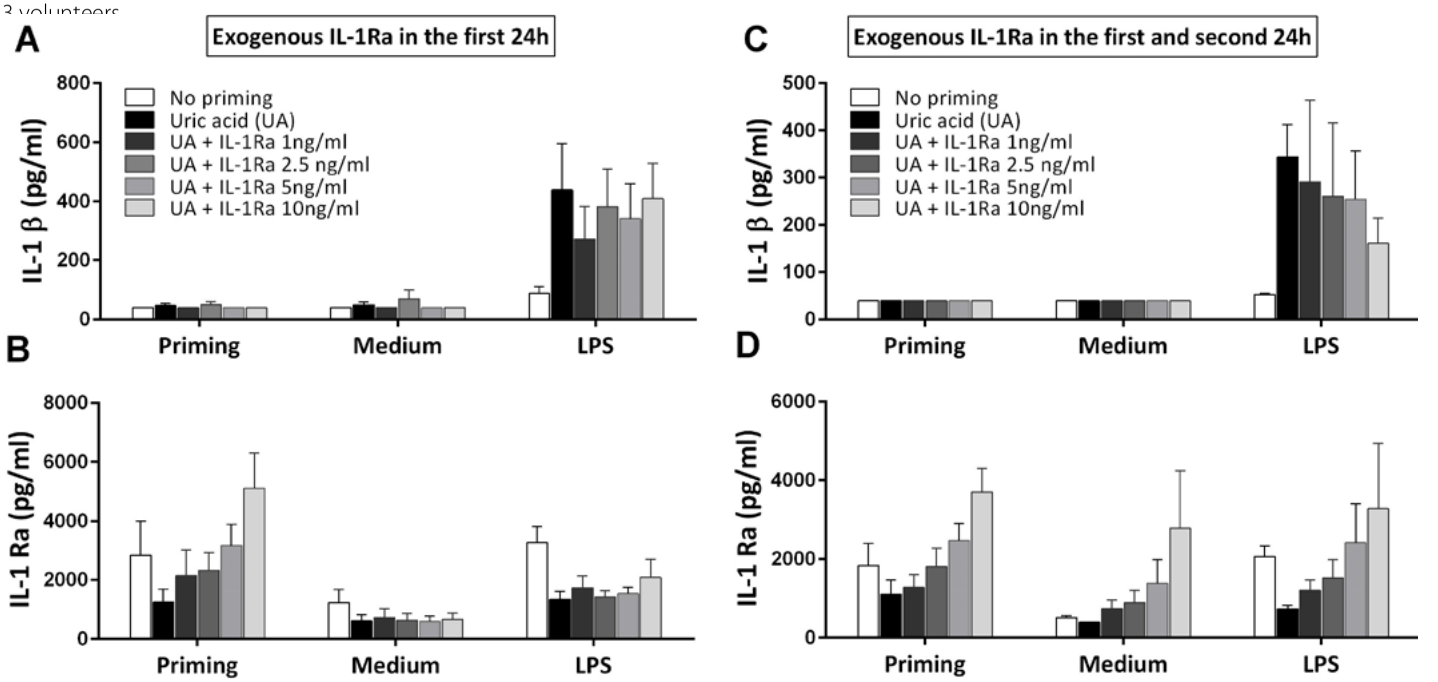


FIGURE S5. Exogenous recombinant IL-1Ra (1-10 ng/ml) was added to uric acid treated cells (50 mg/dl) during the first 24h of priming with 50 mg/dl uric acid (A, B) or both during priming and re-stimulation (C, D) with LPS (10 ng/ml). IL-1β (A, C) and IL-1Ra (B, D) levels are measured in 4 volunteers and are shown as means ± SEM.

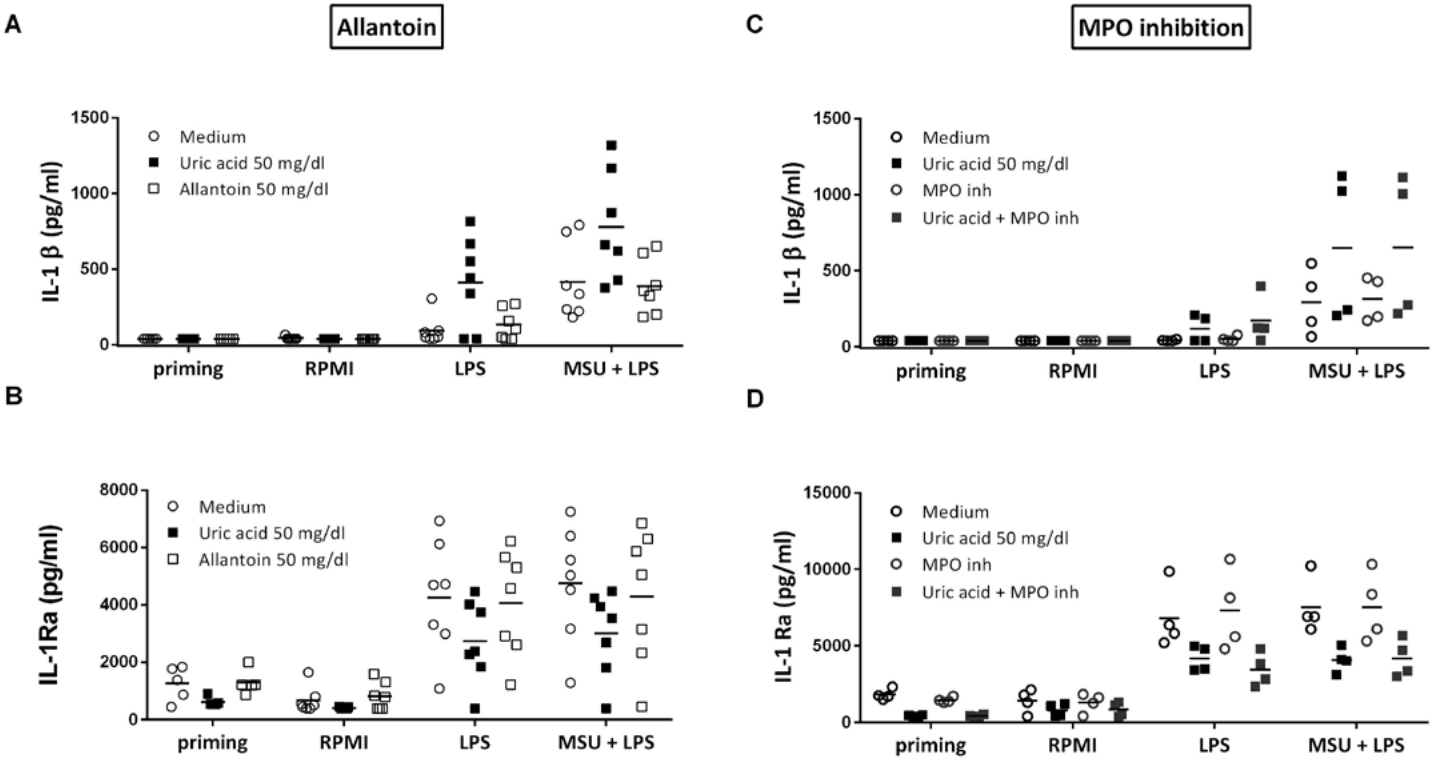
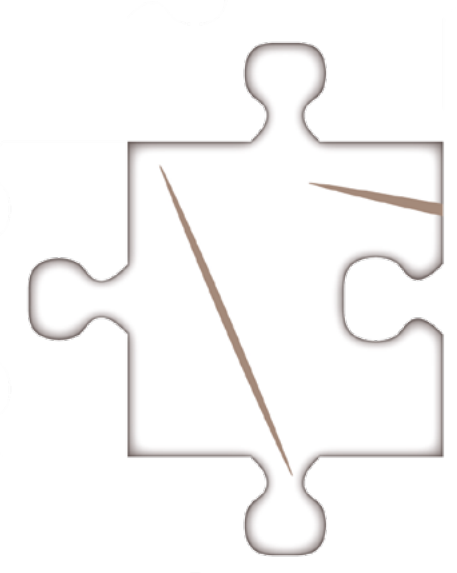


FIGURE S6. Absolute individual values are shown for the cytokine production obtained by priming with uric acid, allantoin (n=7) (A, B) or priming with uric acid in the presence or absence of MPO inhibitor (n=4) (C, D). Restimulation followed with medium or LPS (10 ng/ml) in the presence or absence of MSU crystals (300 µg/ml).



URIC ACID PRIMING IN HUMAN MONOCYTES IS DRIVEN BY THE AKT-PRAS40 AUTOPHAGY PATHWAY

Crişan TO
Cleophas MCP
Novakovic B
Erler K
van de Veerdonk FL
Stunnenberg HG
Netea MG
Dinarello CA
Joosten LAB

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SUMMARY

Metabolic triggers are important inducers of the inflammatory processes in gout. Whereas the high serum urate levels observed in patients with gout predispose to the formation of monosodium urate (MSU) crystals, soluble urate also primes for inflammatory signals in cells responding to gout-related stimuli, but also in other common metabolic diseases. In this study, we investigated the mechanisms through which uric acid selectively lowers human blood monocyte production of the natural inhibitor IL-1 receptor antagonist (IL-1Ra) and shifts production towards the highly inflammatory IL-1β. Monocytes from healthy volunteers were first primed with uric acid for 24 hours and then subjected to stimulation with LPS in the presence or absence of MSU. Transcriptomic analysis revealed broad inflammatory pathways associated to uric acid priming, with NFκB and mammalian target of rapamycin (mTOR) signaling strongly increased. Functional validation did not identify NFκB or AMP activated protein kinase (AMPK) phosphorylation, but uric acid priming induced phosphorylation of AKT and proline rich AKT substrate 40 kDa (PRAS 40) that in turn activate mTOR. Subsequently, Western blot for the autophagic structures LC3-I and LC3-II (microtubule-associated protein 1A/1B-light chain 3) fractions as well as fluorescence microscopy of LC3-GFP overexpressing HeLa cells revealed lower autophagic activity in cells exposed to uric acid compared to control conditions. Interestingly, reactive oxygen species (ROS) production was diminished by uric acid priming. Thus, the Akt-PRAS40 pathway is activated by uric acid, which inhibits autophagy and recapitulates uric acid-induced proinflammatory cytokine phenotype.

INTRODUCTION

Uric acid is a naturally occurring product of purine metabolism. The elevation of serum uric acid levels above the threshold of 0.36 mM is called hyperuricemia (1). Hyperuricemia is the single necessary condition that is significantly associated with gout susceptibility and pathogenesis (2). This is due to the fact that uric acid precipitates to form monosodium urate (MSU) crystals (3), which in turn promote inflammation and arthritic events.

Due to the evolutionary loss of uricase gene functionality, uric acid is the end-product of purine metabolism in humans and higher primates (4). In less evolved organisms, uricase enzyme activity is preserved, enabling uric acid catabolism to the more soluble product allantoin (4). A relatively recent study described that the loss of uricase activity happened gradually during evolution, allowing adaptation to progressively less enzymatic activity and slow rise of uric acid levels in the blood (5). Moreover, 90% of the total filtered urate is reabsorbed in the kidney by the uric acid transporter machinery (6). In line with this, strongest genetic associations with hyperuricemia and gout come from variants in genes encoding urate transporters (7, 8).

The existence of these complementary mechanisms that conserve uric acid is indicative of evolutionary advantages of high uric acid levels in the blood (9, 10). Nevertheless, an increasing body of evidence highlights the role of uric acid in diseases such as chronic kidney disease (11) , fatty liver disease (12), cardio-renal syndrome and hypertension (13, 14), coronary heart disease (15, 16), type 2 diabetes (17), aging (18) or cancer (19), in addition to gout. The many associations of uric acid with common inflammatory diseases lead to a scenario of thrifty genes that currently predispose to fat storage disorders and systemic comorbidities (20).

Inflammatory processes induced by uric acid are initiated after cellular injury and death, causing purines and purine degradation products to be released in the extracellular milieu (21, 22). Uric acid and MSU act as damage associated molecular patterns (DAMPs) and induce sterile inflammation in the involved tissue (21, 22). For a long time uric acid was considered a non-functional byproduct associated to metabolic syndrome, but in the context of increasing numbers of studies investigating the direct effects of uric acid in inflammation and disease pathogenesis, the quest for targetable mechanisms in uric acid mediated inflammation has been reappraised (23).

Recently, our group has discovered that soluble uric acid exerts pro-inflammatory properties through a direct effect on human primary peripheral blood mononuclear cells (PBMCs) (24). We have found that PBMCs of patients with hyperuricemia produce higher amounts of pro-inflammatory cytokines than healthy controls after *ex-vivo* stimulation, but the mechanisms responsible for this inflammatory imbalance are not elucidated (24, 25). In the present study, we report the intracellular mechanisms that underlie uric acid priming effects in human monocytes.

RESULTS

Monocytes show a shift between IL-1 beta and IL-1 receptor antagonist production after uric acid exposure

Modulatory effects of soluble uric acid on cytokine production have been previously described (24). Here we reproduce the cytokine responses induced by uric acid in a highly pure human monocyte population (Figure S1), obtained using sequential steps of Ficoll density gradient centrifugation, Percol density gradient centrifugation, and magnetic micro beads negative selection. Monocytes were subjected to a priming protocol consisting in 24h exposure to medium containing increasing concentrations of solubilized uric acid, followed by washout and stimulation with lipopolysaccharide (LPS) or LPS with monosodium urate(MSU) crystals. Cytokines were measured after 24 hours priming and at the end of the experiment. Uric acid priming induced higher IL-1β production which was visible only after restimulation with LPS or LPS+MSU, while IL-1β could not be measured in supernatants of cells after uric acid priming alone (Figure 1A). IL-1Ra production showed an opposite trend compared to IL-1β, as uric acid down-regulated IL-1Ra (Figure 1B). This phenotype was visible after uric acid treatment alone and it also persisted at 48 hours, following LPS+MSU stimulation . Allantoin used as control priming stimulus was not able to induce any of these effects (Figure 1A-B).

Transcriptomic data reveals broad functional effects of uric acid in monocytes and shows enrichment in candidate pathways in IL1 regulation

In order to extract mechanistic information to explain the inflammatory effects of uric acid, RNA-sequencing was performed in highly purified monocytes (separated by negative selection). Efficacy of monocyte selection was assessed by flow cytometry (Figure S1). The experimental setup is summarized in Figure 2A. Monocytes were exposed to medium or uric acid 50 mg/dl for 20h followed by stimulation with medium or LPS 10 ng/ml for another 4h (cytokines were measured in the supernatants of cells used for RNA-seq –Figure S1) and RNA sequencing was performed in fresh monocytes and 24h samples. Known cytokine and mRNA patterns for *IL1B*, *IL1RN* and *IL6* were confirmed by RNA-seq (Figure 2B-D). Principal component analysis shows moderate effects of uric acid in its capacity to segregate the samples (approximately 8% of variance explained by PC2) and genes that contribute to PC2 were extracted for further pathway enrichment analysis. Pathway enrichment analysis was performed using the KEGG pathway database starting from significantly regulated genes (full list depicted in Table S1) and top 100 contributors to PC2 in Figure 2E. GO terms associated to up-regulated and down-regulated genes are depicted in Figure S2. Of note EIF4EBP3 is up-regulated in uric acid treated samples, suggesting implications of mTOR pathway in uric acid effects (Figure 2E, Figure S2 and Table S2).

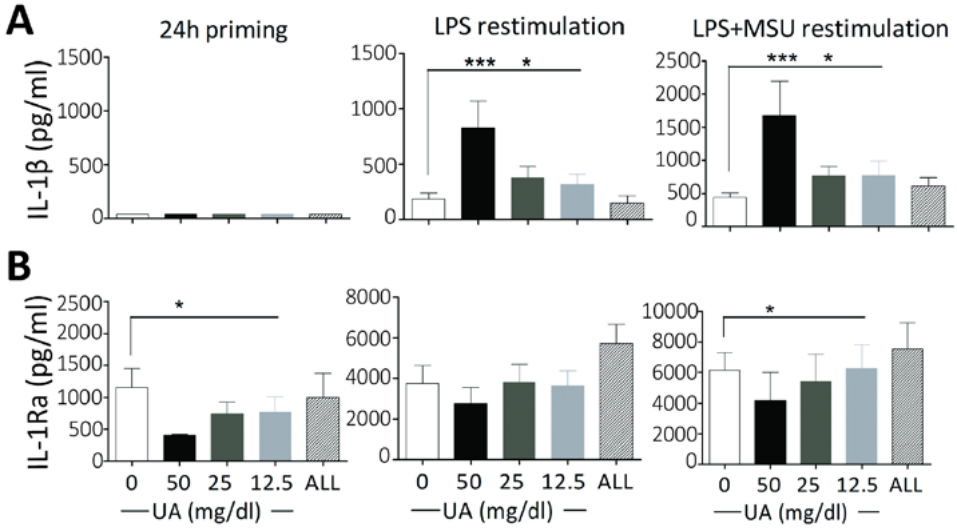

FIGURE 1. Enhanced IL-1β and diminished IL-1Ra production in uric acid primed monocytes.

Monocytes were freshly isolated by differential centrifugation and magnetic beads negative selection and were stimulated in vitro with increasing concentrations of uric acid solubilized in RPMI supplemented with 10% human serum. Monocytes were exposed to increasing concentrations of uric acid (UA), allantoin 50 mg/dl(ALL) or medium alone for 24h (24h priming). Afterwards, the medium was refreshed and the cells were stimulated with LPS 10 ng/ml or LPS+MSU crystals 300 µg/ml for another 24h. IL-1β (panel A) and IL-1Ra (panel B) cytokine production in the supernatant of cells was measured by ELISA after 24h priming and after another 24h stimulation. Data are shown as means +/- SEM of at least 6 donors from 3 independent experiments, data was analysed using Friedman test, *p<.05, ***p<.001

The analysis shows enrichment in pathways involving innate immunity, cytokine and chemokine interactions, Toll-like receptor signaling as well as pathways that could indicate mechanistic leads in uric acid induced proinflammatory status. Focusing on possible targets regulating IL-1β and IL-1Ra in response to uric acid, terms such as NFκB were highlighted in both down-regulated and up-regulated gene analysis, indicating that this was a common term associated with the general process and less likely to explain the shift in cytokines. Other pathways that were relevant in the context of uric acid signaling such as FoxO (transcription factors downstream AKT) or lysosomal mediated processes were indicative of intracellular pathways that are involved in modulating IL-1β production and were further explored. A full list of GO terms and significance coefficients as well as genes associated to terms is presented in Table S2 for up-regulated genes.

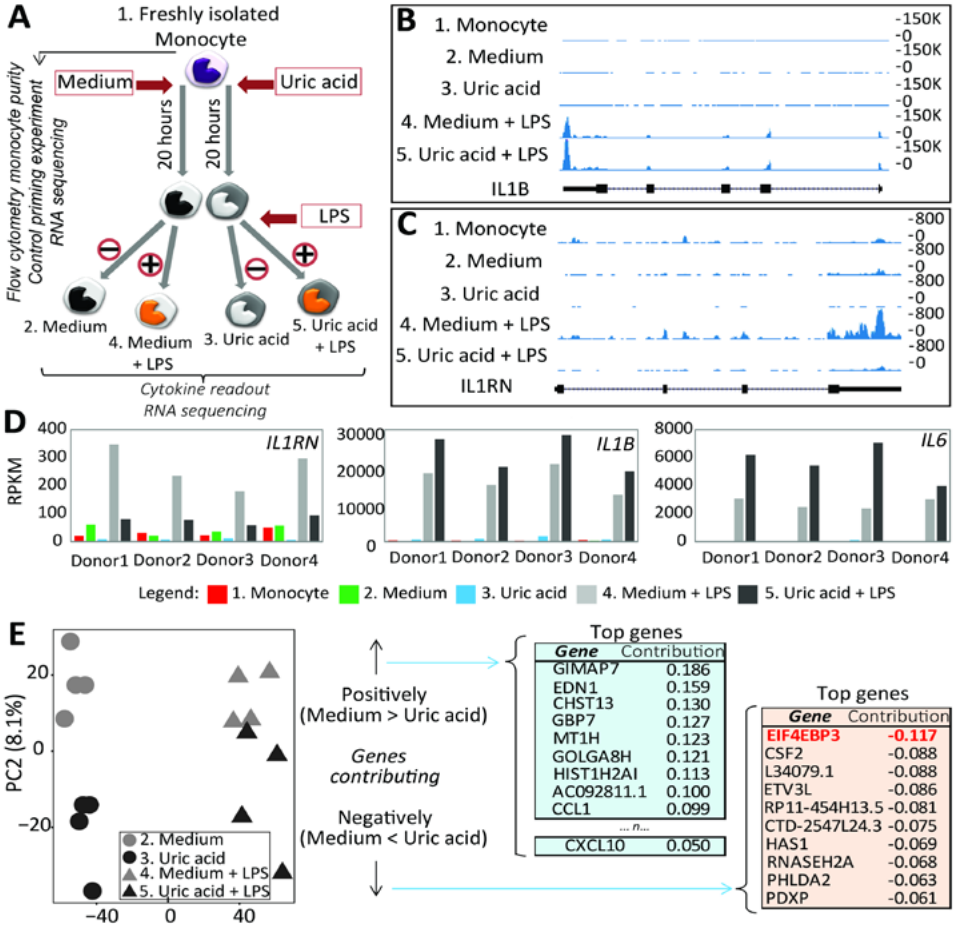

FIGURE 2. Transcriptomic analysis in uric acid primed monocytes.

Monocytes were enriched using sequential steps of Ficoll density gradient centrifugation, Percol density gradient centrifugation, and negative selection using magnetic beads. Cells were exposed to medium or uric acid 50 mg/dl for 20h followed by stimulation with medium or LPS 10 ng/ml for another 4h and RNA sequencing was performed in fresh monocytes (sample 1) and 24h samples (samples 2-5) (panel A). Example UCSC genome browser screenshot of a single donor at IL1B (panel B) and IL1RN (panel C) gene tracks showing higher IL1B peaks and lower IL1RN peaks in uric acid samples versus control (sample 3 versus sample 2, or samples 5 versus sample 4). Plots depicting RPKM (reads per kilobase per million mapped reads) values (from left to right) for IL1RN, IL1B, and IL6 genes (panel D) showing that normalized read counts are lower for IL1RN and higher for IL1B and IL6 in uric acid treated samples compared to controls. Principal component analysis of samples at day 1 shows segregation of control samples from uric acid treated samples; genes that contribute to PC2 were extracted for further pathway enrichment analysis (positive contribution refers to genes that are up-regulated in medium control compared to uric acid, thus down-regulated in uric acid samples, and vice versa for negatively contributing genes) (panel E).

AKT is phosphorylated in uric acid primed monocytes in the absence of ROS induction

To describe the intracellular signaling related to uric acid priming, we sought to validate targets revealed by the transcriptomic analysis. The involvement of NFκB in uric acid priming effects was assessed by Western blot of phosphorylated p65. Uric acid 50 mg/dl was unable to modify phosphorylated p65 levels after 30 min, 1 hour or 2 hours (Figure S3. This suggests that NFκB is associated with transcription of genes involved in the general processes of the inflammatory response, explaining its appearance as a common hit in transcriptomic data (Figure S2), but not likely directly activated and mediating the IL-1β-shifted production pattern.

Additional targets indicated by the transcriptomic analysis were the terms mTOR (up-regulated) and FoxO (down-regulated), both downstream of AKT. AKT has been associated previously with effects of uric acid, such as oxidative stress and AKT inhibition which could be an explanation for reduced IL-1Ra (26). Uric acid has also been linked to AMPK inhibition through ROS induction, and this effect was described to induce mTOR (27). We have investigated these pathways in human monocytes. The dependence of uric acid effects on ROS production has been studied by measurement of ROS using a Luminol based assay and in PBMCs of a patient with chronic granulomatous disease (CGD). Uric acid co-stimulation diminished ROS production as measured by 1h kinetics in response to zymosan or phorbol-12-mystrate-13-acetate (PMA) stimulation (receptor-dependent and receptor-independent ROS induction) (Figure 3A-B). CGD patients have NADPH oxidase mutations leading to inability to produce cytosolic ROS. In order to determine whether ROS are required for uric acid modulation of cytokines, the priming setup was applied on PBMCs isolated from a CGD patient and one healthy subject. Uric acid could still prime production of IL-1β (Figure 3C) and reduction of IL-1Ra (Figure S4), indicating that these effects do not require cytosolic ROS production.

Phosphorylation of AMPK and AKT was assessed by Western blot in cells treated with inducers of AMPK (Metformin or AICAR) or AKT (β-glucan or insulin), respectively (Figure 3D-E). AMPK was not modified by uric acid treatment. In contrast, uric acid priming induced phosphorylated AKT in the monocytes (Figure 3E). This effect of AKT induction was observed in a time dependent manner and was reversible using phosphatidyl-inositol 3 kinase (PI3K) inhibitor wortmannin (Figure 3F and S5).

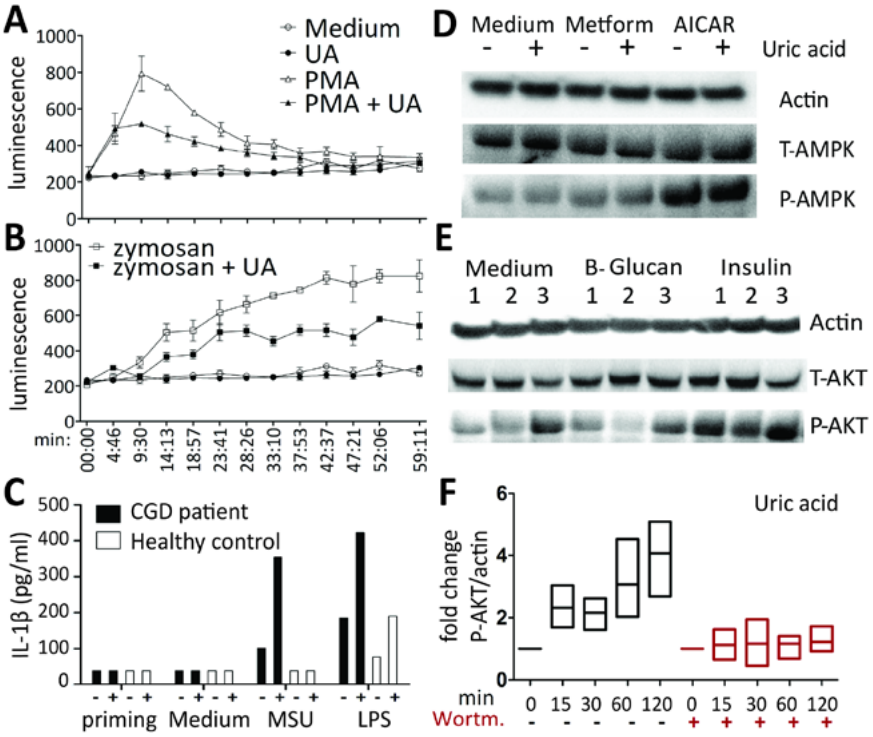

FIGURE 3. Phosphorylation of AKT and lack of ROS or AMPK induction by uric acid.

Percol enriched monocytes (10^5/well) were stimulated with Zymosan 50µg/ml (panel A) or PMA 250 ng/ml (panel B) in order to induce receptor dependent or receptor independent ROS production, in the presence or absence of uric acid 50 mg/dl. Kinetics of ROS production was documented for 1 hour and is representative of 2 independent experiments using 5 donors. PBMCs of CGD patient and control were isolated and 0.5 x 10^6 cells were stimulated per well with MSU 300 µg/ml or LPS 10 ng/ml after priming with medium or uric acid and IL-1β levels were measured (Panel C). 10^6 Monocytes were pre-stimulated with medium or AMPK inducers such as Metformin 30 mM or AICAR 0.5 mM followed by 2 hours in the presence or absence of uric acid 50 mg/dl(panel D). Monocytes were pre-stimulated with medium or AKT inducers such as β-glucan 5 µg/ml or insulin 100 nM followed by 2 hours in the presence or absence of wortmannin 100 nM (AKT inhibitor) or uric acid 50 mg/dl (panel E). 10^6 monocytes were treated with uric acid 50 mg/dl for increasing durations in the presence or absence of wortmannin 100 nM in 4 donors (panel F).

AKT-PRAS40 transduces effects to autophagy inhibition, which in turn recapitulates uric acid-induced cytokine pattern

To further determine which signaling pathway is important for uric acid inflammatory effects, phospho-kinase activity was scanned in monocytes using human proteome profiler – phospho-kinase array (R&D) and percent change of spotted proteins was calculated. Consistently throughout the 3 experiments performing this assay, PRAS40 (proline rich AKT substrate 40 kDa) was identified as being phosphorylated by uric acid (Figure S6). This was further validated by western blot in a similar experimental setup (Figure 4A).

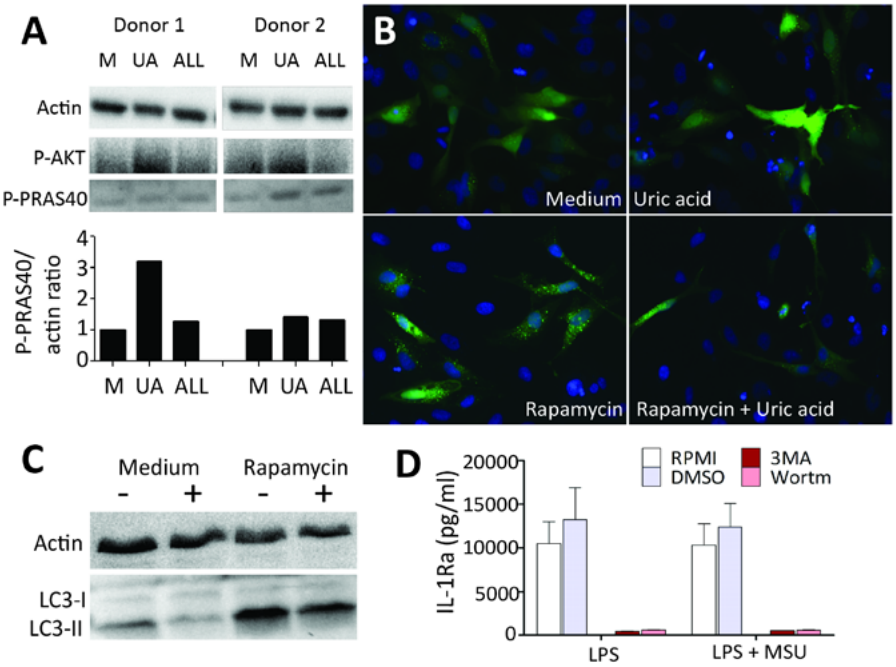

Figure 4. Identification of PRAS40 as intermediate in AKT signal transduction to inhibit autophagy in uric acid primed cells.

Negatively selected monocytes were primed with or without uric acid (UA) or allantoin (ALL) for 24h, followed by 7 minutes short exposure to high concentrations of LPS 1µg/ml. Cells were lysed and PRAS40 was identified through western blot (panel A). The effects downstream PRAS40 and mTOR activation on autophagy were examined by means of fluorescence microscopy in HeLa cells overexpressing LC3 coupled with GFP (panel B) and by western blotting for LC3-I and LC3-II in HeLa cells (panel C). Fresh PBMCs were stimulated with LPS 10 ng/ml or LPS+MSU 300 µg/ml in the presence or absence of autophagy inhibitors 3MA 10 mM and wortmannin 100 nM (panel D).

It is known that PRAS40 phosphorylation has an inhibitory effect on regulatory-associated protein of mTOR (raptor). Raptor dissociates from mTOR complex 1 and induces mTOR (28), which in turn has inhibitory effects on autophagy. The effects of uric acid on autophagy were investigated using complementary assays in HeLa cells. HeLa cells overexpressing LC3-GFP were stimulated with rapamycin in the presence or absence of uric acid. Green fluorescent punctae were microscopically assessed to determine autophagy activity (Figure 4B). Higher numbers of punctae were visible after positive autophagy control rapamycin, which was inhibited by uric acid (Figure 4B). Western blot assessment of endogenous production of LC3 autophagy marker in response to the same treatments depicted similar inhibitory effects of uric acid on LC3-II levels (lower band, migrates faster) compared to LC3-I (migrates slower than the lipidated LC3-II) (Figure 4C). To observe whether uric acid inhibition could explain the uric acid-specific cytokine pattern involving induction of IL-1β and repression of IL-1Ra, pharmacological inhibitors of autophagy were used to recapitulate uric acid effects.

Inhibition of autophagy by PI3K inhibitors 3MA or wortmannin, is known to significantly enhance IL-1β as previously demonstrated (29, 30), whereas IL-1Ra was reduced (Figure 4D), enforcing that this signaling pathway is likely mediating the effects on cytokine production by uric acid priming.

Uricase inhibition in mice potentiates joint inflammation in an in vivo model of gout

To further explore the effects of high uric acid levels on inflammation in gout, an *in vivo* model of hyperuricaemia in mice was used. Mice were administered exogenous uric acid in addition to oxonic acid (uricase inhibitor) to promote hyperuricemia, and were given i.a. injections with MSU crystals and palmitic acid (C16) to induce gout. Inflammation was significantly enhanced in the oxonic acid group compared to controls as observed by macroscopic scoring of joints and histology (Figure 5).

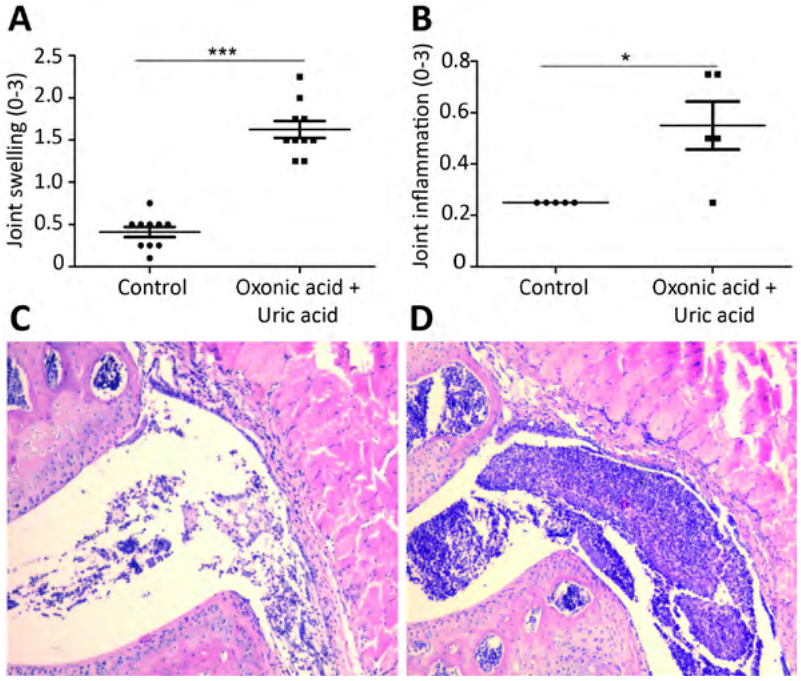

FIGURE 5. Uricase inhibition in mice exacerbates joint inflammation in a model of acute gout arthritis.

Macroscopic (Panel A) and microscopic (Panel B) scores of the knees in mice treated with Vehicle control or Oxonic acid + Uric acid following intraarticular injection of MSU+C16. Histology (H&E staining) of joints treated with MSU+C16 in control (panel C) and oxonic acid (panel D) mice. (A) 10 knees/group, Mann-Whitney, ***p<0.001, (B) 5 knees/group, Mann-Whitney, ***p<0.05

DISCUSSION

In the current study we investigated the mechanisms through which uric acid primes human monocytes. The previous findings that higher concentrations of uric acid promote IL-1 production and inhibit IL-1Ra synthesis were confirmed. This uric acid effect is unique because it shifts the IL-1/IL-1Ra balance to a proinflammatory phenotype by strong reduction of IL-1Ra through a yet unclear mechanism. Very high concentrations of uric acid have been used in this setup and previously (24) in order to obtain the maximum effect and allow in vitro manipulation. While we cannot exclude that uric acid microcrystals that were undetectable by polarized light microscopy are also involved in this effect, we see a clearly distinct pattern of cytokines induced by soluble uric acid compared to MSU crystals (which in turn induce both IL-1β and IL-1RA (Figure 1)).

We generated transcriptomic data through RNA-sequencing (RNA-seq) in highly pure human monocytes after 24h treatment with medium or uric acid. LPS stimulation for 4 hours was used to boost the potential differences observed between medium and uric acid exposure. As summarized in Figure 2 B-D, the expected effects could be demonstrated in RNA-seq samples: *IL1B* and *IL6* RNA levels were higher in uric acid compared to medium control after 24 hours; *IL1RN* RNA levels were lower in uric acid compared to medium control after 24 hours; and these differences were amplified by LPS stimulation. This was in line with cytokine data (Figure 1) showing that uric acid effects are not visible unless cells are challenged with a pattern recognition receptor ligand, such as LPS. The PCA analysis (Figure 2E) revealed a moderate effect of uric acid in segregating the 24 hours samples, most of the variance being attributable to LPS. This effect is also not surprising, as LPS determines a strong transcriptional program, whereas uric acid had a less dramatic but highly consistent effect.

Pathway enrichment analysis was performed using the KEGG database starting fromgenes that varied significantly in uric acid compared to medium control at 24 hours (Table S1), combined with top genes that made a contribution to principal component 2(Figure S2). The analysis revealed the enrichment of several pathways (Table S2), among which those providing mechanistic links for modulating cytokine production (NFκB, mTOR, FoxO, lysosome) were further investigated. Uric acid was previously found to induce NFκB and to promote cell death in pancreatic β cells (31), but we were unable to detect changes in phosphorylation of NFκB component p65 in monocytes treated with uric acid (Figure S3), indicating that NFκB is likely a common term associated to the cytokine genes regulated by uric acid, and not reflecting its mechanism of action.

We further investigated the AKT pathway which was indicated by RNA-seq data showing up-regulated mTOR and down-regulated FoxO transcription factors. AKT has also been studied in relation to uric acid effects in a model of potassium oxonate induced hyperuricemia in mice where AKT phosphorylation was inhibited and this diminished

insulin sensitivity in cardiomyocytes (32) and in liver cells (26) through a ROS dependent pathway. In our experimental setup, uric acid potently inhibited reactive oxygen species production (Figure 3A-B) and could still prime the cells for high IL-1β and low IL-1Ra in cells of CGD patient (Figure 3C, S4). These findings exclude a NADPH oxidase dependent mechanism for the observed findings and demonstrate an anti-oxidant role of uric acid in human primary monocytes. This finding adds information to existing evidence showing the dual role of uric acid in oxidative stress(20). In line with our findings, a study investigating the expression of NFkB p65 and NADPH oxidase $p47^{phox}$ in brachial artery endothelial cells found no correlation with serum uric acid levels (33). Moreover, AKT was induced by uric acid (Figure 3E-F) making AKT an important player in uric acid signal transduction. Whereas the induction of ROS in other studies also led to AMPK inhibition (27, 34), our assessment did not reveal changes in AMPK phosphorylation in monocytes (Figure3D), in line with the observed antioxidant effect of uric acid in our experimental setup.

Finally, we discovered that AKT induction is followed by downstream PRAS40 phosphorylation which couples this signaling pathway to decreased LC3-II autophagosome formation in uric acid treated HeLa cells (Figure 4). Autophagy is a conserved housekeeping lysosomal process involving LC3 and its phosphatidyl ethanolamine lipidated form LC3-II, that has the role of degrading long-lived intracellular cargo but is also closely linked to inflammation (35). A newly characterized non-canonical pathway termed LC3-associated phagocytosis (LAP) is still difficult to dissociate from classical autophagy (36). Autophagy defects have largely been inflicted in human inflammatory diseases (37) due to processes such as inflammasome activation (38), lack of pro-IL-1β (39) degradation or enhanced transcription of IL-1β (29). A discordance in IL-1β and IL-1Ra cytokines was described in chronic granulomatous disease where patients are deficient in autophagy due to inability to produce ROS (40). This deficiency in autophagy led to IL-1 mediated inflammation that could be rescued by IL-1Ra administration (40). Moreover, pharmacological inhibition of autophagy in human cells also showed downregulation of IL-1Ra (Figure 4D). .This shows that autophagy inhibition is an important mechanism that can explain the high levels of IL-1β observed in uric acid primed monocytes while uniquely discoupling IL-1 from the natural antagonist IL-1Ra. The further characterization and validation of these findings in humans *in vivo* is of high relevance for the regulatory mechanisms of gout. On the one hand, autophagy deficiency can lead to proinflammatory signals that increase the vulnerability of patients to stimuli that precipitate gout. On the other hand, boosting autophagy, as well as restoring IL-1Ra levels can limit the degree of inflammation or facilitate the resolution of attacks.

Nevertheless, the effects on cytokine production are likely to be influenced by other pathways in addition to AKT, as revealed by other kinases such as ERK which was phosphorylated upon uric acid treatment (Figure S5). This diversity of uric acid induced pathways illustrates that uric acid can impact on many different biological processes and affect different phenotypes

of the cell reflecting a large group of diseases for which hyperuricemia and DAMP mediated responses are of relevance.

In conclusion, our data show that uric acid has complex effects in monocytes. Firstly, it induced the phosphorylation of AKT in line with the effects of uric acid on phosphorylation of PRAS40, which is an mTOR inhibitory molecule downstream AKT. Subsequently, activation of the AKT-PRAS40 pathway led to a decrease in LC3-II formation and less fluorescent green punctae on GFP-LC3 overexpressing HeLa cells. This coincides with diminished lysosomal mediated pathways such as autophagy or non-canonical LC3 associated phagocytosis (LAP). Finally, autophagy blockade is known to induce higher IL-1β production and discordance between IL-1β and IL-1Ra, whereas normal autophagy activity has been associated with basal inhibition of inflammation. Moreover, blocking of uricase and exogenous administration of uric acid in mice determined a higher inflammatory reaction to intra-articular injection of MSU and C16, providing proof of concept that high uric acid levels can increase inflammatory responses in acute gout models *in vivo*.

Pathways with importance in common metabolic disorders and innate immunity implications have been highlighted by this approach and are warranted investigation in subsequent studies. The novel link between high uric acid exposure and autophagy is likely to be an important new susceptibility factor in conditions associating hyperuricemia and the validity and utility of this finding are to be assessed in future patient studies.

MATERIALS AND METHODS

Volunteers

The study was approved by the Arnhem-Nijmegen ethical review board. All human experiments were conducted according to the Declaration of Helsinki. Informed written consent of each human subject was provided.

PBMC and monocyte isolation

PBMCs were separated using Ficoll-Paque (Pharmacia Biotech) and suspended in RPMI (Roswell Park Memorial Institute 1640). Monocytes were enriched using hyperosmotic Percol solution followed by negative selection using the Pan Monocyte Isolation kit (Miltenyi Biotec) and efficacy of selection was verified by flow cytometry.

Stimulation experiments

Experiments were performed in culture medium containing RPMI, supplemented with 10% human pooled serum. Cells were primed for 24 hours with medium, uric acid or allantoin, cells were washed with warm PBS (phosphate buffered saline) and remaining adherent cells

were stimulated as described. For RNA-seq experiments, 0.5x10^6 monocytes were seeded per well in 6-well plates (Corning) and were primed for 20 hours followed by addition of medium or LPS 10 ng/ml for another 4 h.

Cytokine measurements

Cytokines were measured using ELISA kits for interleukin-1β (IL-1β) and IL-1Ra (receptor antagonist) (R&D Systems).

Western blot

Western blotting was performed using a Trans Turbo Blot system (Bio-Rad) according to the manufacturer's instructions. Protein was loaded and separated on SDS-PAGE using 4–15% gradient precast gels and transfered to PVDF (polyvinylidene fluoride) membranes using the semi-dry method (Bio-Rad). Proteome profiler – human phospho-kinase array kit (R&D Systems) was used to assess phospho-kinase activity upon uric acid treatment in monocytes.

Reactive oxygen species measurement

ROS formation was measured by a chemiluminescence assay using luminol over 1 hour exposure of cells to stimuli.

Fluorescence microscopy

HeLa cells overexpressing GFP-LC3 were obtained as previously described (30) Green fluorescent punctae were observed to assess autophagy activity after uric acid stimulation.

RNA sequencing preparation and analysis

Preparation of RNA for sequencing was performed as described in (41). Total RNA was extracted from cells using the Qiagen RNeasy RNA extraction kit (Qiagen, Netherlands). Ribosomal RNA was removed using the riboZero rRNA removal kit (Illumina), RNA was fragmented to 200bp fragments, followed by first and second strand cDNA synthesis. Library preparation was performed using the KAPA hyperprep kit (KAPA Biosystems). RNA-seq reads were aligned to the hg19 reference genome using bwa (42). Dynamic genes were identified using DESEQ and were used for Principal Component Analysis (PCA). Genes contributing to Uric acid diferences were extracted using the package prcomp and were used for Gene ontology analysis.

Mouse model

Gout arthritis was induced in mice through intra-articular injection of MSU and palmitate (C16) as previously described(43, 44). Hyperuricemia was induced in mice through uricase inhibition with oxonic acid (45, 46).

Data availability

A detailed version of this section is described in the Supporting Information of this manuscript. Data used for this manuscript will be made available to readers upon request.

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Competing interests

The authors declare no competing interests.

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SUPPORTING INFORMATION

Materials and methods

Volunteers

Venous blood was drawn from the cubital vein of healthy volunteers or patient with chronic granulomatous disease (CGD) into 10-ml EDTA tubes. Experiments requiring large amounts of cells were performed using cells isolated from buffy coats after overnight storage at room temperature (Sanquin blood bank, Nijmegen, The Netherlands). All human experiments were conducted according to the principles of the Declaration of Helsinki. Informed written consent of each human subject was provided before drawing blood. The study was approved by the Arnhem-Nijmegen ethical review board.

Reagents

Uric acid, lipopolysaccharide (LPS, *E. coli* serotype 055:B5), allantoin, 3-methyl-adenine (3MA), AICAR, PMA (phorbol-12-mystrate-13-acetate, Sigma) and zymosan were purchased from Sigma. LPS was subjected to ultra-purification before cell culture experiments. β-glucan (from *Candida albicans*) was kindly provided by D. Williams (East Tennessee State University). Other reagents were: wortmannin (Cayla Invivogen), metformin (RnD), rapamycin (LC-laboratories), insulin (Novorapid). Monosodium urate (MSU) crystals were prepared in house as previously described (43).

PBMC and monocyte isolation

PBMCs were separated using Ficoll-Paque (Pharmacia Biotech) and suspended in culture medium RPMI (Roswell Park Memorial Institute 1640). Monocytes were enriched using hyperosmotic Percol solution (47) and were subsequently purified by negative selection using the Pan Monocyte Isolation kit (Miltenyi Biotec) according to the manufacturer's instructions.

Flow cytometry

Purity of cells obtained through MACS isolation was assessed by 30 minutes staining on ice in the dark of $1x10^5$ freshly isolated cells with CD14-FITC, CD56-PE, CD19-ECD, CD3-PC5, CD45-PC7, followed by 1 washing step with 1% BSA. Fluorescence was measured using Cytomics FC500 (Beckman Coulter).

Stimulation experiments

Experiments were performed in culture medium containing RPMI, supplemented with 50 µg/ml gentamicin, 2 mM L-glutamine, 1 mM pyruvate and 10% human pooled serum.

Cells were incubated for 24 hours at 37 °C in 5% CO2 with culture medium, uric acid or allantoin (priming). Uric acid or allantoin were sollubilised in culture medium by vortexing and heating followind by filter sterilization using 0.2 µm filters. After priming, culture medium was removed, cells were washed with warm PBS (phosphate buffered saline) and remaining adherent cells were stimulated with medium or LPS with or without MSU crystals. Supernatants were checked for presence of urate microcrystals using polarized light microscopy. Experiments were performed using 0.5 x 10^6 PBMCs/well or 0.1 x 10^6 monocytes/well in 96 flat well plates (Corning) in a volume of 200 µl. Experiments were performed in duplicate and replicates were pooled at the end of the experiments. Samples were stored frozen at -20°C until measured. In experiments where pharmacological inhibitors were used, priming was performed after a variable pre-incubation period as indicated. For RNA-seq experiments, 0.5x10^6 monocytes were seeded per well in 6-well plates (Corning) and were primed for 20 hours followed by addition of medium or LPS 10 ng/ml for another 4 h. Cells from all donors used for RNA-seq were used in control experiments in 96-well plates to assess cytokine production and all donors showed an efficient uric acid priming phenotype (Figure 1). Cytokines were also measured in the wells of cells used for RNA-seq (Supporting Information Figure S1, H-I). Stimulation of HeLa cells for autophagy assessment experiments was performed using culture medium RPMI, 10%FCS (fetal calf serum) supplemented with penicillin/streptomycin combination instead of gentamycin, in the presence of ammonium chloride (NH4Cl) inhibitor of lysososomal fusion.

Cytokine measurements

Cytokine concentrations were determined in supernatants of cell culture using specific sandwich ELISA kits for interleukin-1β (IL-1β) and IL-1Ra (receptor antagonist) (R&D Systems) according to the manufacturer's instructions. The differences between groups were compared using paired or non-paired nonparametric statistical tests as appropriate according to experimental design, at α 0.05.

Western blot

Western blotting was performed using a Trans Turbo Blot system (Bio-Rad) according to the manufacturer's instructions. 0.5 × 10^6 monocytes or HeLa cells were stimulated and lysed in 50-100 µL lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 40 mM β-glycerophosphate, 50 mM sodium fluoride, 200 µM sodium orthovanadate, complete mini EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche)). The cell homogenate was frozen at -80°C. When needed, samples were thawed and centrifuged shortly at 14000 rpm, and the supernatant was taken for western blotting. Protein was loaded and separated on SDS-

PAGE using 4–15% gradient precast polyacrylamide gels (Bio-Rad). Separated proteins were transferred to ethanol-activated PVDF (polyvinylidene fluoride) membranes using the semi-dry method (Bio-Rad). The membrane was blocked with 5% (w/v) milk powder or bovine serum albumin (BSA) in Tris-buffered saline/Tween 20 (TBST) for 1 hour at room temperature followed by incubation over night at 4°C with the primary antibody 1:1000 in 5% (w/v) milk powder or BSA /TBST. After overnight incubation the blots were washed three times with TBST and incubated with HRP-conjugated anti-rabbit or anti-mouse antibody at a dilution of 1:5000 in 5% (w/v) milk powder in TBST for 1 hour at room temperature. After washing three times with TBST the blots were developed using ECL (Bio-Rad) or using the Super Signal® West Femto Maximum Sensitivity Substrate (Thermo Scientific) according to the manufacturer's instructions. Quantitative assessment of band intensity was performed by Image Lab software (Bio-Rad, CA, USA). Primary antibodies were used to detect: total and phosphorylated p65 Ser536 (Cell Signaling), phosphorylated AKT Ser473 (Cell Signaling), total and phosphorylated AMPK Thr172 (Cell Signaling), phosphorylated PRAS-40 Thr26 (R&D Systems), LC3B (Novus Biologicals), actin (Sigma). Proteome profiler – human phospho-kinase array kit (R&D Systems) was used to assess phospho-kinase activity upon uric acid treatment in monocytes. 0.5x10^6 negatively selected monocytes were primed with medium or uric acid for 24h and subjected to short high dose LPS stimulation for 7 min at 1µg/ml. Cells were immediately lysed and subjected to incubation with membrane according to manufacturer's instructions. Data was analysed using Image Lab software after correction of intensity for background and control spots on each of the membranes.

Reactive oxygen species measurement

PBMCs were suspended in RPMI and exposed to uric acid in the presence or absence of ROS inducers. ROS formation was measured by a chemiluminescence assay using luminol (5 mM, 5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) over 1 hour exposure of cells to stimuli.

Fluorescence microscopy

HeLa cells overexpressing GFP-LC3 were obtained as previously described(30). 5x10^4 cells were seeded on coverslips in 24 well plates and were adhered for 24 hours in medium free of antibiotics. Cells were transfected using FuGENE HD transfection reagent and 0.5 µg plasmid DNA per well and were rested for another 24 h. Stimulation was performed with medium or uric acid 50 mg/dl for 1 hour followed by addition of medium or rapamycin 50 µM for 4 hours. Green fluorescent punctae were observed using fluorescence microscopy to assess autophagy activity after stimulation.

RNA sequencing preparation and analysis

Preparation of RNA for sequencing was performed as described in (41). Total RNA was extracted from cells using the Qiagen RNeasy RNA extraction kit (Qiagen, Netherlands), with on-column DNAseI treatment. Ribosomal RNA was removed using the riboZero rRNA removal kit (Illumina), RNA was fragmented to 200bp fragments, followed by first and second strand cDNA synthesis. Library preparation was performed using the KAPA hyperprep kit (KAPA Biosystems). For visualization on the UCSC Genome Browser, RNA-seq reads were aligned to the hg19 reference genome using bwa (42). To infer gene expression levels, RNA-seq reads were aligned to the Ensembl v68 human transcriptome using Bowtie. Dynamic genes were identified using DESEQ, with pairwise comparisons between each of the 5 treatments shown in Figure 2A (i.e. 1 vs 2, 2 vs 4, etc), at cut-offs of p value <0.05, log(47) fold change >=1 and RPKM >=1. Dynamic genes were used for Principal Component Analysis (PCA) shown in Figure 2E, and top genes contributing to differences between medium and Uric acid samples were extracted, using the package prcomp.

Gene ontology analysis

Pathway enrichment analysis was performed using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database and Biological Processes database by means of Cytoscape application version 3.4.0 and ClueGO plug in. Genes that were significantly up- or down-regulated were combined with genes contributing to principal component that differentiates samples based on uric acid treatment. Terms enriched were graphically depicted based on GO term p-values and number of genes associated.

Animal model

Male C57Bl/6J mice at 10-12 weeks of age were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). Uricase was inhibited using oxonic acid and uric acid was administred in order to increase serum uric acid levels in mice according to previously described protocol (45, 46). Briefly, mice were given oxonic acid orally 140 mg/kg, 2 times/day combined with uric acid 4 mg/kg, 2 times/day intra-peritoneally. Joint inflammation was induced by intra-articular injection (i.a.) of 300 µg MSU cruystals and 200 µM palmitic acid (C16) in a volume of 10 µl phosphate buffered saline (PBS) as previously described (43, 44). 24h after injection, mice were sacrificed, knees were macroscopically scored for thickness of joint after removal of skin (scores ranging from 0 to 3), followed by harvesting of joints for histology. Histology was performed as previously described (43).

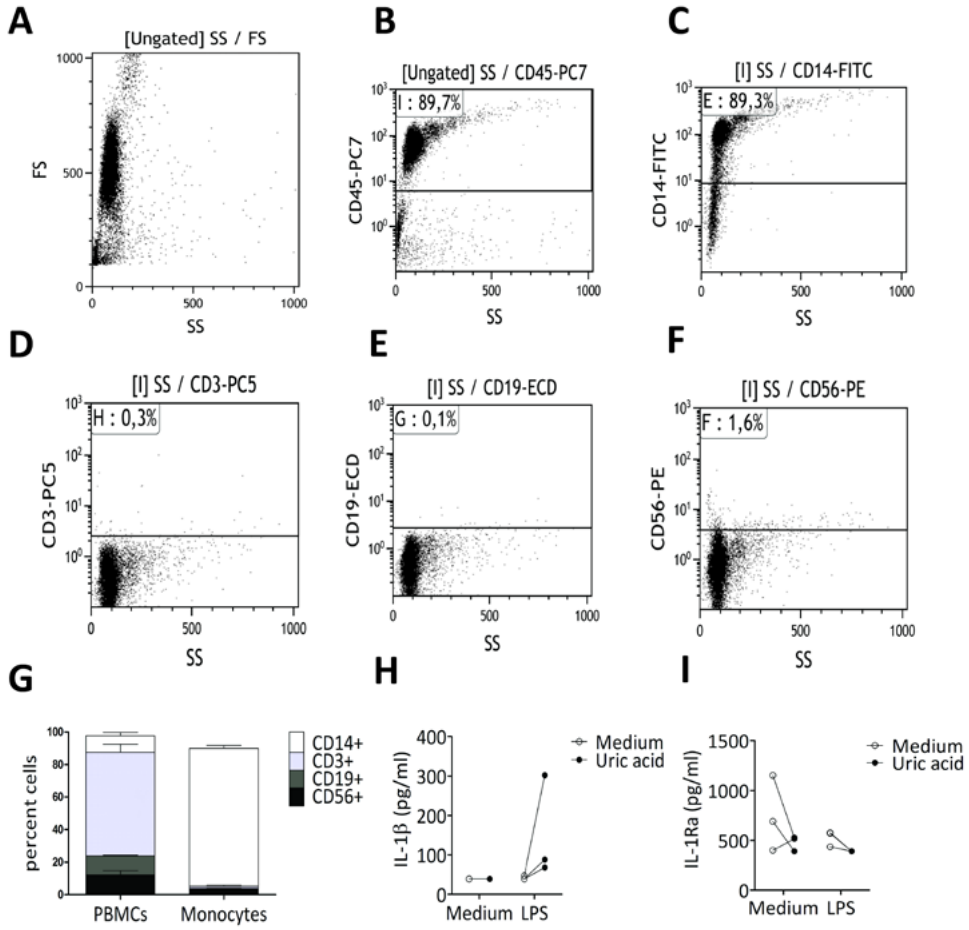


FIGURE S1. Flow cytometry and cytokine control data for negatively selected monocytes.

Representative plots depicting cell populations present in the cell suspensions after negative selection of monocytes (panels A-B) and gating on CD45+ cells (panels C-F). Means of cell fractions in all 4 donors used for RNA-seq (panel G). Cytokines were measured as control in the wells of cells used for RNA seq in 3 donors (0.5x10^6 in 6-well plate).

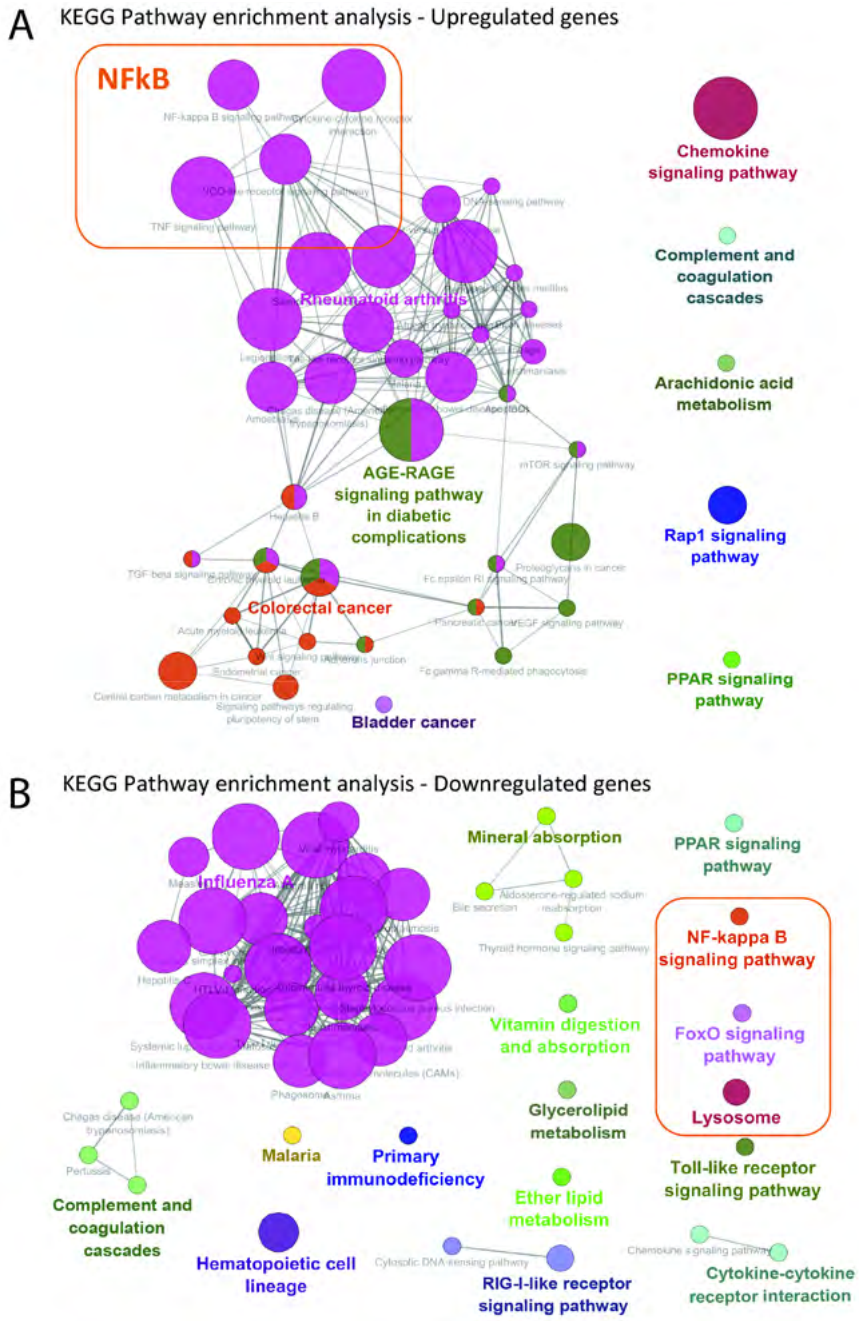


FIGURE S2. Pathway enrichment analysis was performed using significantly regulated genes and top 100 contributors to PC2 in panel D. GO terms associated to up-regulated (panel A) and down-regulated (panel B) genes according to the KEGG pathway database are depicted.

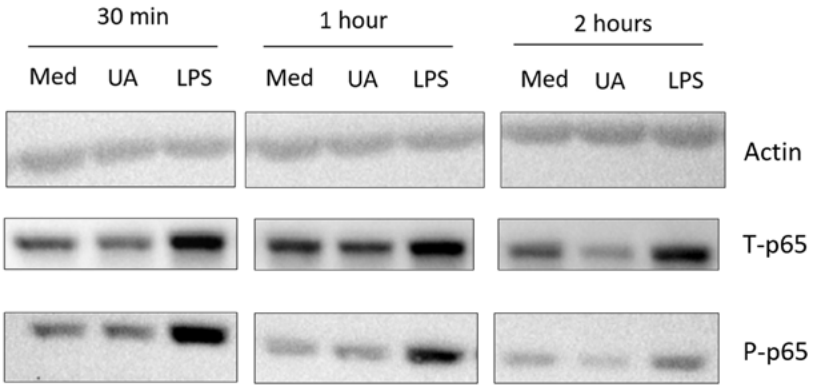


FIGURE S3. Western blot of p65 component of NF-κB in uric acid priming.

PBMCs were freshly isolated and 10^6 cells were stimulated for 30 min, 1 hour and 2 hours with medium (10% serum RPMI), uric acid 50 mg/dl or LPS 10 ng/ml. Samples were lysed in 100 µl lysis buffer and subjected to SDS-PAGE and semi-dry blotting. Results are representative for 3 donors.

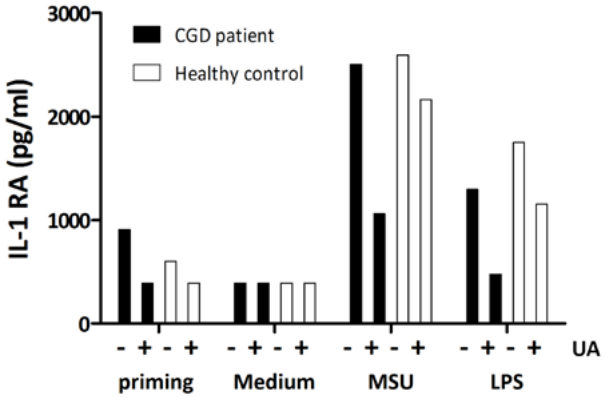


FIGURE S4. PBMCs of CGD patient and control were isolated and 0.5 x 10^6 cells were stimulated per well with MSU 300 µg/ml or LPS 10 ng/ml after priming with medium or uric acid and IL-1RA levels were measured by ELISA.

Uric acid		P-AKT/Actin			
Wortmannin	**Min**	Donor 1	Donor 2	Donor 3	Donor 4
-	**0**	1,00	1	1,00	1,00
-	**15**	1,97	1,69	3,04	2,58
-	**30**	2,62	1,95	1,61	2,47
-	**60**	2,25	2,03	3,44	4,54
-	**120**	3,44	2,68	5,06	5,10
+	**0**	1,00	1,00	1,00	1,00
+	**15**	1,63	1,51	0,66	0,72
+	**30**	1,94	1,65	0,46	0,62
+	**60**	1,24	1,34	1,41	0,69
+	**120**	1,28	1,72	0,92	0,99

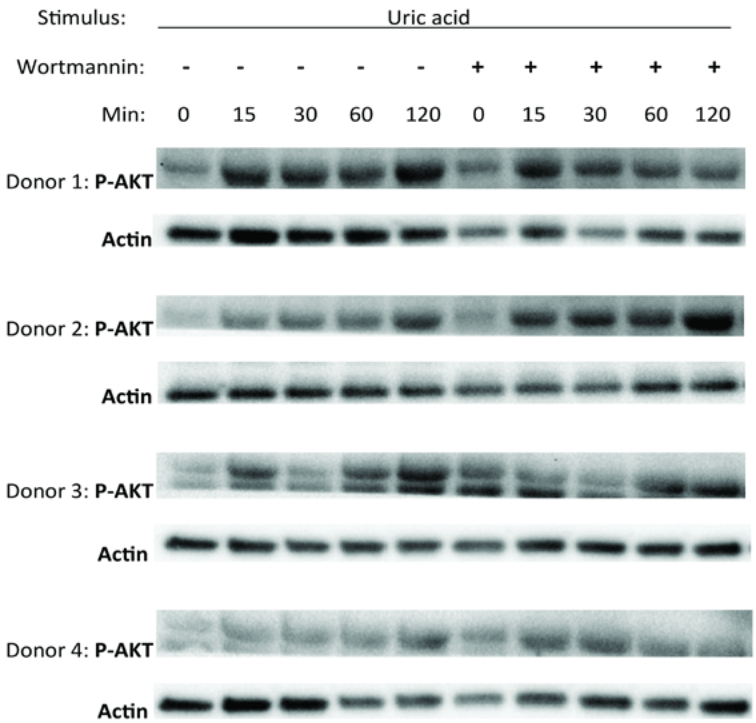
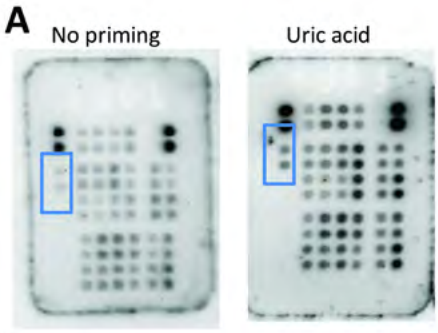


FIGURE S5. 10^6 monocytes were treated with uric acid 50 mg/dl for increasing durations in the presence or absence of wortmannin 100 nM in 4 donors and results were quantified based on pixel density and compared to actin loading control. Corrected ratios are depicted in the table above, followed by western blot in 4 donors.



B

Kinase	Site	%change
Fyn	Y420	-77
HcK	Y411	-76
p27	T198	-73
GSK-3 alpha/beta	S21/S9	42
ERK 1/2	T202/Y204, T185/Y187	54
PRAS40	T246	72

FIGURE S6. Negatively selected monocytes were primed with or without uric acid for 24h, followed by 7 minutes short exposure to high concentrations of LPS 1µg/ml. Cells were lysed and lysates were exposed to phosphokinase array spotted membranes in order to evaluate the downstream effects of AKT phosphorylation. PRAS40 was identified and was consistent in 3 experiments using the phosphokinase array (panel A). Results were quantified using the Image Lab software (panel B).

TABLE S1. Differential expression analysis in uric acid samples compared to control conditions.

ENSEMBL_GENE_ID	Name	Associated_Gene_Name	foldChange	log2FoldChange	pval	padj
ENSG00000196460	hypothetical protein LOC731220	RFX8	7.828883982	2.968806664	9.31E-08	0.000501603
ENSG00000125538	interleukin 1, beta	IL1B	6.964252923	2.799968599	6.24E-05	0.045243847
ENSG00000148737	transcription factor 7-like 2 (T-cell specific, HMG-box)	TCF7L2	5.290478721	2.403398274	2.96E-05	0.027957091
ENSG00000106089	syntaxin 1A (brain)	STX1A	4.860235491	2.281026218	1.28E-05	0.01615319
ENSG00000169429	interleukin 8	IL8	4.251778875	2.088066568	6.51E-06	0.009816684
ENSG00000108688	chemokine (C-C motif) ligand 7	CCL7	3.884742518	1.957818979	5.30E-07	0.001665109
ENSG00000163814	CUB domain containing protein 1	CDCP1	3.8430716	1.942259856	1.17E-06	0.002766214
ENSG00000132965	arachidonate 5-lipoxygenase-activating protein	ALOX5AP	3.091663552	1.628383328	3.30E-06	0.005933258
ENSG00000135318	5'-nucleotidase, ecto (CD73)	NT5E	3.033587692	1.601025016	7.84E-07	0.002275635
ENSG00000185022	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	MAFF	2.840509574	1.506149765	7.21E-05	0.050348895
ENSG00000112715	vascular endothelial growth factor A	VEGFA	2.693852556	1.429670889	5.64E-05	0.042515061
ENSG00000108691	chemokine (C-C motif) ligand 2	CCL2	2.627520301	1.393701911	3.23E-05	0.02972007
ENSG00000074416	monoglyceride lipase	MGLL	0.362661618	-1.463304029	5.43E-05	0.041807142
ENSG00000104763	N-acylsphingosine amidohydrolase (acid ceramidase) 1	ASAH1	0.356794446	-1.486834938	3.65E-05	0.031977329
ENSG00000138642	hect domain and RLD 6	HERC6	0.352987591	-1.502310628	1.02E-05	0.013787693
ENSG00000185432	methyltransferase like 7A	METTL7A	0.318666116	-1.649882471	7.68E-06	0.010726304
ENSG00000019582	CD74 molecule, major histocompatibility complex, class II invariant chain	CD74	0.314002709	-1.67115109	4.49E-05	0.037622658
ENSG00000171115	GTPase, IMAP family member 8	GIMAP8	0.306271203	-1.707118371	2.23E-05	0.023904018
ENSG00000164125	chromosome 4 open reading frame 18	FAM198B	0.305061574	-1.712827628	7.50E-06	0.010726304

TABLE S1. Differential expression analysis in uric acid samples compared to control conditions. *(Continued)*

ENSEMBL_GENE_ID	Name	Associated_Gene_Name	foldChange	log2FoldChange	pval	padj
ENSG00000136634	interleukin 10	IL10	0.299402731	-1.739840716	5.40E-05	0.041807142
ENSG00000145703	IQ motif containing GTPase activating protein 2	IQGAP2	0.292308919	-1.774434246	2.02E-05	0.022398402
ENSG00000196743	GM2 ganglioside activator	GM2A	0.289258816	-1.789567166	2.18E-06	0.004322456
ENSG00000074964	Rho guanine nucleotide exchange factor (GEF) 10-like	ARHGEF10L	0.279086377	-1.841216393	1.17E-06	0.002766214
ENSG00000115896	phospholipase C-like 1	PLCL1	0.27442808	-1.865499987	2.34E-05	0.023904018
ENSG00000158473	CD1d molecule	CD1D	0.270697697	-1.885245479	4.75E-05	0.038941898
ENSG00000014257	acid phosphatase, prostate	ACPP	0.261254237	-1.936473658	3.94E-05	0.033803606
ENSG00000123836	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	PFKFB2	0.261197926	-1.936784651	2.82E-05	0.027432543
ENSG00000105612	deoxyribonuclease II, lysosomal	DNASE2	0.256812983	-1.961209954	5.36E-06	0.008804521
ENSG00000110077	membrane-spanning 4-domains, subfamily A, member 6A	MS4A6A	0.244247792	-2.033582573	1.07E-06	0.002766214
ENSG00000137965	interferon-induced protein 44	IFI44	0.239462685	-2.062127232	2.32E-06	0.004369454
ENSG00000111331	2'-5'-oligoadenylate synthetase 3, 100kDa	OAS3	0.23849616	-2.067962058	1.42E-09	1.79E-05
	ENSG00000242498	C15orf38	0.231719067	-2.109551332	5.87E-05	0.043425999
ENSG00000106066	carboxypeptidase, vitellogenic-like	CPVL	0.230554672	-2.116819196	1.23E-07	0.000580282
ENSG00000185909	kelch domain containing 8B	KLHDC8B	0.226269032	-2.14388895	3.53E-05	0.031745009
ENSG00000137841	phospholipase C, beta 2	PLCB2	0.224149991	-2.157463652	5.64E-08	0.000398307
ENSG00000187554	toll-like receptor 5	TLR5	0.223855507	-2.159360283	2.34E-05	0.023904018
ENSG00000126351	thyroid hormone receptor, alpha (erythroblastic leukemia viral (v-erb-a) oncogene homolog, avian)	THRA	0.217448782	-2.201252467	4.33E-07	0.001484985
ENSG00000112799	lymphocyte antigen 86	LY86	0.209726208	-2.253420939	2.08E-07	0.000782856
ENSG00000111335	2'-5'-oligoadenylate synthetase 2, 69/71kDa	OAS2	0.189865215	-2.396952481	5.34E-05	0.041807142

TABLE S1. Differential expression analysis in uric acid samples compared to control conditions. *(Continued)*

ENSEMBL_GENE_ID	Name	Associated_Gene_Name	foldChange	log2FoldChange	pval	padj
ENSG00000157168	neuregulin 1	NRG1	0.181321359	-2.463379217	1.06E-05	0.013791558
ENSG00000010610	CD4 molecule	CD4	0.179535508	-2.477658891	6.50E-09	6.13E-05
ENSG00000110079	membrane-spanning 4-domains, subfamily A, member 4	MS4A4A	0.177332037	-2.495474897	6.21E-06	0.009764507
	ENSG00000242574	HLA-DMB	0.176548141	-2.501866465	1.78E-06	0.003950855
ENSG00000136689	interleukin 1 receptor antagonist	IL1RN	0.165868925	-2.591884463	1.85E-05	0.021101038
ENSG00000088827	sialic acid binding Ig-like lectin 1, sialoadhesin	SIGLEC1	0.133775145	-2.902118	6.49E-05	0.046181687
ENSG00000095970	triggering receptor expressed on myeloid cells 2	TREM2	0.124222742	-3.008998781	1.66E-07	0.000695424
ENSG00000178199	zinc finger CCCH-type containing 12D	ZC3H12D	0.09259206	-3.432967709	5.37E-06	0.008804521
ENSG00000019991	hepatocyte growth factor (hepapoietin A; scatter factor)	HGF	0.082874408	-3.592929523	7.28E-12	2.75E-07
ENSG00000180767	carbohydrate (chondroitin 4) sulfotransferase 13	CHST13	0.080061474	-3.642748007	1.40E-05	0.01701873
ENSG00000136235	glycoprotein (transmembrane) nmb	GPNMB	0.054288662	-4.203205255	1.31E-10	2.47E-06
ENSG00000173369	complement component 1, q subcomponent, B chain	C1QB	0.045270458	-4.465286294	2.03E-06	0.004261044
ENSG00000174370	chromosome 11 open reading frame 45	C11orf45	0.025142778	-5.313712108	2.84E-05	0.027432543

All samples were compared to one another and all dynamic protein coding genes (~5000 genes) were selected at a threshold of adjusted p-value <0.05, fold change >2.5, RPKM (reads per kilobase of transcript per million mapped reads) >1). Biologically relevant comparisons have been performed thereafter in order to study the research question regarding the uric acid effect: the comparison d1_RPMI vs d1_Uricacid yielded the following set of statistically significant hits (up-regulated genes in red, down-regulated genes in blue)

TABLE S2. GO Terms and p-values associated to KEGG pathway enrichment analysis for genes upregulated in uric acid contributing to principal component 2 in PCA analysis.

GOID	GOTerm	Term PValue	Term PValue Corrected with Bonferroni step down	Group PValue	Group PValue Corrected with Bonferroni step down	% Associated Genes	Nr. Genes	Associated Genes Found
GO:0000590	Arachidonic acid metabolism	8.7E-3	150.0E-3	8.7E-3	26.0E-3	6.45	4.00	[EPHX2, GPX2, PTGES, PTGS2]
GO:0003320	PPAR signaling pathway	62.0E-3	120.0E-3	62.0E-3	62.0E-3	4.35	3.00	[ACSL6, ANGPTL4, APOA2]
GO:0004610	Complement and coagulation cascades	62.0E-3	120.0E-3	62.0E-3	62.0E-3	4.35	3.00	[C4A, F3, THBD]
GO:0004015	Rap1 signaling pathway	420.0E-6	11.0E-3	420.0E-6	1.6E-3	4.74	10.00	[ADORA2A, ADORA2B, ARAP3, FGFR1, FLT1, LAT, PFN2, PIK3CD, RAC2, VEGFA]
GO:0004060	Cytokine-cytokine receptor interaction	7.0E-9	280.0E-9	230.0E-9	1.8E-6	6.79	18.00	[CCL20, CCL3, CCL4L1, CLCF1, CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, FLT1, IL1A, IL1B, IL23A, IL6, LIF, TNF, TNFSF15, VEGFA]
GO:0004064	NF-kappa B signaling pathway	27.0E-6	890.0E-6	230.0E-9	1.8E-6	8.60	8.00	[BCL2A1, CCL4L1, CXCL2, CXCL8, IL1B, LAT, PTGS2, TNF]
GO:0004150	mTOR signaling pathway	44.0E-3	170.0E-3	230.0E-9	1.8E-6	5.00	3.00	[PIK3CD, TNF, VEGFA]
GO:0004210	Apoptosis	25.0E-3	220.0E-3	230.0E-9	1.8E-6	4.71	4.00	[IL1A, IL1B, PIK3CD, TNF]
GO:0004350	TGF-beta signaling pathway	24.0E-3	240.0E-3	230.0E-9	1.8E-6	4.76	4.00	[MYC, SMAD3, TNF, ZFYVE9]
GO:0004620	Toll-like receptor signaling pathway	69.0E-6	2.0E-3	230.0E-9	1.8E-6	7.55	8.00	[CCL3, CCL4L1, CXCL8, IL1B, IL6, PIK3CD, TLR2, TNF]
GO:0004621	NOD-like receptor signaling pathway	93.0E-6	2.7E-3	230.0E-9	1.8E-6	10.53	6.00	[CXCL1, CXCL2, CXCL8, IL1B, IL6, TNF]

TABLE S2. GO Terms and p-values associated to KEGG pathway enrichment analysis for genes upregulated in uric acid contributing to principal component 2 in PCA analysis. *(Continued)*

GOID	GOTerm	Term PValue	Term PValue Corrected with Bonferroni step down	Group PValue	Group PValue Corrected with Bonferroni step down	% Associated Genes	Nr. Genes	Associated Genes Found
GO:0004623	Cytosolic DNA-sensing pathway	51.0E-3	150.0E-3	230.0E-9	1.8E-6	4.69	3.00	[CCL4L1, IL1B, IL6]
GO:0004640	Hematopoietic cell lineage	28.0E-3	220.0E-3	230.0E-9	1.8E-6	4.55	4.00	[IL1A, IL1B, IL6, TNF]
GO:0004664	Fc epsilon RI signaling pathway	12.0E-3	160.0E-3	230.0E-9	1.8E-6	5.88	4.00	[LAT, PIK3CD, RAC2, TNF]
GO:0004668	TNF signaling pathway	160.0E-9	6.4E-6	230.0E-9	1.8E-6	10.00	11.00	[CCL20, CXCL1, CXCL2, CXCL3, CXCL5, IL1B, IL6, LIF, PIK3CD, PTGS2, TNF]
GO:0004933	AGE-RAGE signaling pathway in diabetic complications	680.0E-9	25.0E-6	230.0E-9	1.8E-6	9.90	10.00	[CXCL8, F3, IL1A, IL1B, IL6, PIK3CD, SMAD3, THBD, TNF, VEGFA]
GO:0004940	Type I diabetes mellitus	18.0E-3	200.0E-3	230.0E-9	1.8E-6	6.98	3.00	[IL1A, IL1B, TNF]
GO:0005020	Prion diseases	10.0E-3	180.0E-3	230.0E-9	1.8E-6	8.57	3.00	[IL1A, IL1B, IL6]
GO:0005132	Salmonella infection	12.0E-9	490.0E-9	230.0E-9	1.8E-6	12.79	11.00	[CCL3, CCL4L1, CXCL1, CXCL2, CXCL3, CXCL8, FLNB, IL1A, IL1B, IL6, PFN2]
GO:0005133	Pertussis	5.4E-6	190.0E-6	230.0E-9	1.8E-6	10.67	8.00	[C4A, CXCL5, CXCL8, IL1A, IL1B, IL23A, IL6, TNF]
GO:0005134	Legionellosis	480.0E-9	18.0E-6	230.0E-9	1.8E-6	14.55	8.00	[CXCL1, CXCL2, CXCL3, CXCL8, IL1B, IL6, TLR2, TNF]
GO:0005140	Leishmaniasis	2.6E-3	55.0E-3	230.0E-9	1.8E-6	6.85	5.00	[IL1A, IL1B, PTGS2, TLR2, TNF]
GO:0005142	Chagas disease (American trypanosomiasis)	61.0E-6	1.8E-3	230.0E-9	1.8E-6	7.69	8.00	[CCL3, CXCL8, IL1B, IL6, PIK3CD, SMAD3, TLR2, TNF]

TABLE S2. GO Terms and p-values associated to KEGG pathway enrichment analysis for genes upregulated in uric acid contributing to principal component 2 in PCA analysis. *(Continued)*

GOID	GOTerm	Term PValue	Term PValue Corrected with Bonferroni step down	Group PValue	Group PValue Corrected with Bonferroni step down	% Associated Genes	Nr. Genes	Associated Genes Found
GO:0005143	African trypanosomiasis	10.0E-3	180.0E-3	230.0E-9	1.8E-6	8.57	3.00	[IL1B, IL6, TNF]
GO:0005144	Malaria	420.0E-6	11.0E-3	230.0E-9	1.8E-6	10.20	5.00	[CXCL8, IL1B, IL6, TLR2, TNF]
GO:0005146	Amoebiasis	49.0E-6	1.5E-3	230.0E-9	1.8E-6	7.92	8.00	[CXCL1, CXCL8, IL1B, IL6, PIK3CD, SERPINB2, TLR2, TNF]
GO:0005161	Hepatitis B	3.0E-3	60.0E-3	230.0E-9	1.8E-6	4.79	7.00	[CXCL8, IL6, MYC, PIK3CD, SMAD3, TLR2, TNF]
GO:0005210	Colorectal cancer	1.2E-3	31.0E-3	230.0E-9	1.8E-6	8.06	5.00	[MYC, PIK3CD, RAC2, SMAD3, TCF7L2]
GO:0005220	Chronic myeloid leukemia	71.0E-3	71.0E-3	230.0E-9	1.8E-6	4.11	3.00	[MYC, PIK3CD, SMAD3]
GO:0005321	Inflammatory bowel disease (IBD)	20.0E-6	690.0E-6	230.0E-9	1.8E-6	10.77	7.00	[IL1A, IL1B, IL23A, IL6, SMAD3, TLR2, TNF]
GO:0005323	Rheumatoid arthritis	100.0E-12	4.5E-9	230.0E-9	1.8E-6	14.44	13.00	[CCL20, CCL3, CXCL1, CXCL5, CXCL8, FLT1, IL1A, IL1B, IL23A, IL6, TLR2, TNF, VEGFA]
GO:0005332	Graft-versus-host disease	1.9E-3	45.0E-3	230.0E-9	1.8E-6	9.76	4.00	[IL1A, IL1B, IL6, TNF]
GO:0004310	Wnt signaling pathway	10.0E-3	170.0E-3	160.0E-6	800.0E-6	4.23	6.00	[FOSL1, MYC, RAC2, SMAD3, TCF7L2, WNT2B]
GO:0004350	TGF-beta signaling pathway	24.0E-3	240.0E-3	160.0E-6	800.0E-6	4.76	4.00	[MYC, SMAD3, TNF, ZFYVE9]
GO:0004520	Adherens junction	16.0E-3	200.0E-3	160.0E-6	800.0E-6	5.41	4.00	[FGFR1, RAC2, SMAD3, TCF7L2]
GO:0004550	Signaling pathways regulating pluripotency of stem cells	2.5E-3	56.0E-3	160.0E-6	800.0E-6	4.93	7.00	[FGFR1, KLF4, LIF, MYC, PIK3CD, SMAD3, WNT2B]

TABLE S2. GO Terms and p-values associated to KEGG pathway enrichment analysis for genes upregulated in uric acid contributing to principal component 2 in PCA analysis. *(Continued)*

GOID	GOTerm	Term PValue	Term PValue Corrected with Bonferroni step down	Group PValue	Group PValue Corrected with Bonferroni step down	% Associated Genes	Nr. Genes	Associated Genes Found
GO:0005161	Hepatitis B	3.0E-3	60.0E-3	160.0E-6	800.0E-6	4.79	7.00	[CXCL8, IL6, MYC, PIK3CD, SMAD3, TLR2, TNF]
GO:0005210	Colorectal cancer	1.2E-3	31.0E-3	160.0E-6	800.0E-6	8.06	5.00	[MYC, PIK3CD, RAC2, SMAD3, TCF7L2]
GO:0005212	Pancreatic cancer	10.0E-3	160.0E-3	160.0E-6	800.0E-6	6.06	4.00	[PIK3CD, RAC2, SMAD3, VEGFA]
GO:0005213	Endometrial cancer	30.0E-3	210.0E-3	160.0E-6	800.0E-6	5.77	3.00	[MYC, PIK3CD, TCF7L2]
GO:0005220	Chronic myeloid leukemia	71.0E-3	71.0E-3	160.0E-6	800.0E-6	4.11	3.00	[MYC, PIK3CD, SMAD3]
GO:0005221	Acute myeloid leukemia	38.0E-3	190.0E-3	160.0E-6	800.0E-6	5.26	3.00	[MYC, PIK3CD, TCF7L2]
GO:0005230	Central carbon metabolism in cancer	1.8E-3	43.0E-3	160.0E-6	800.0E-6	7.46	5.00	[FGFR1, GLS2, MYC, PIK3CD, SLC7A5]
GO:0005219	Bladder cancer	16.0E-3	190.0E-3	16.0E-3	32.0E-3	7.32	3.00	[CXCL8, MYC, VEGFA]
GO:0004062	Chemokine signaling pathway	5.3E-6	190.0E-6	5.3E-6	31.0E-6	6.42	12.00	[CCL20, CCL3, CCL4L1, CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, GNG11, GNG2, PIK3CD, RAC2]
GO:0004150	mTOR signaling pathway	44.0E-3	170.0E-3	980.0E-9	6.9E-6	5.00	3.00	[PIK3CD, TNF, VEGFA]
GO:0004210	Apoptosis	25.0E-3	220.0E-3	980.0E-9	6.9E-6	4.71	4.00	[IL1A, IL1B, PIK3CD, TNF]
GO:0004370	VEGF signaling pathway	8.3E-3	150.0E-3	980.0E-9	6.9E-6	6.56	4.00	[PIK3CD, PTGS2, RAC2, VEGFA]
GO:0004520	Adherens junction	16.0E-3	200.0E-3	980.0E-9	6.9E-6	5.41	4.00	[FGFR1, RAC2, SMAD3, TCF7L2]
GO:0004664	Fc epsilon RI signaling pathway	12.0E-3	160.0E-3	980.0E-9	6.9E-6	5.88	4.00	[LAT, PIK3CD, RAC2, TNF]

TABLE S2. GO Terms and p-values associated to KEGG pathway enrichment analysis for genes upregulated in uric acid contributing to principal component 2 in PCA analysis. *(Continued)*

GOID	GOTerm	Term PValue	Term PValue Corrected with Bonferroni step down	Group PValue	Group PValue Corrected with Bonferroni step down	% Associated Genes	Nr. Genes	Associated Genes Found
GO:0004666	Fc gamma R-mediated phagocytosis	33.0E-3	200.0E-3	980.0E-9	6.9E-6	4.30	4.00	[LAT, PIK3CD, PLPP1, RAC2]
GO:0004933	AGE-RAGE signaling pathway in diabetic complications	680.0E-9	25.0E-6	980.0E-9	6.9E-6	9.90	10.00	[CXCL8, F3, IL1A, IL1B, IL6, PIK3CD, SMAD3, THBD, TNF, VEGFA]
GO:0005205	Proteoglycans in cancer	310.0E-6	8.7E-3	980.0E-9	6.9E-6	4.93	10.00	[FGFR1, FLNB, HPSE, MYC, PIK3CD, SDC4, TLR2, TNF, VEGFA, WNT2B]
GO:0005210	Colorectal cancer	1.2E-3	31.0E-3	980.0E-9	6.9E-6	8.06	5.00	[MYC, PIK3CD, RAC2, SMAD3, TCF7L2]
GO:0005212	Pancreatic cancer	10.0E-3	160.0E-3	980.0E-9	6.9E-6	6.06	4.00	[PIK3CD, RAC2, SMAD3, VEGFA]
GO:0005220	Chronic myeloid leukemia	71.0E-3	71.0E-3	980.0E-9	6.9E-6	4.11	3.00	[MYC, PIK3CD, SMAD3]

URIC ACID – INDUCED EPIGENETIC REPROGRAMMING IN MYELOID CELLS

Crişan TO
Cleophas MC
Klück V
Davar R
Habibi E
Keating S
Novakovic B
Helsen M
Dalbeth N
Stamp L
Macartney-Coxson D
Phipps-Green A
Xavier R
Stunnenberg HG
Dinarello CA
Merriman T
Netea MG
Joosten LAB

Manuscript in preparation

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SUMMARY

Hyperuricemia is a metabolic condition that is intrinsically linked to gouty arthritis. Studies show that serum uric acid levels are associated also with other common metabolic inflammatory diseases. In human monocytes, uric acid treatment primes the cytokine responses to determine a moderate, but persistent, shift in the balance of IL-1 cytokine production. This consists in elevated levels of proinflammatory cytokines and low anti-inflammatory IL-1 receptor antagonist. We show in in vitro and in vivo studies that high levels of uric acid enhance inflammation and that broad-spectrum methylation inhibitors reverse uric acid effects. Surprisingly, genome wide assessment of two main candidate histone posttranslational modifications (histone 3 lysine 4 trimethylation, H3K4me3, and histone 3 lysine 27 acetylation, H3K27ac) did not show significant differences in the epigenetic landscape for these marks after in vitro uric acid treatment in human monocytes. In contrast, we describe possible roles for DNA methylation following prolonged uric acid exposure in vivo in humans. Differentially methylated regions were found in patients with high serum urate concentrations in genes relevant for inflammatory cytokine signaling cascades. Among them, higher methylation levels were observed for the *SOCS3* gene in hyperuricemic patients of Maori ancestry. *In vitro* validation in monocytes showed that uric acid reduced phosphorylation of STAT3. Further integration and validation of these observations are important in studying the extent to which diseases that associate hyperuricemia (such as gout or other common metabolic disorders) are mediated by epigenetic changes of myeloid cells, and whether this is a novel therapeutic target.

INTRODUCTION

Uric acid is the end-point metabolite in purine catabolism in humans and is regarded as an alarmin released from disintegrating cells at time of stress or cell death (1, 2). Abnormally high uric acid concentrations in serum define the condition of hyperuricemia, at which point uric acid crystals can precipitate in peripheral tissues, mounting inflammation. The main disease associated to persistent hyperuricemia is gout, an inflammatory and painful arthritis caused by deposition of monosodium urate crystals (MSU) at the level of the synovial cavity (3). MSU crystals have been shown to induce IL-1β release in studies demonstrating that MSU crystals activate the NLRP3 inflammasome in THP1 cells (4) or that they recruit ASC (Inflammasome Adaptor Protein Apoptosis-Associated Speck-Like Protein Containing CARD) at the inflammasome formation site through polymerization of tubulin (5). It has also been proven that MSU crystals alone are insufficient for the full-blown gouty attack and that second signals are required to act in synergy with MSU crystals. Such second signals can be either pathogen related ligands such as LPS (6) or Pam3Cys(7), or sterile stimuli such as stearate (8) or complement C5a component (9).

Despite a widely accepted pathogenesis model for gout, stemming from long-lasting hyperuricemia that determines the formation of MSU crystals, many questions remain to address the clinical observations of uric acid related inflammation(10). The reasons why not all patients with hyperuricemia develop gout, or why some patients with MSU crystals in synovial fluids do not have any signs of inflammation (11, 12) are still unknown. Large scale genetic studies and the use of hyperuricemic controls in genetic research are likely to bring more evidence on the additional factors that specifically lead to gout in patients with similar risk background (13, 14). Nevertheless, environmental factors or differential responses to elevated serum uric acid concentrations can also contribute to explain the appearance or lack of marked inflammation in patients with hyperuricemia. Moreover, other epidemiological studies suggest that MSU crystals and uric acid itself also play a role in signaling danger in diseases other than gout, ranging from aging (15), common metabolic disorders (16), to cancer (17). This further suggests that uric acid could play broader roles that are intertwined with low grade chronic inflammation.

Previously, we have described priming effects of high concentrations of soluble uric acid on primary human peripheral blood mononuclear cells (PBMC)s and monocytes (18). In addition, we have found that PBMCs of patients with hyperuricemia produce higher amounts of pro-inflammatory cytokines than healthy controls after ex-vivo stimulation(18). This could be reproduced in vitro by pre-treating cells with increasing uric acid doses followed by washout and re-stimulation with TLR ligands and MSU crystals (18). Interestingly, the high proinflammatory capacity coincided with a reduction in IL-1 receptor antagonist (Ra) production(18) which is at least in part mediated by AKT phosphorylation and autophagy repression in primary human monocytes (19). In this study, we show that uric acid exposure

can have persistent effects in vitro, which is in line with previous data showing that cells of gout patients retain their capacity to produce higher cytokines in absence of hyperuricemia (7, 18).

Several stimuli have been proven to exert long-term effects on innate immunity through epigenetic modifications, a process termed trained immunity (20). This has been shown to be induced by microbial stimuli such as Candida albicans or β-glucan (cell wall component of C. albicans) (21, 22), BCG and muramyl dipeptide (ref), but also in response to sterile stimuli such as oxidized cholesterol or phospholipids (23, 24), or hyperglycemia (25). In this study, we present complementary approaches aiming to establish the molecular basis of uric acid-mediated long term proinflammatory status of human monocytes. We reveal that DNA methylation could be a molecular substrate for the effects of hyperuricemia, and we discuss the implications and future perspectives of these findings for the treatment of hyperuricemia associated diseases, such as gout.

MATERIALS AND METHODS

Volunteers

Venous blood was drawn from the cubital vein of healthy volunteers into EDTA tubes. Experiments requiring large amounts of cells were performed using cells isolated from buffy coats after overnight storage at room temperature (Sanquin blood bank, Nijmegen, The Netherlands). Stimulation experiments were approved by the Arnhem-Nijmegen ethical review board and were conducted according to the principles of the Declaration of Helsinki. The DNA methylation study was approved by the The Health and Disability Ethics Committee of New Zealand. The study was performed in 80 individuals of Maori ancestry, 40 men and 40 women, with varying degrees of serum uric acid values (average 0.37 mmol/L, +/- standard deviation 0.12 mmol/L), who for further analysis were segregated into a hyperuricemia group (0.42 mmol/L or higher) or a normouricemia group. Informed written consent of each human subject was provided before drawing blood.

Reagents

Uric acid, lipopolysaccharide (LPS, E. coli serotype 055:B5), palmitic acid (C16) and methyl-thio-adenosine (MTA) were purchased from Sigma. LPS was subjected to ultra-purification before cell culture experiments. Monosodium urate (MSU) crystals were prepared in house as previously described (8). A library of pharmacological epigenetic inhibitors was kindly provided by Prof. R. Xavier, Broad Institute, Boston, USA.

PBMC and monocyte isolation

Human PBMCs were separated using Ficoll-Paque (Pharmacia Biotech) and suspended in culture medium RPMI (Roswell Park Memorial Institute 1640). Monocytes were enriched using hyperosmotic Percol solution (26) and were subsequently purified by negative selection using the Pan Monocyte Isolation kit (Miltenyi Biotec) according to the manufacturer's instructions.

Stimulation experiments

Experiments were performed in culture medium containing RPMI 1640, supplemented with 50 µg/ml gentamicin, 2 mM L-glutamine, 1 mM pyruvate and 10% human pooled serum following an in vitro uric acid priming protocol described extensively elsewhere (19). For ChIP-seq experiments, 5x10^6 human monocytes were seeded in Petri dishes (Corning) and were primed for 20 hours followed by addition of medium or LPS 10 ng/ml for another 4 h. Cells from all donors used for ChIP-seq were also used in a separate experiment conducted in parallel for RNA-seq assessment and for control experiments to test if the expected uric acid priming phenotype was present. The experimental setup, purity of cells and cytokine control experiments were previously described (19).

Cytokine measurements

Cytokine concentrations were determined in supernatants of cell culture using specific sandwich ELISA kits for interleukin-1β (IL-1β), IL-1Ra (receptor antagonist) (R&D Systems), and IL-6 (Sanquin) according to the manufacturer's instructions.

Animal model

Male C57Bl/6J mice at 10-12 weeks of age were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). Uricase was inhibited using oxonic acid and uric acid was administered in order to increase serum uric acid levels in mice according to a previously described protocol (19). Briefly, mice were given oxonic acid orally 140 mg/kg, 2 times/day combined with uric acid 4 mg/kg, 2 times/day intraperitoneally, in the presence of MTA 30 mg/kg/twice a day intra-peritoneally or equivalent volumes of vehicle control. Gouty arthritis was induced by intra-articular injection (i.a.) of 300 µg MSU crystals and 200 µM palmitic acid (C16) in a volume of 10 µl phosphate buffered saline (PBS) as previously described (8, 27). 24h after injection, mice were sacrificed, knees were macroscopically scored for thickness of joint after removal of skin (scores ranging from 0 to 3), followed by harvesting of joints for histology. Histology was performed as previously described (8).

Chromatin immunoprecipitation – quantitative PCR (ChIP-qPCR)

In experiments where specific histone marks were investigated in a targeted manner, ChIP-qPCR was used. 5-10x10^6 monocytes were plated and treated with uric acid as described above in vitro in 10 cm petri dishes (Corning) in 10 mL RPMI 1640 medium volumes. After 24 h culture, the cells were detached from the plate by gentle scraping in PBS and fixed in methanol-free 1% formaldehyde for 10 minutes followed by treatment with 1.25 mol/L glycine for another 3 minutes. Cells were then sonicated for 5 minutes and immunoprecipitation was performed using antibodies against H3K9me3 and H3K4me3 (Diagenode) and agarose beads. After ChIP, DNA was purified and subjected to qPCR analysis using the SYBR green method. Samples were analyzed using the comparative Ct method. Amplified interest regions were located at the promoters of the IL1B and IL1RN genes. For H3K9me3 EIF4A2 was used as a negative control and ZNF UTR as a positive control according to the manufacturer's instructions. For H3K4me3 myoglobin was used as a negative control and H2B as a positive control according to the manufacturer's instructions. Primers are listed in Table S2.

ChIP sequencing preparation and analysis

After stimulation, samples were subjected to DNA-histone crosslinking by treatment with 1% formaldehyde for 10 minutes followed by treatment with 1.25 mol/L glycine for another 3 minutes. Floating medium and cells were removed followed by three cycles of scraping and recovering adherent fixated cells (2.4-4.8 x 10^6 cells could be recovered from 5x10^6 plated monocytes). Samples were stored at 4°C in PBS containing complete phosphate inhibitor cocktail until sonicated and subjected to chromatin immunoprecipitation as previously described (28). Briefly, fixed cell preparations were sonicated using a Diagenode Bioruptor UCD-300 for 3x 10 min (30 s on; 30 s off). 67 µl of chromatin (1 million cells) was incubated with 229 µl dilution buffer, 3 µl protease inhibitor cocktail and 0.5-1µg of H3K27ac or H3K4me3 antibodies (Diagenode) and incubated overnight at 4°C with rotation. Protein A/G magnetic beads were washed in dilution buffer with 0.15% SDS and 0.1% BSA, added to the chromatin/antibody mix and rotated for 60 min at 4°C. Beads were washed with 400µl buffer for 5 min at 4°C with five rounds of washes. Chromatin was eluted using elution buffer for 20 min. Supernatant was collected, 8 µl 5M NaCl, 3µl proteinase K were added and samples were incubated for 4 hr at 65°C. Finally, samples were purified using QIAGEN; Qiaquick MinElute PCR purification Kit and eluted in 20 µl EB. Detailed protocols can be found on the Blueprint website (http://www.blueprint-epigenome.eu/UserFiles/file/Protocols/Histone_ChIP_May2013.pdf).

Illumina library preparation was done as previously described (28), using the Kapa Hyper Prep Kit. Samples were purified using the QIAquick MinElute PCR purification kit and 300bp

fragments selected using E-gel. Correct size selection was confirmed by BioAnalyzer analysis. Sequencing was performed using Illumina HiSeq 2000 machines and generated 43bp single end reads. Sequencing reads were aligned to human genome assembly hg19 (NCBI version 37) using bwa. Duplicate reads were removed after the alignment with the Picard tools. For peak calling the BAM files were first filtered to remove the reads with mapping quality less than 15, followed by fragment size modeling (https://code.google.com/archive/p/phantompeakqualtools/) and MACS2 (https://github.com/taoliu/MACS/) was used to call the peaks. For each histone mark dataset, the data were normalized using the R package DESeq2 and then pair-wise comparisons were performed (fold change 3, adjusted p-adjvalue < 0.05 and RPKM ≥ 2 in at least in any condition) to determine the differentially expressed genes per condition. The results from all possible pairwise comparisons (within each condition and similar time points across all conditions per mark) were pooled and merged to define the dynamic set of enriched regions.

DNA methylation analysis

Genomic DNA was isolated from peripheral blood and DNA methylation was assessed in whole blood of 80 individuals of Maori ancestry with varying serum uric acid levels using Illumina EPIC platform (~850K CpG sites interrogated). Differentially methylated regions (DMR) were identified using the ChAMP package (29) under default settings. The differentially methylated position (DMP) output from ChAMP consisted in 193 significant DMPs. After filtering for delta beta values > 0.05, 34 DMPs were selected. Genes with >=3 DMP that matched these criteria were defined as DMR.

Intracellular STAT3 flow cytometry

Following the protocol for uric acid priming described above, cells were detached from the plates using Versene (Gibco). Cells were fixed and permeabilized with Cytofix/Cytoperm solution (eBioscience) according to the manufacturer's recommendations. Cells were stained intracellularly with Anti-human/mouse p-STAT3 (Y705) – PE (eBioscience) according to the manufacturer's recommendations. Cells were measured using a FC500 flow cytometer (Beckman Coulter).

RESULTS

Uric acid treatment of cells in vitro results in a specific and persistent cytokine production phenotype

Previous data demonstrated that human PBMCs or monocytes exposed to uric acid show marked increases in proinflammatory cytokines, of which IL-1β is crucial for gout, but also

decrease the natural IL-1 antagonist, IL-1Ra (IL-1 Receptor antagonist). Here we investigated whether these priming effects last longer than the initial 24h uric acid priming protocol followed by 24h stimulation with TLR ligands. Cells were incubated for 24h with uric acid and subjected to increasing resting times (up to 5 days) in culture medium before being restimulated with LPS (10 ng/mL) and MSU crystals (300µg/mL). While IL-1β production capacity was very diminished after 48h of culture (Fig.1A – 24h rest and onwards), persistent effects are observed for reduction of IL-1Ra (Fig.1B) and for induction of IL-6 (Fig.1C).

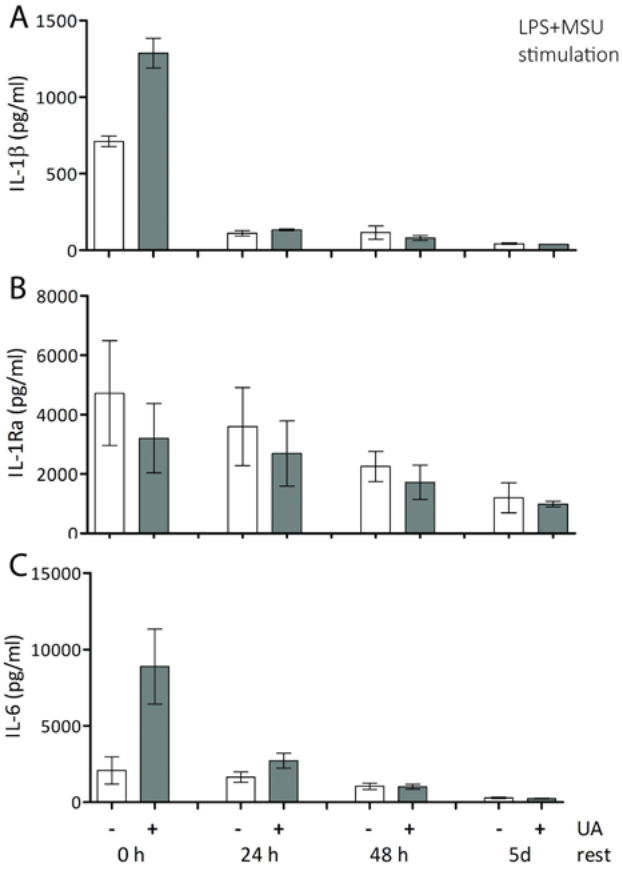

FIGURE 1. Persistence of uric acid priming effects in vitro.

Freshly isolated PBMCs from healthy volunteers were exposed to culture medium (RPMI 1640 supplemented with 10% human pooled serum) in the presence or absence of uric acid (UA) 50 mg/dL. After 24 hours, uric acid was removed and cells were stimulated with LPS 10 ng/mL in addition to MSU crystals (300µg/mL). The second stimulation was performed at several times after uric acid washout: immediately (0 h resting time), or after increasing number of days of resting in 10% serum RPMI. IL-1β (A), IL-1Ra (B), IL-6 (C) were measured in the supernatants of cells, data is representative of 2 independent experiments and 4 different volunteers, graphs depict means+/-SEM.

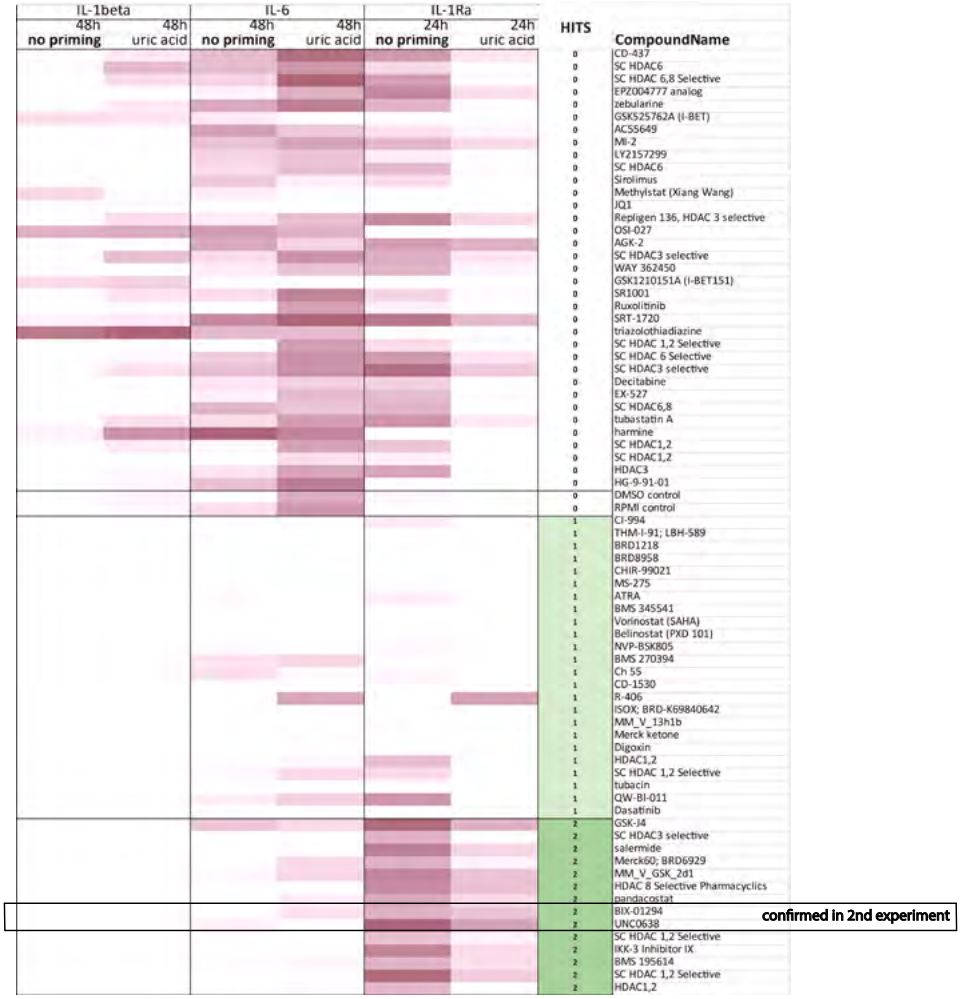

FIGURE 2. Pharmacological epigenetic inhibitor library and uric acid priming effects in vitro.

Freshly isolated human PBMCs were further separated over Percol hyperosmotic solution for monocyte enrichment and were subjected to the in vitro uric acid priming protocol in the presence of medium, vehicle control or each of the pharmacological compounds in the library above. Colors indicate IL-1β, IL-6 or IL-1Ra levels at the indicated times. Hits were defined as compounds that reverse the effect of uric acid with at least 50% IL-1β reduction or at least 50% IL-1Ra increase (code 1), compounds with both effects received code 2. The experiment was performed twice with 2 compounds shown to have the biological effects in both experiments.

A broad epigenetic inhibitor library identifies methylation inhibitors as partially reversing agents of the cytokine shift induced by uric acid in vitro

A pharmacological library of 75 epigenetic modulators has been screened for their capacity to modulate the in vitro model of uric acid-induced cytokine production (SI Table 1). Hits

were defined as compounds that had the capacity to reduce proinflammatory cytokines IL-1β by at least 50% and that recovered IL-1Ra secretion by at least 50% (Fig.2). After validating the experiment, the hits that were consistent between experiments were: BIX-01294 (pharmacological modulator of cellular process; histone lysine methylation; HIV Infections; EHMT2, Euchromatic Histone Lysine Methyltransferase 2), and UNC0638 (pharmacological inhibitor of G9a and GLP methyltransferase activity in cells). Absolute and fold change values are shown separately (Fig.3) for IL-1β production (Fig.3 A-B) and IL-1Ra levels (Fig.3 C-D). Since both compounds target the EHMT2, we investigated H3K9me3 levels at IL1B and IL1RN promoter regions in uric acid primed cells using ChIP-qPCR. Enrichment of this mark at the targeted regions was variable even at neighboring sites within same promoter (Fig.S1 A-B).

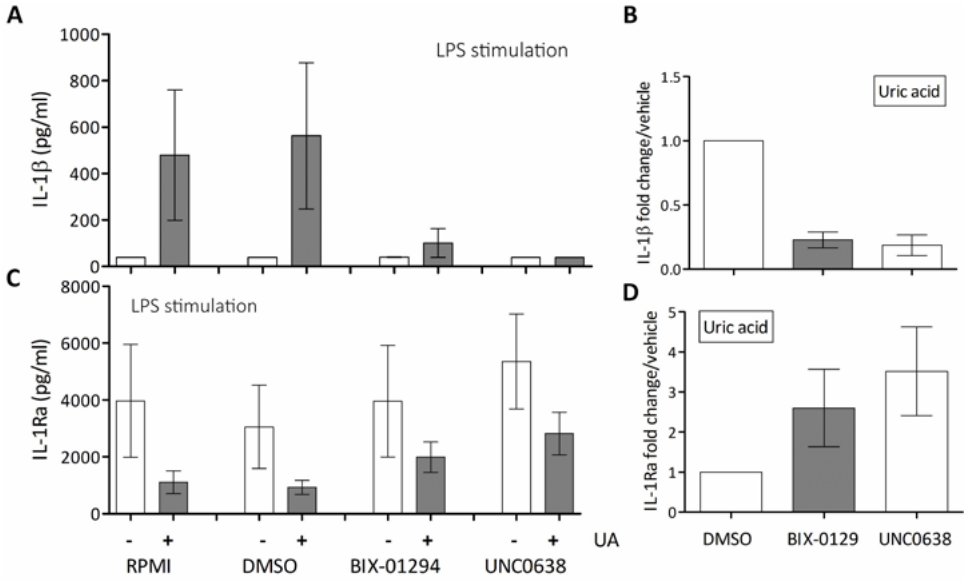

FIGURE 3. Methyl transferase inhibitors limit uric acid priming effects in vitro.

Absolute cytokine levels for IL-1β (A) and IL1-Ra (C) and fold change due (B and D, respectively) due to compounds selected after using the epigenetic inhibitor library. Cells were primed with uric acid for 24h in the presence or absence of inhibitors, followed by medium washout and stimulation with LPS. IL-1β is shown at 48h (after LPS stimulation) and IL-1Ra is shown at 24h (after uric acid priming alone). IBIX-01294 (cellular process; histone lysine methylation; HIV Infections; EHMT2, Euchromatic Histone Lysine Methyltransferase 2), UNC0638 (inhibition of G9a and GLP methytransferase activity in cells). Data is representative of 2 independent experiments and 4 different volunteers, graphs depict means+/-SEM.

Pharmacological inhibition of methyl transferases also inhibits uric acid effects in an in vivo murine model of gout

To validate the in vitro results of methyl transferases on uric acid induced epigenetic modifications, mice were administered exogenous uric acid in addition to pharmacological uricase inhibitor oxonic acid to induce hyperuricemia. Acute gout was induced by intra-articular (i.a.) injections with MSU crystals and palmitic acid (C16). Inflammation was significantly enhanced in the oxonic acid group compared with controls as observed by inflammation scoring macroscopically (Fig. 4A). Addition of broad methyl-transferase inhibitor MTA potently inhibited this effect of hyperuricemia on the enhanced joint inflammation and histology at 24h post i.a. injections (Fig.4A-C). To confirm the uric acid driven histone modifications, we assessed H3K4me3 levels using ChIP-qPCR at the IL1B and IL1RN gene promoters and also found large variability within promoter sites (Fig.S1-C), similarly to H3K9me3, that was not able to provide a consistent picture.

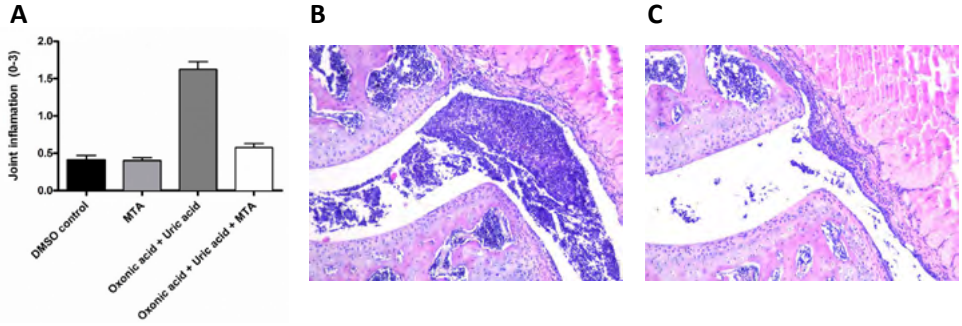

FIGURE 4. Methyl transferase inhibition limits gout inflammation in hyperuricemic mice.

Macroscopic (Panel A) scores of the knees in mice treated with vehicle control or Oxonic acid + Uric acid in the presence or absence of methyl transferase inhibitor MTA (methyl-thio-adenosine) followed by intraarticular injection of MSU+C16. Inflammation was scored at 24 h. Histology (H&E staining) of joints treated with MSU+C16 in Oxonic acid + Uric acid mice (panel B) and in the presence of MTA (panel C).

Histone 3 Lysine 4 trimethylation (H3K4me3) or Histone 3 Lysine 27 acetylation (H3K27ac) are not markedly affected by uric acid treatment of human monocytes in vitro

To test whether certain histone marks are indeed involved in the persistent effects of uric acid priming, we designed a ChIP-sequencing experiment to investigate two histone modifications with relevance for long-term effects of sterile stimuli (23) and following on the broad indications obtained in our experiments, namely H3K4me3 and H3K27ac. Monocytes were purified from buffy coat cells using a sequential isolation protocol that consisted in: isolation of PBMCs from buffy coat by density gradient centrifugation, enrichment of

the monocyte cell fraction by centrifugation over hyperosmotic Percoll solution, negative selection of monocytes by magnetic bead cell separation. This lead to a cell suspension containing monocytes with 85-95% purity. Monocytes were then subjected to uric acid priming following the standard protocol described above. This was followed by stimulation of cells with LPS (10ng/ml) which served as a proinflammatory trigger to validate the effects of uric acid pre-exposure. RNA sequencing (reported elsewhere (19)) and ChIP sequencing for H3K27ac and H3K4me3 histone marks was performed in untreated monocytes, 24h primed cells and controls, 24h primed + 4h stimulated cells and non-primed stimulated controls.

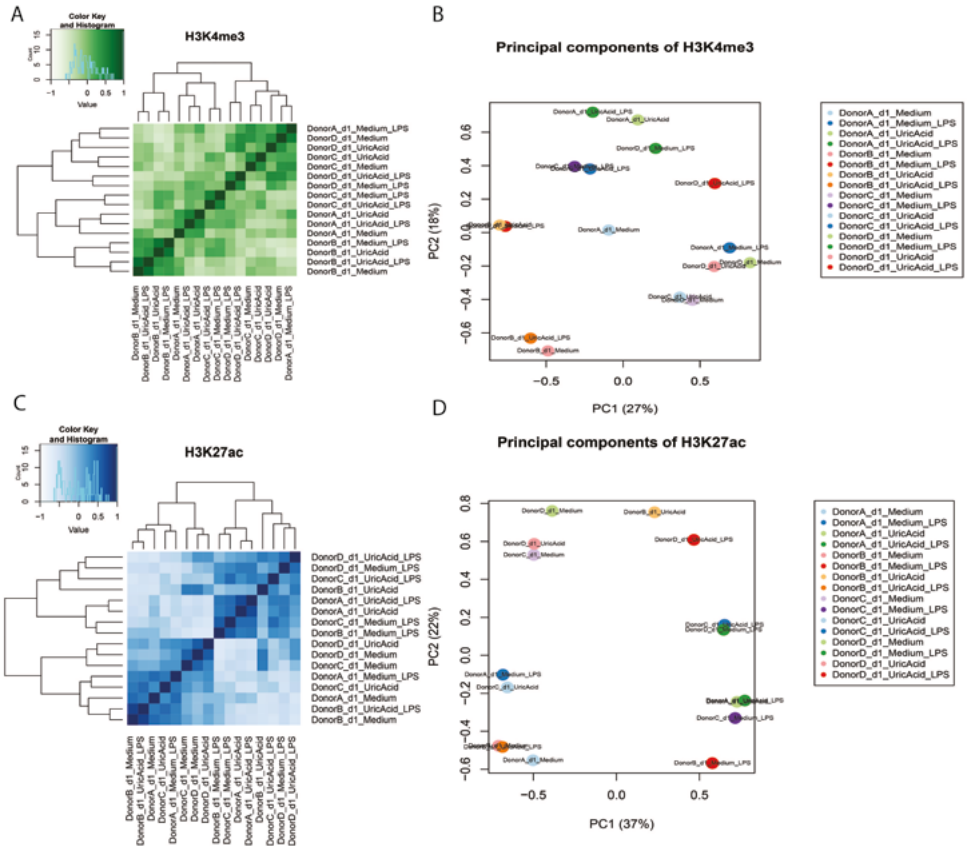

FIGURE 5. ChIP sequencing in uric acid stimulated monocytes reveals no clustering based on uric acid induced phenotype for H3K4 trimethylation and H3K27 acetylation.

Cluster and principal component analysis of datasets obtained on ChIP sequencing for H3K4me3 (A and B) or H3K27ac (C and D) in 4 different donors (labeled DonorA-D) and 4 different conditions (24h medium – d1_Medium, 24h uric acid - d1_Uric acid, 20h medium + 4h LPS – d1_Medium_LPS, 20h uric acid + 4h LPS – d1_Uric acid_LPS).

The ChIPseq analysis was based on all dynamic genes that were identified by comparing all samples to each other. A gene was considered dynamic if the comparison to at least another samples gave at least a 2-fold difference after accounting for the variability among replicates, and RPKM (reads per kilobase per mapped reads) >3. After filtering the data based on these cutoffs, no clustering of samples was evident to indicate that uric acid induced differences create strong effects on these two studied marks (Fig.5). No gene variance was found to be significant between uric acid and control after multiple testing correction. The genes showing variability for H3K4me3 enrichment are listed in Table S3, and for H3K27ac in Table S4.

DNA methylation profiling reveals important candidates for effects of serum uric acid levels in vivo in humans

Another important process that could function as basis for uric acid imprinting is DNA methylation. In a population of Maori descent, DNA methylation was determined and assessed between hyperuricemic and normouricemic volunteers. Approximately 850K CpG sites were studied among the two groups and revealed 193 significantly different DMPs, of which 34 consisted in a difference in DNA methylation of at least 5%. Regions that depicted significant differential methylation over the two groups of patients were found in intergenic or intragenic regions of certain genes. One notable example is gene *SOCS3*, which showed three adjacent intragenic differentially methylated positions (Fig.6 B-C) and which showed higher methylation in hyperuricemic compared to normouricemic patients (Fig.6 D). Publicly available data from ENCODE database show that this region presents TF (transcription factor), HDAC (histone deacetylases), or CTCF (CCCTC-binding factor) binding sites, that suggest the region to be influenced by chromatin accessibility and to be methylation-sensitive (Fig.6 E).

Uric acid priming dose-dependently suppresses STAT3 phosphorylation

To assess whether the differential methylation in the *SOCS3* gene can be functionally validated, downstream levels of STAT3 phosphorylation were assessed. Freshly isolated monocytes were subjected to an in vitro protocol of uric acid priming with 10 mg/dL uric acid (UA10) or 50 mg/dL uric acid (UA50) for 24 h, and cells were washed and restimulated with LPS 10 ng/mL (Fig.7 A) or LPS+MSU 300μg/mL (Fig.7 B). Phosphorylation levels of STAT3 were determined by intracellular staining and flow cytometry in 3 individuals. Of high interest, a remarkable dose-dependent effect of uric acid in decreasing phosphorylated STAT3 was observed.

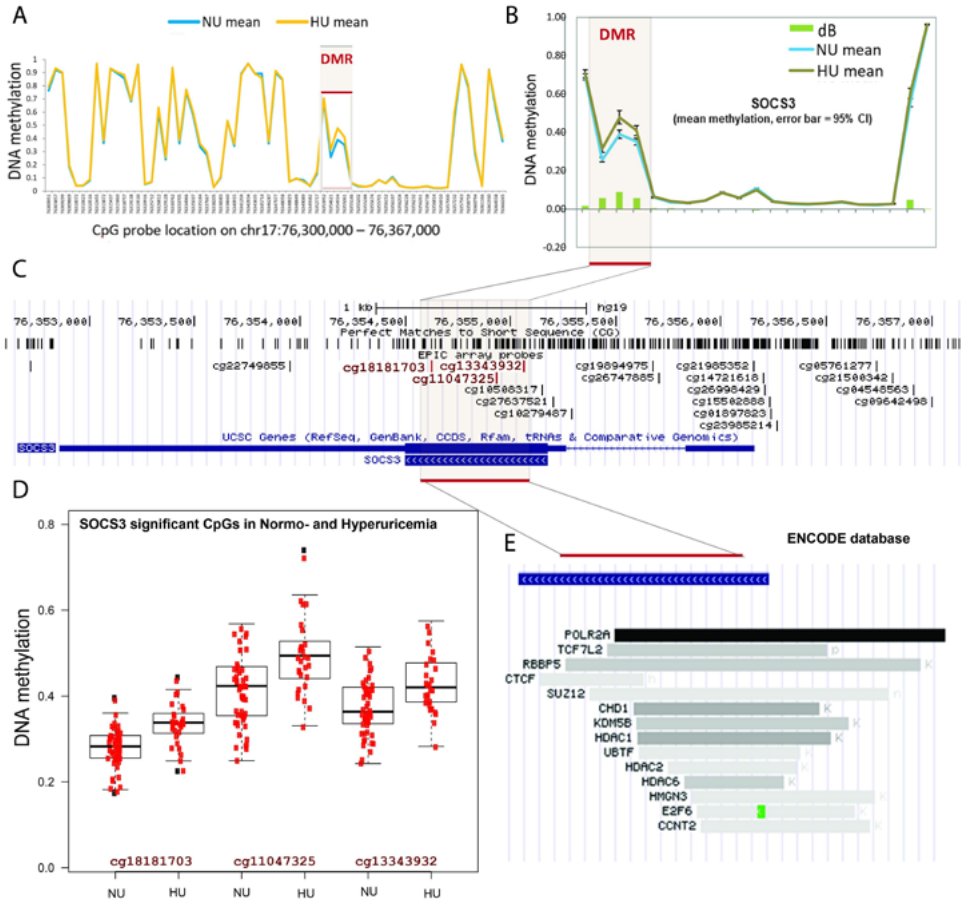

FIGURE 6. Differentially methylated region (DMR) in patients with high serum uric acid levels compared to normal serum levels.

DNA methylation was assessed in 80 individuals of Maori ancestry with varying serum uric acid levels, hyperuricemia (HU) was defined as serum uric acid levels >=0.42 mmol/L, and DNA methylation was assessed in whole blood using Illumina EPIC platform (~850K CpG sites interrogated). Significantly differentially methylated positions (DMP) were found at the level of CpG islands (CGI) in various intergenic or intragenic regions. CpG probes located on a wider area on chromosome 17 are shown, with one highlighted DMR region A. DNA methylation levels (B) and localization (C) of 3 intragenic DMPs at the level of the *SOCS3* gene (dB, delta beta values, DNA methylation change). Individual methylation levels in NU versus HU patients for the 3 positions identified (D). Transcription factors and regulatory proteins known to bind the interest regions according to ENCODE database (E).

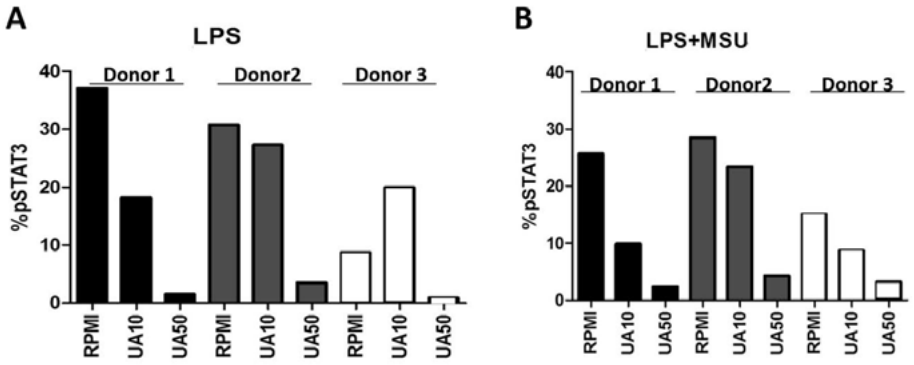

FIGURE 7. Uric acid priming dose-dependently suppresses STAT3 phosphorylation in response to LPS or LPS+MSU stimulation.

Freshly isolated enriched monocytes were subjected to an in vitro protocol of uric acid priming with 10 mg/dL uric acid (UA10) or 50 mg/dL uric acid (UA50) in RPMI supplemented with 10%human serum. After 24 h cells were washed and restimulated with LPS 10 ng/mL (A) or LPS+MSU 300µg/mL (B). Phosphorylation levels of STAT3 were determined by intracellular staining and flow cytometry, cells were gated on the monocytic cell population based on forward and side scatter plot, results are shown in three individuals.

DISCUSSION

In this study, we investigate the persistent effects of primary myeloid cell treatment with soluble uric acid in vitro. Cells have been treated with uric acid for 24h, followed by medium removal and stimulation with LPS+MSU crystals in the absence of uric acid for another 24h. The behavior of monocytes following this 48h uric acid priming protocol has been extensively described previously, demonstrating that the cytokine production capacity of the cells switches to higher IL-1β production and lower IL-1Ra basal concentrations(18) in an AKT- and autophagy-dependent manner(19). Here, we show that even after extended resting times between priming and restimulation, modified cytokine levels persist in primary cells exposed to uric acid: IL-6 increase was still present when cells were rested for 24h while IL-1Ra remained downregulated after 5 days resting time (Fig.1 B-C). IL-1β levels, however, were markedly reduced after the initial 48 hours protocol (Fig.1 A – 0 h resting time). This is in accordance with the restricted IL-1β production in differentiated macrophages after longer in vitro culture of primary monocytes (30).

To test for the involvement of enzymes that modulate the epigenetic state of the uric acid exposed cells, a pharmacological library was used which identified two compounds that inhibit methylation and target EHMT2 (Euchromatic Histone Lysine Methyltransferase 2). The EHMT2 gene encodes the G9a enzyme, a histone lysine methyltransferase known to be involved together with the GLP protein in laying down H3K9 methylation histone marks (31). In addition, G9a and GLP have also been independently linked to DNA methylation

(32). This indicates that methylation of histones (specifically H3K9me) or DNA could be involved in uric acid priming.

ChIP-qPCR assessment of H3K9me3 enrichment at IL1B or IL1RN promoters showed variable non-consistent results for same promoter regions even at adjacent sites. This indicated that either H3K9me3 is not a major factor modified by uric acid at 24 h, or that broader assays are needed to more accurately determine whether significant areas are dynamic for this histone mark in our setup. Moreover, broad methylation inhibitor MTA was previously shown to reverse uric acid effect in vitro (18) and here we show reversal of inflammation in an in vivo model of gout in mice where uricase activity was inhibited (Fig. 4). In light of this data and other reports (23), another important methylation histone mark that could be a candidate for mediating uric acid effects is H3K4me3. We assessed H3K4me3 levels using ChIP-qPCR at the IL1B and IL1RN gene promoters but also found inconclusive results due to variability within promoter sites (Fig.S1-C).

To further decipher the involvement of histone marks in uric acid priming and reminiscence of effects, a genome wide approach was undertaken in the pursuit of assessing epigenetic modifications globally. Two histone marks were analysed in uric acid priming setting: trimethylation of lysine 4 of histone 3 (H3K4me3) and acetylation of lysine 27 of histone 3 (H3K27ac). Both marks are associated with activation of gene expression across different cell types (33), have been previously studied in relation to other trained immunity stimuli in recent reports (22, 34), and are known to be present at the promoters of IL1B and IL1RN in monocytes (35). Bioinformatics analysis of the ChIPseq data (Fig. 5 A-D) shows that the samples cluster in a time and donor-dependent manner as the strongest determinant of the two histone marks that were assessed in this experiment. While the histone modification differences due to uric acid might still add biological information in the context of data integration with other genome wide approaches, it could be stated that, in terms of H3K4me3 and H3K27ac, the effect of uric acid is stochastic and these marks are less likely to be the substrate of uric acid priming.

Since transcriptional and protein regulation is observed due to uric acid treatment in the absence of a major effect on the initial histone marks assessed so far, it is possible that other underlying mechanisms could be at play to induce this pattern of cytokine production. On the one hand, other histone marks could probably have a higher influence in the context of uric acid, such as H3K9 methylation marks, as suggested by pharmacological data. Future strategies to investigate whether epigenetic alterations are the biological background of uric acid effects also include more general approaches based on assays such as DNase or transposase accessibility of chromatin. On the other hand, despite a large body of literature linking the activity of lysine methyl transferases with histone modifications and chromatin dynamics, increasing evidence pinpoints to the importance of other protein lysine methylation processes, such as transcription factor methylation (36), that could further

explain the biological effects of methyl transferase inhibitors. Last but not least, dynamic RNA modification processes (37) could play a role in the transcriptomic differences that we have observed, while the histone modification landscape might remain relatively stable. Integrating the ChIP and RNA sequencing data in uric acid primed monocytes is crucial to extract more mechanistic leads.

Finally, we have studied the possibility of DNA methylation involvement in hyperuricemia in a cohort of Maori ancestry. Polynesian populations are known to have a high prevalence of hyperuricemia, with an approximately three times higher risk of gout compared to populations of European descent (38), due to possible genetic susceptibility alleles that have been selected in the Pacific through still debated mechanisms (39). By comparing DNA methylation status in hyperuricemic versus normouricemic volunteers within this cohort, several differentially methylated regions have been identified, among which some were functionally linked to IL-1 and IL-1Ra production. Since IL-Ra downregulation is one of the most intriguing findings caused by uric acid exposure, potential candidates that modulate this pathway are important to be investigated (Fig 6. A). One example is *SOCS3* (Suppressor of cytokine signaling 3), a regulator of STAT3 signaling with broad roles in cytokine signaling (40, 41), mostly associated with negative feedback inhibition of proinflammatory cytokines (42, 43). Our data shows higher DNA methylation in hyperuricemic individuals at 3 neighboring *SOCS3* intragenic positions (Fig.6 B-D). Since the functionality of this higher methylation status is not known, future validation studies are necessary to show whether SOCS3 levels are indeed modified in the context of uric acid exposure in vitro or in vivo. If SOCS3 levels are diminished, this could typically coincide with less STAT3 inhibition and promotion of proinflammatory cytokines production (42, 43). Interestingly, STAT3 inhibition has been reported to mediate IL-1Ra downregulation by reducing chromatin accessibility at the IL1-Ra gene locus (44). Therefore, STAT3 inhibition could mediate IL-1Ra downregulation in uric acid exposure and represents a candidate to be functionally validated. We have assessed this possibility by studying the effects of uric acid on STAT3 phosphorylation (Figure 7). Interestingly, phosphorylated STAT3 is markedly diminished by uric acid pretreatment, which, based on previous studies (44), could be a mechanism for IL-1Ra downregulation. As observed in the publicly available data from ENCODE database (Fig.6 E), this region presents binding sites for various transcription factors, but also HDACs (histone deacetylases), or CTCF (CCCTC-binding factor), all of which can have functional importance. Nevertheless, CTCF is known to have complex roles as insulator protein, chromatin remodeler or transcription factor which are highly dependent on the methylation status of the binding region (45). Further validating SOCS3 and STAT3 regulation in uric acid induced inflammation is warranted.

In conclusion, we have generated extensive datasets involving epigenomic and functional immunological experiments to pinpoint the major possible mechanisms involved in uric

acid priming of myeloid cells. The integration of epigenetic data with genetic susceptibility data is likely to elucidate to which extent diseases such as gout or other common metabolic disorders that associate hyperuricemia are genetically or environmentally determined. Based on complementary methods we argue that epigenetic alterations could be also at role in mediating the persistent effects of uric acid exposure on innate immune cells. While histone modifications were a strong initial candidate, we were not able to observe major differences for H3K4me3 or H3K27ac at genome-wide level. Other histone posttranslational modifications could be involved. On the other hand, DNA methylation is a promising mechanism that depicts differences in hyperuricemic compared to normouricemic patients. Further validation of these differentially methylated targets and assessment of their function in patients may represent an important novel mechanism in understanding gout and hyperuricemia and providing a platform for therapy research in low grade chronic inflammation.

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Conflict of interest

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

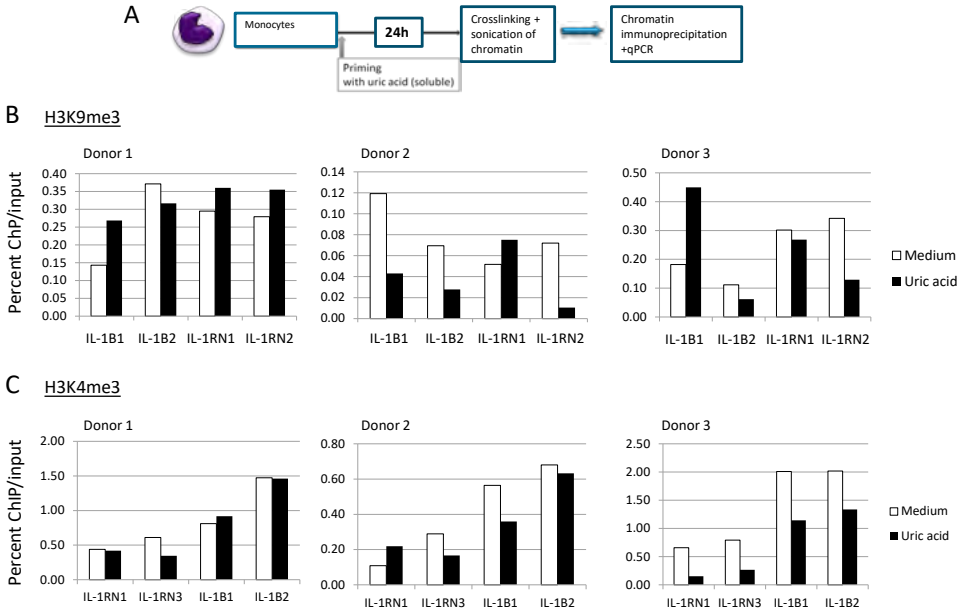


FIGURE S1. ChIP-qPCR in uric acid stimulated monocytes reveals variability at promoter regions of IL-1B and IL-1RN genes for H3K9 trimethylation and H3K4 trimethylation.

Monocytes were treated with culture medium or uric acid for 24h followed by fixation, sonication, chromatin immunoprecipitation and quantitative PCR for target regions at the level of IL1B and IL1RN gene promoters (A). Results are shown as percent ChIP over input in 3 individual donors for 2 separate regions both located at the promotors of each of the IL1B and IL1RN genes, for H3K9me3 (B) and for H3K4me3 (C).

TABLE S1. Complete list and target or activity of epigenetic pharmacological library compounds.

Compound	Compound Name	TARGET OR ACTIVITY
1	GSK-J4	JMJD3 inhibitor (from GSK)
2	Merck60; BRD6929	HDAC; histone deacetylase activity; histone acetylation
3	CD-437	Pleiotropic
4	SC HDAC6	HDAC6
5	CI-994	HDAC
6	SC HDAC 6,8 Selective	HDAC 6,8
7	THM-I-91; LBH-589	HDAC
8	EPZ004777 analog	selective inhibitor of DOT1L; selectively inhibits H3K79 methylation
9	zebularine	DNA methylation; DNA-methyltransferase activity; cytidine deaminase activity; CDA; DNMT1
10	GSK525762A (I-BET)	BRD2/3/4 Inhibitor
11	SC HDAC3 selective	HDAC3
12	BRD1218	HDAC6;tubulin deacetylation
13	AC55649	RARB
14	BRD8958	p300 inhibitor
15	MM_V_GSK_2d1	SAM-competitive EZH2 inhibitor
16	MI-2	Menin:MLL inhibitor
17	LY2157299	TGF-b receptor kinase inhibitor
18	SC HDAC6	HDAC6
19	HDAC 8 Selective Pharmacyclics	HDAC 8
20	Sirolimus	mTOR inhibitor
21	Methylstat	JMJD annotation
22	CHIR-99021	GSK-3 inhibitor
23	pandacostat	HDAC; Non-selective among all biochemically active human HDAC enzymes (HDAC1-9)
24	JQ1	inhibitor of BET bromodomains
25	Repligen 136, HDAC 3 selective	HDAC 3
26	MS-275	HDAC
27	OSI-027	mTORC1 & mTORC2
28	BIX-01294	cellular process; histone lysine methylation; HIV Infections; EHMT2
29	ATRA	cell differentiation
30	BMS 345541	nfkb
31	UNC0638	inhibition of G9a and GLP methytransferase activity in cells
32	Vorinostat (SAHA)	HDAC1, HDAC2, HDAC3, HDAC6, HDAC8, ACUC1
33	SC HDAC 1,2 Selective	HDAC
34	IKK-3 Inhibitor IX	IKK3
35	Belinostat (PXD 101)	HDACs

TABLE S1. Complete list and target or activity of epigenetic pharmacological library compounds. (*Continued*)

Compound	Compound Name	TARGET OR ACTIVITY
36	NVP-BSK805	JAK2
37	AGK-2	SIRT2
38	SC HDAC3 selective	HDAC3
39	BMS 195614	RAR-selective antagonist
40	BMS 270394	Nuclear retinoic acid receptor (RAR) gamma agonist
41	Ch 55	Potent retinoic acid receptor (RAR) agonist
42	WAY 362450	farnesoid X receptor (FXR)
43	GSK1210151A (I-BET151)	inhibitor of BET family (BRD2, BRD3, BRD4)
44	SR1001	agonists of RORα and RORγt
45	Ruxolitinib	JAK1, JAK2
46	SRT-1720	mitochondrion morphogenesis; mitochondrion morphogenesis; glucose homeostasis; cellular respiration; cellular metabolic process; SIRT1; response to oxidative stress
47	triazolothiadiazine	PDE4A inhibitor
48	SC HDAC 1,2 Selective	HDAC
49	SC HDAC 1,2 Selective	HDAC
50	SC HDAC 6 Selective	HDAC 6
51	salermide	SIRT1 and SIRT2 inhibitor
52	SC HDAC3 selective	HDAC3
53	Decitabine	DNA, DNMT1
54	EX-527	SIRT1
55	SC HDAC6,8	HDAC6,8
56	CD-1530	RARG
57	R-406	Syk inhibitor
58	ISOX; BRD-K69840642	HDAC6 inhibitor
59	tubastatin A	HDAC6
60	MM_V_13h1b	non-SAM-competitive EZH2 inhibitor
61	Merck ketone	HDAC
62	harmine	Dyrk1a and MOA inhibitor
63	Digoxin	Ror-γt antagonist
64	SC HDAC1,2	HDAC1,2
65	HDAC1,2	Broad HDAC1,2-selective inhibitor
66	SC HDAC 1,2 Selective	HDAC
67	SC HDAC1,2	HDAC1,2
68	HDAC3	HDAC 3 Selective DOS cpd
69	HDAC Neg control	HDAC Neg control
70	tubacin	HDAC6-selective inhibitor

TABLE S1. Complete list and target or activity of epigenetic pharmacological library compounds. (*Continued*)

Compound	Compound Name	TARGET OR ACTIVITY
71	QW-BI-011	must effective version in cells, (use BI-012 for biochemical assay); BI-011 inhibition is not specific for G9a but also for EZH2
72	HDAC1,2	Broad HDAC1,2-selective inhibitor
73	Dasatinib	Tyrosine kinase inhibitor
74	HG-9-91-01	pan-SIK inhibitor

TABLE S2. Primer sequences used in ChIP-qPCR experiments at promoters of indicated genes.

Gene	Primer code	Fw/Rv	5′-3′ primer sequence
IL-1RN	IL-1RN1	FW	TTGCGACACTTAGTGGGGTT
IL-1RN	IL-1RN1	RV	CGGAAATACCCTCCTCGCAT
IL-1RN	IL-1RN2	FW	ACCAGAGAGGCCCTTTACCA
IL-1RN	IL-1RN2	RV	CCTTGCAGCTAACTCGCTCT
IL-1RN	IL-1RN3	FW	TGCACAAACCCTAGGTGCAA
IL-1RN	IL-1RN3	RV	CTGCCCTCATATGTGCCCTT
IL-1B	IL-1B1	FW	AATCCCAGAGCAGCCTGTTG
IL-1B	IL-1B1	RV	AACAGCGAGGGAGAAACTGG
IL-1B	IL-1B2	FW	CATGGCTGCTTCAGACACCT
IL-1B	IL-1B2	RV	ACACATGAACGTAGCCGTCA
ZNF UTR	ZNF UTR	FW	AAGCACTTTGACAACCGTGA
ZNF UTR	ZNF UTR	RV	GGAGGAATTTTGTGGAGCAA
EIF4A2	hEIF4A2	FW	GGGGAAAGCGAGGTTTAACT
EIF4A2	hEIF4A2	RV	TTACAGGGTCGCTGGAAATC
Myoglobulin	Myo	FW	AGCATGGTGCCACTGTGCT
Myoglobulin	Myo	RV	GGCTTAATCTCTGCCTCATGAT
H2B	H2B	FW	TGTACTTGGTGACGGCCTTA
H2B	H2B	RV	CATTACAACAAGCGCTCGAC

TABLE S3. Genes showing enrichment of histone modification H3K4me3.

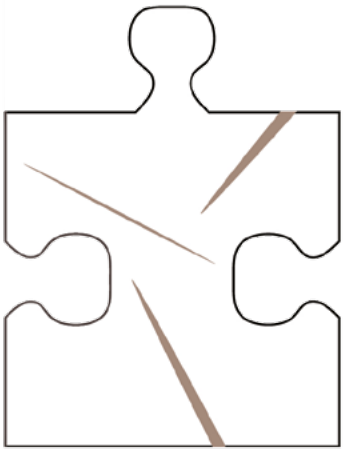
Log 2 Fold change	p-val	chr	gene name	function	ENSEMBL gene ID
3.453114728	0.003647337	chr17	MED24	protein_coding	ENSG00000008838
3.453114728	0.003647337	chr17	CSF3	protein_coding	ENSG00000108342
3.261089137	0.001644928	chr16	TAF1C	protein_coding	ENSG00000103168
3.261089137	0.001644928	chr16	DNAAF1	protein_coding	ENSG00000154099
2.789250238	0.003494836	chr12	HCAR2	protein_coding	ENSG00000182782
2.439116042	0.005077587	chr12	HCAR3	protein_coding	ENSG00000255398
2.439116042	0.005077587	chr12	RP11-324E6.6	lincRNA	ENSG00000256249
2.195723898	0.017238977	chr6	C2	protein_coding	ENSG00000166278
1.894915497	0.010902149	chr7	AC073072.5	antisense	ENSG00000179428
1.798834019	0.016175879	chr8	IDO1	protein_coding	ENSG00000131203
1.798834019	0.016175879	chr8	RP11-44K6.2	sense_intronic	ENSG00000253838
1.742004675	0.04436532	chr9	IFNB1	protein_coding	ENSG00000171855
1.708204243	0.031737693	chr9	RP11-370F5.4	lincRNA	ENSG00000203364
1.468300149	0.011794183	chr2	CCL20	protein_coding	ENSG00000115009
1.468300149	0.011794183	chr2	AC073065.3	pseudogene	ENSG00000229172
1.458881832	0.030495842	chr8	KB-1507C5.2	protein_coding	ENSG00000253320
1.399798558	0.043826601	chr1	RP4-620F22.2	antisense	ENSG00000237568
1.362218093	0.018699699	chr15	BCL2A1	protein_coding	ENSG00000140379
1.345967414	0.038410059	chr8	RP11-44K6.5	pseudogene	ENSG00000253790
-1.465971184	0.019156236	chr6	TREM2	protein_coding	ENSG00000095970
-1.560001921	0.000921444	chr6	SNRPC	protein_coding	ENSG00000124562
-1.62527138	0.014555958	chr19	APOE	protein_coding	ENSG00000130203

TABLE S4. Genes showing enrichment of histone modification H3K27ac.

Log 2 Fold change	p-val	chr	gene name	function	ENSEMBL gene ID
2.660679085	0.049415298	chr9	Metazoa_SRP	misc_RNA	ENSG00000240481
2.4833987	0.008343537	chr22	MN1	protein_coding	ENSG00000169184
2.331095586	0.029818526	chr9	RP11-370F5.4	lincRNA	ENSG00000203364
2.317025595	0.023650622	chr7	AC002486.2	processed_pseudogene	ENSG00000231525
2.288963236	0.033411237	chr14	RP11-45P15.2	processed_pseudogene	ENSG00000240624
2.25651627	0.037984468	chr1	MRPS21P1	processed_pseudogene	ENSG00000234496
2.248719451	0.031445481	chr17	MED24	protein_coding	ENSG00000008838
2.248719451	0.031445481	chr17	CSF3	protein_coding	ENSG00000108342
2.248719451	0.031445481	chr17	SNORD124	snoRNA	ENSG00000238793
2.12713601	0.029778725	chr8	IDO1	protein_coding	ENSG00000131203
2.12713601	0.029778725	chr8	RP11-44K6.2	sense_intronic	ENSG00000253838
2.112693825	0.024122425	chr20	MAP1LC3A	protein_coding	ENSG00000101460
2.112693825	0.024122425	chr20	PIGU	protein_coding	ENSG00000101464
2.057983805	0.036224137	chr10	IL2RA	protein_coding	ENSG00000134460
2.057983805	0.036224137	chr10	SNORA14	snoRNA	ENSG00000251922
2.019280739	0.035081165	chr7	ITGB8	retained_intron	ENSG00000105855
2.0036205	0.029821686	chr4	RP11-377G16.2	antisense	ENSG00000248719
1.988134229	0.031778894	chr7	AC073072.5	antisense	ENSG00000179428
1.987089151	0.048185373	chr8	IDO2	processed_transcript	ENSG00000188676
1.987089151	0.048185373	chr8	RP11-44K6.5	processed_pseudogene	ENSG00000253790
1.975253497	0.022740122	chr16	TAF1C	protein_coding	ENSG00000103168
1.975253497	0.022740122	chr16	DNAAF1	retained_intron	ENSG00000154099
1.937312246	0.019288718	chr2	IL1A	protein_coding	ENSG00000115008
1.937312246	0.019288718	chr2	IL1B	protein_coding	ENSG00000125538
1.937312246	0.019288718	chr2	AC079753.1	miRNA	ENSG00000221541
1.882679399	0.009843247	chr12	Metazoa_SRP	misc_RNA	ENSG00000244104
1.855958285	0.030549533	chr22	RP3-439F8.1	antisense	ENSG00000234869
1.847756122	0.03698174	chr17	CCL4	protein_coding	ENSG00000129277
1.847756122	0.03698174	chr17	Metazoa_SRP	misc_RNA	ENSG00000263488
1.82584124	0.038435114	chr2	MIR4773-2	miRNA	ENSG00000264684
1.82210199	0.021945742	chr6	RP11-356I2.1	lincRNA	ENSG00000234956
1.767001404	0.004999522	chr10	RP5-1031D4.2	lincRNA	ENSG00000232591
1.767001404	0.040816855	chr1	RP1-313L4.4	unprocessed_pseudogene	ENSG00000250762
1.707678185	0.037799945	chr14	RP11-173D9.5	processed_pseudogene	ENSG00000259090
1.624220426	0.016611913	chr22	ADORA2A	processed_transcript	ENSG00000128271
1.624220426	0.016611913	chr22	ADORA2A-AS1	protein_coding	ENSG00000178803
1.624220426	0.016611913	chr22	SPECC1L	nonsense_mediated_decay	ENSG00000258555
1.594327437	0.028926028	chr14	C14orf183	protein_coding	ENSG00000168260
1.594327437	0.028926028	chr14	Y_RNA	misc_RNA	ENSG00000251792

TABLE S4. Genes showing enrichment of histone modification H3K27ac. *(Continued)*

Log 2 Fold change	p-val	chr	gene name	function	ENSEMBL gene ID
1.578449394	0.012112987	chr11	EHD1	protein_coding	ENSG00000110047
1.576074942	0.016481953	chr22	RP11-375H17.1	antisense	ENSG00000226169
1.518260468	0.046382489	chr4	RP11-701P16.5	processed_transcript	ENSG00000251230
1.518260468	0.046382489	chr4	MIR3945	miRNA	ENSG00000266698
1.505171147	0.046901668	chr6	RP11-356I2.2	processed_transcript	ENSG00000235842
1.501702784	0.015347594	chr9	CACFD1	protein_coding	ENSG00000160325
1.501702784	0.015347594	chr9	SLC2A6	protein_coding	ENSG00000160326
1.501702784	0.015347594	chr9	RP13-100B2.4	antisense	ENSG00000227898
1.49495818	0.049556532	chr22	AP000355.2	antisense	ENSG00000228923
1.467430144	0.028457946	chr11	RP11-58K22.5	antisense	ENSG00000254693
1.456100065	0.049415298	chr9	RP11-96L7.2	lincRNA	ENSG00000227269
1.456100065	0.016541538	chr9	Metazoa_SRP	misc_RNA	ENSG00000240235
1.444454268	0.030761321	chr19	AC006262.5	lincRNA	ENSG00000268621
1.441021384	0.001085335	chr6	C6orf223	retained_intron	ENSG00000181577
1.432593211	0.035080838	chr19	snoU13	snoRNA	ENSG00000238486
1.430883727	0.042912192	chr3	XCR1	protein_coding	ENSG00000173578
1.409296074	0.034337489	chr12	HCAR2	protein_coding	ENSG00000182782
1.379969683	0.043211828	chr1	PDE4B	protein_coding	ENSG00000184588
1.358261441	0.020657629	chr4	IL8	protein_coding	ENSG00000169429
1.358261441	0.020657629	chr4	AC112518.3	lincRNA	ENSG00000228277
1.347227679	0.005361996	chr20	TM9SF4	protein_coding	ENSG00000101337
-1.322056969	0.048660256	chr7	AC002480.3	processed_transcript	ENSG00000232759
-1.350060501	0.002737099	chr1	AL138930.1	miRNA	ENSG00000223009
-1.350752668	0.046680949	chr9	RP11-344B5.4	lincRNA	ENSG00000235007
-1.363846275	0.01333402	chr6	SNRPC	protein_coding	ENSG00000124562
-1.410199621	0.008181413	chr6	RP1-293L8.5	antisense	ENSG00000232131
-1.438088747	0.017730138	chr19	APOE	protein_coding	ENSG00000130203
-1.439789416	0.021650591	chr17	RP11-120M18.2	processed_transcript	ENSG00000267009
-1.450802183	0.004685896	chr11	MS4A4A	protein_coding	ENSG00000110079
-1.475016524	0.006456502	chr16	RP11-680G24.5	antisense	ENSG00000260872
-1.497297212	0.009168295	chr8	FABP4	protein_coding	ENSG00000170323
-1.526278807	0.023133039	chr17	SKAP1	protein_coding	ENSG00000141293
-1.574186014	0.04185702	chr19	CTC-490E21.10	lincRNA	ENSG00000269843
-1.601140817	0.000431541	chr11	OR8G7P	unprocessed_pseudogene	ENSG00000197849
-1.601140817	0.000431541	chr11	OR8G1	unprocessed_pseudogene	ENSG00000255298
-1.648621286	0.042950836	chr2	AC092653.5	unprocessed_pseudogene	ENSG00000204872
-1.696480009	0.016851342	chr13	RP11-141M1.3	lincRNA	ENSG00000230490
-1.698362905	0.011217538	chr6	RP1-303A1.1	processed_pseudogene	ENSG00000217746
-1.933742919	0.000134422	chr6	RP11-345L23.1	antisense	ENSG00000235899



SUPPRESSION OF MONOSODIUM URATE CRYSTAL-INDUCED CYTOKINE PRODUCTION BY BUTYRATE IS MEDIATED BY THE INHIBITION OF CLASS I HISTONE DEACETYLASES

Cleophas MC
Crişan TO
Lemmers H
Toenhake-Dijkstra H
Fossati G
Jansen TL
Dinarello CA
Netea MG
Joosten LA

8

Ann Rheum Dis. 2016 Mar;75(3):593-600.

SUMMARY

Objectives: Acute gouty arthritis is caused by endogenously formed monosodium urate (MSU) crystals, which are potent activators of the NLRP3 inflammasome. However, to induce the release of active interleukin (IL)-1β, an additional stimulus is needed. Saturated long-chain free fatty acids (FFA) can provide such a signal and stimulate transcription of pro-IL-1β. In contrast, the short-chain fatty acid butyrate possesses anti-inflammatory effects. One of the mechanisms involved is inhibition of histone deacetylases (HDACs). Here, we explored the effects of butyrate on MSU+FFA-induced cytokine production and its inhibition of specific HDACs.

Methods: Freshly isolated peripheral blood mononuclear cells (PBMCs) from healthy donors were stimulated with MSU and palmitic acid (C16.0) in the presence or absence of butyrate or a synthetic HDAC inhibitor. Cytokine responses were measured with ELISA and quantitative PCR. HDAC activity was measured with fluorimetric assays.

Results: Butyrate decreased C16.0+MSU-induced production of IL-1β, IL-6, IL-8, and IL-1β mRNA in PBMCs from healthy donors. Similar results were obtained in PBMCs isolated from gout patients. Butyrate specifically inhibited class I HDACs. The HDAC inhibitor Panobinostat and the potent HDAC inhibitor ITF-B also decreased ex vivo C16.0+MSU-induced IL-1β production.

Conclusions: In agreement with the reported low inhibitory potency of butyrate, a high concentration was needed for cytokine suppression, while synthetic HDAC inhibitors showed potent anti-inflammatory effects at nanomolar concentrations. These novel HDAC inhibitors could be effective in the treatment of acute gout. Moreover, the use of specific HDAC inhibitors could even improve the efficacy and reduce any potential adverse effects.

INTRODUCTION

Gout is a crystal-induced disease with an increasing prevalence that currently affects up to 4% of adults in developed countries.[1,2] Acute gout is characterized by recurrent self-limiting attacks of joint inflammation. A prerequisite for the acute joint inflammation is the presence of monosodium urate crystals (MSU) with additional inflammatory signals providing a second hit. Formation of MSU crystals is a result of chronic hyperuricemia in selected patients.[3]

Acute gout attacks are dominated by the production of the classical pro-inflammatory cytokine interleukin-1β (IL-1β)[4,5], which is produced by monocytes as inactive pro-IL-1β. Pro-IL-1β can be cleaved to its mature form via activation of the NLRP3 inflammasome and caspase-1, or via other IL-1β-converting enzymes, such as proteinase-3 and elastase.[6-8] MSU crystals are potent activators of the NLRP3 inflammasome and can mediate caspase-1-depentent activation of IL-1β.[8] However, a second signal is required to induce the production of pro-IL-1β. Interestingly, such signal can be induced by saturated long-chain fatty acids[9], which are abundantly present in the blood.

In contrast to long-chain fatty acids, short-chain fatty acids have been reported to exert various opposite anti-inflammatory effects. They are produced in the colon by bacterial fermentation of indigestible dietary fibers. High dose butyrate in particular was found to have immune-modulatory effects; it decreases lipopolysaccharide (LPS)-induced cytokine production and NF-κB activation in human peripheral blood mononuclear cells (PBMCs).[10-12] One of the mechanisms by which butyrate exerts its anti-inflammatory effects is inhibition of histone deacetylases (HDACs).[13,14] Recently, the synthetic pan-HDAC inhibitor Givinostat was shown to have a broad anti-inflammatory activity with beneficial effects in experimental models of arthritis and even led to attenuation of clinical scores in a trial with juvenile idiopathic arthritis patients.[15,16] Inhibition of HDACs might therefore also have beneficial effects in acute gouty arthritis.

In this study, we explore the suppressive effects of butyrate on MSU crystal-induced cytokine production. We confirm that butyrate specifically inhibits class I HDACs and show that butyrate has highest specificity for HDAC8. Additionally, we show the effects of the pan-HDAC inhibitors Givinostat and Panobinostat, as well as those of a selective HDAC8 inhibitor and a potent HDAC inhibitor devoided of class IIa inhibitory activity, on LPS- and MSU crystal-induced cytokine production. With these results, we provide a rationale for further exploring the beneficial effects of specific HDAC inhibitors in gouty arthritis.

METHODS

Human samples

Gout patients visited the outpatient Rheumatology department of the Radboud University Medical Center in Nijmegen, the Netherlands. All patients were diagnosed with crystal-proven gout by an experienced rheumatologist (TJ). The gout patient cohort consisted of 117 volunteers. Written informed consent was received from all donors. Experiments with human blood were performed in accordance with the Declaration of Helsinki.

Reagents

Uric acid, butyric acid and palmitic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). *E. coli* LPS was purchased from Invitrogen (Bleiswijk, the Netherlands) and sodium hydroxide from Merck (Darmstadt, Germany). Human albumin (Albuman) was purchased from Sanquin (Amsterdam, the Netherlands). Panobinostat was purchased from Selleckchem (Munich, Germany), Givinostat and other ITF compounds were kindly provided by Dr. Fossati. Synthetic HDAC inhibitors were dissolved in DMSO, which was present in the cell culture at a maximal concentration of 0.01%.

Palmitic acid and albumin conjugation

Stock palmitic acid was dissolved in 100% ethanol. Palmitic acid (C16.0) and human albumin were conjugated by warming to 37° C in a water bath before adding together in a 1:5 ratio. The mixture was sonicated for 20-25 minutes and kept at 37° C until use. The vehicle control for 50 μM C16.0 consisted of 0.025% albumin and 0.025% ethanol.

Monosodium urate crystal formation

In 400 mL of sterile water, 1.0 gram of uric acid and 0.48 grams sodium hydroxide were dissolved. The pH was adjusted to 7.2 and the solution was sterilized by heating it for 6 hours at 120° C. No LPS-contamination was detected by LAL-assay.

PBMC isolation and stimulation experiments

Venous blood was drawn from healthy donors and gout patients. PBMCs were isolated after Ficoll-Paque density centrifugation and plated in a U-bottom 96-wells plate at $5x10^5$ cells per well. They were cultured for 24 hours with either culture medium, 10 ng/mL *E. coli* LPS, 50 μM C16.0, or a combination of C16.0 with 300 μg/mL MSU crystals. Cells were pre-incubated with butyrate or HDAC inhibitors for 1 hour. In experiments with PBMCs from gout patients, butyrate was added to the cells without pre-incubation.

Cytokine measurements

IL-1β, IL-1Ra, IL-6, IL-8, TNF-α, IL-10, and TGF-β1 protein concentrations were determined with commercially available ELISA kits (R&D Systems, Abingdon, UK; or Sanquin, Amsterdam, the Netherlands), according to the manufacturer's protocol. Intracellular IL-1β was determined after lysing the cells via three freeze-thaw cycles.

RNA isolation, cDNA synthesis and qPCR

RNA was extracted by phase separation with 400 µL TRIzol reagent (Life Technologies, Paisley, UK) and 80 µL of chloroform (Merck, Darmstadt, Germany). RNA was precipitated using 200 µL 2-propanol (Merck, Darmstadt, Germany). Complementary DNA was obtained using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA USA).
Quantitative PCR was performed with SYBR Green PCR Master Mix (Life Technologies, Paisley, UK). Primers were as follows: IL-1β forward primer 5'-GCCCTAAACAGA TGAAGTGCTC-3'; IL-1β reverse primer 5'-GAACCAGCATCTTCCTCAG-3'; GAPDH forward primer 5'-AGGGGAGATTCAGTGTGGTG-3'; and GAPDH reverse primer 5'-CGACCACTTTGTCAAGCTCA-3' (BioLegio, Nijmegen, the Netherlands).

Flow cytometry

Cell death was determined by means of flow cytometry. Cells were stained with Annexin V-FITC (BioVision, Milpitas, CA USA). Annexin V-FITC was diluted 1/100 in $CaCl_2$-enriched RPMI (6 µM) and 100 µL of the diluted Annexin V staining was added to each sample. This was incubated for 15 minutes in the dark on ice. Fluorescence was measured with the Cytomics FC500 (Beckman Coulter, Woerden, the Netherlands).

HDAC activity assay

Soluble human recombinant HDAC enzymes were purchased (HDAC1-10: BPS Bioscience, San Diego, CA; HDAC11: Farmingdale, NY, USA). Fluor de Lys Deacetylase Substrate (Enzo Life Sciences) was used to assay activity of HDAC1, -3, -6, -10 and -11; Fluor de Lys Green Deacetylase Substrate (Enzo Life Science) was sued for HDAC8; and Nε-Trifluoroacetyl-L-lysine was used to assay HDAC4, -5, -7, and -9. Recombinant enzymes were pre-incubated with butyrate at 30 °C in a total volume of 25 µL. Next, 25 µL of substrate was added. A fluorescent signal was generated after adding 50 µl Fluor de Lys Developer (Enzo LifeScience) containing 2 µM Trichostatin A (Sigma-Aldrich, St. Louis, MO, USA). Conditions were optimized for each assay. Positive control consisted of enzyme plus substrate. Fluorescence was measured using a Victor multilabel plate reader (Perkin Elmer, Waltham, MA, USA).

Statistics

In dose-response experiments with butyrate or synthetic HDAC inhibitors, experimental conditions were compared to the shaded control bar using with Friedman's test followed by Dunn's test for multiple comparisons. The same test was used for the gout patient analysis of different treatment groups and time points. All groups were compared to the total gout patient group or time point t = 0, respectively. In gout patient stratification by serum urate levels, the two groups were compared with the Mann-Whitney U test. In all other graphs, means were compared with the Wilcoxon signed-rank test. *p<0.05, **p<0.01, ***p<0.001. Statistical analysis was performed with GraphPad Prism Software (version 6.0f).

RESULTS

Butyrate suppresses C16.0+MSU-induced cytokine production in a dose-dependent manner

A combination MSU crystals and palmitic acid (C16.0) was used to induce potent production of active IL-1β. C16.0 alone induced the production of IL-1β and IL-6, and MSU crystals synergistically amplified this effect (Figures 1A and 1D). Similar results were found for IL-8 (see online supplementary figure S1).

Butyrate suppressed C16.0+MSU-induced IL-1β production in a dose-dependent manner (Figure 1B). An IC50 value of 0,485 mM was calculated based on figure 1B using non-linear regression. This effect of butyrate was specific for C16.0+MSU stimulation. Butyrate even increased the LPS-induced IL-1β production at 5 mM (Figure 1C). Butyrate did not induce cell death in combination with C16.0+MSU (Figure 1E) or LPS (Figure 1F).

Butyrate suppresses C16.0+MSU-induced IL-1β mRNA at 1 mM

Butyrate decreased C16.0+MSU-induced IL-1β, IL-6, and IL-8 production (Figures 2A-C). This suppressive effect was not caused by an increased production of the IL-1 receptor antagonist (IL-1Ra), since the IL-1Ra release was also suppressed by butyrate. In addition, IL-10 or TGF-β1 production was not increased upon stimulation with C16.0+MSU crystals (see online supplementary figure S2).

No difference was observed in intracellular IL-1β between stimulation with C16.0 or C16.0+MSU (Figure 2D). However, there was an increase in IL-1β mRNA and extracellular IL-1β with the combination of C16.0 and MSU crystals compared to C16.0 alone (Figure 2A and F). Butyrate decreased C16.0+MSU-induced IL-1β mRNA levels, as well as intracellular IL-1β (Figure 2D and E). PBMC stimulation with C16.0+MSU induced a dramatic increase of IL-1β mRNA, which reached a plateau at around 16 hours of culture (Figure 2F).

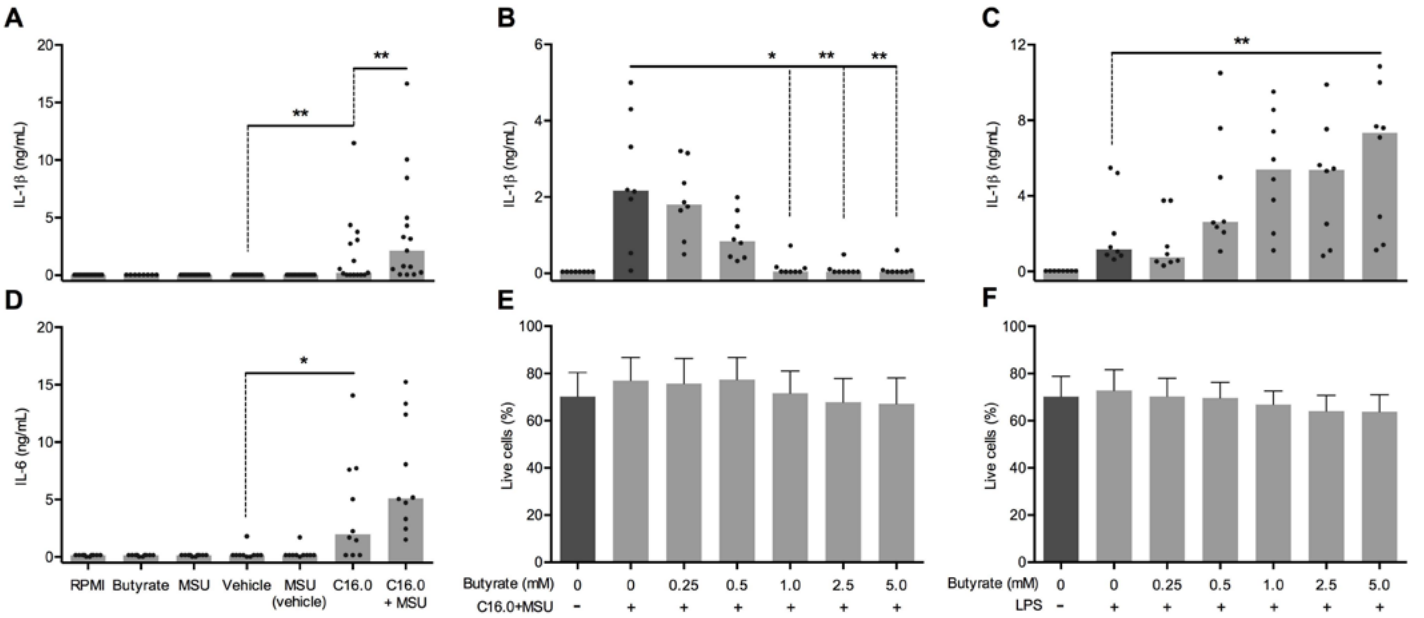


FIGURE 1. Butyrate differentially inhibits C16.0+MSU- and LPS-induced IL-1β production in a dose-dependent manner.

A, B. Freshly isolated PBMCs from healthy volunteers were stimulated for 24 hours with 1 mM butyrate, C16.0, MSU crystals, or a combination of C16.0+MSU. B, C, E, F. PBMCs were pre-incubated for 1 hour with butyrate, and subsequently stimulated for 24 hours with LPS or C16.0+MSU. Release of IL-1== (A-C) and IL-6 (D) in the cell culture supernatant was measured by ELISA. Cell death (E, F) was determined by flow cytometry with Annexn V-FITC. Data in A-D are shown as individual points and median. Data in E and F are shown as mean +/- SD. Panels all include n=6.

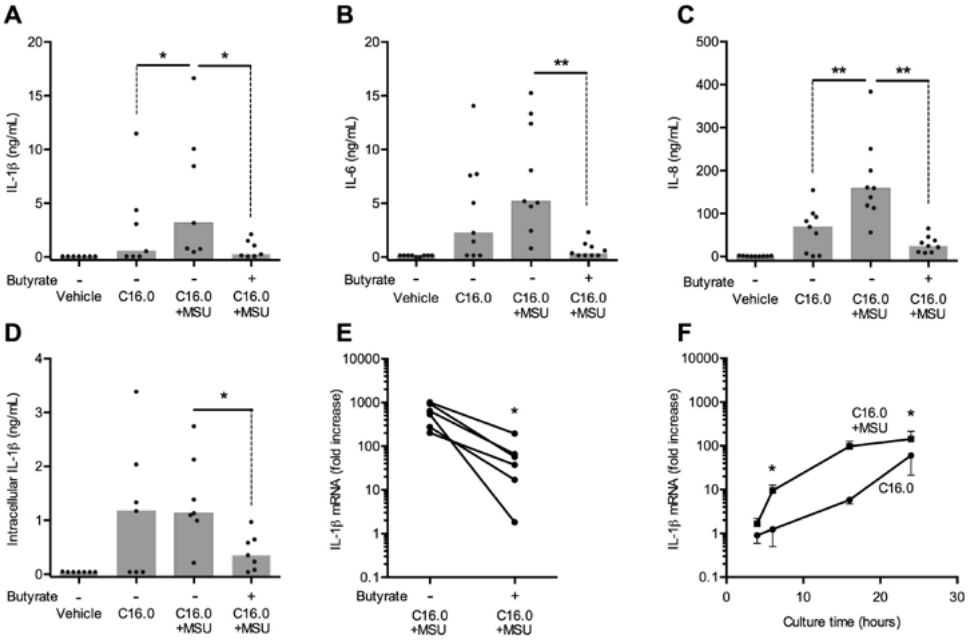


FIGURE 2. Butyrate inhibits both C16.0+MSU-induced intracellular IL-1β and IL-1β mRNA.

Freshly isolated PBMCs from healthy volunteers were pre-incubated for 1 hour with culture medium or 1 mM butyrate, and subsequently stimulated with C16.0 or C16.0+MSU for 24 hours. A-C. Release of IL-1β, IL-6, and IL-8 in the cell culture supernatant was measured by ELISA. D. Intracellular IL-1β was measured after cell lysis by means of three freeze-thaw cycles. E. PBMCs were cultured for 24 hours with C16.0+MSU with or without 1 hour pre-incubation with butyrate. F. PBMCs were cultured for 4, 6, 16 or 24 hours with C16.0 or C16.0+MSU. ELISA data are shown as individual points and median. Data in panel F are shown as mean ± SD. All panels include n=6.

Butyrate inhibits C16.0+MSU-induced IL-1β production in PBMCs from gout patients, regardless of treatment, uric acid level or phase of the disease

The suppressive effects of butyrate were also studied in PBMCs of gout patients. There was no difference in the effect of butyrate on C16.0+MSU-induced IL-1β or IL-6 production between healthy controls and gout patients (Figure 3A and D). Neither did we observe differences when stratifying for medication, serum urate levels or time after last flare (Figure 3B, C, E, F).

Butyrate specifically inhibits class I histone deacetylases

Next, we determined the inhibitory effect of butyrate on the deacetylase activity of all class I, II and IV HDACs separately. Butyrate specifically inhibited class I HDACs with an IC50 between 100-700 μM (Table 1). HDAC10 and 11 were inhibited by butyrate at a much higher concentration, with IC50 values of 1,9 and 2,8 mM, respectively. These results are in

agreement with previously published data on HDAC1-9[17], although it is worth noting that IC50 values in this work are higher due to higher substrate concentrations used in the HDAC assay to assure proper enzyme saturation. Furthermore, our experimental conditions show that HDAC8 is the most sensitive isoform to butyrate inhibition.

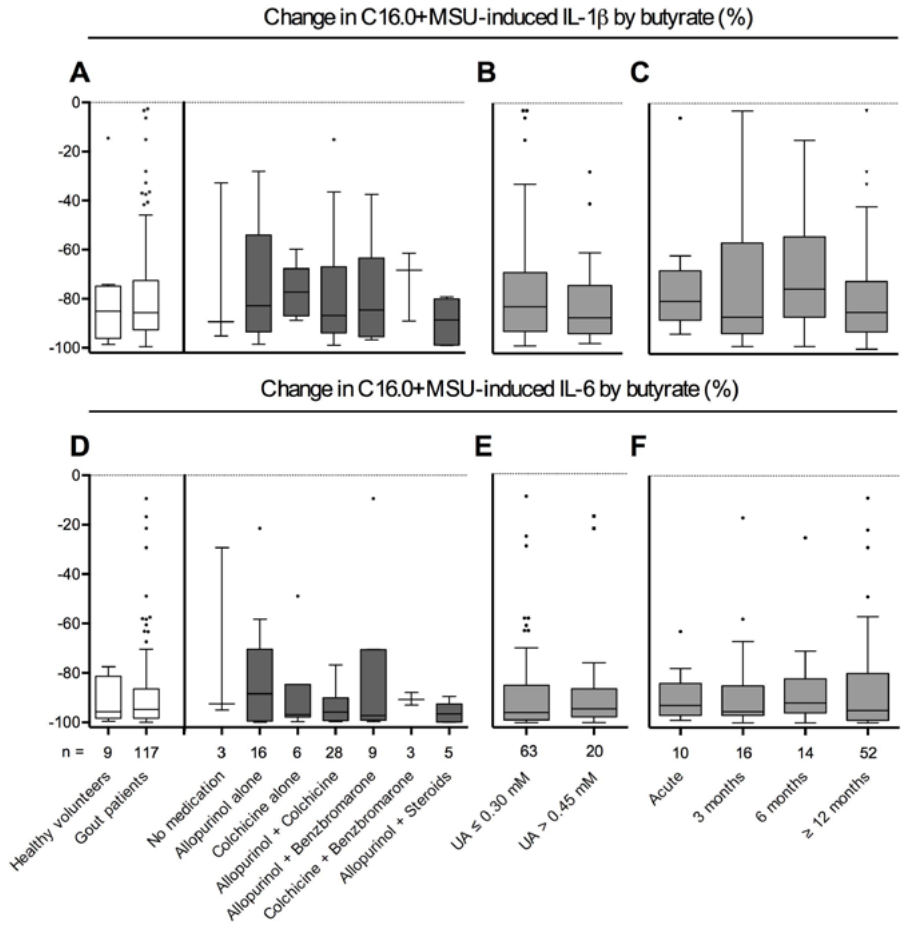


FIGURE 3. Butyrate inhibits C16.0+MSU-induced IL-1β in PBMCs from gout patients regardless of treatment, serum urate levels or phase of the disease.

Freshly isolated PBMCs from gout patients (n=117, mean age 62 ± 14 SD, 85% male) and healthy controls (n=9, mean age 48 ± 14 SD, 78% male) were stimulated with C16.0+MSU alone or in combination with 1 mM of butyrate. Release of IL-1β (A-C) and IL-6 (D-F) in the cell culture supernatant was measured by ELISA. Data are shown as percentage decrease in C16.0+MSU-induced IL-1β or IL-6 by butyrate in Tukey boxplots. Data are stratified by treatment (A, D), serum urate levels (B, E) or months after last gout flare (C, F). The treatment stratification groups in figures 3A and 3D include patients using only the indicated medication. The number of patients in each group is shown beneath the X-axis.

TABLE 1. Inhibition of specific histone deacetylases by butyrate and synthetic HDAC inhibitors

	Class I				Class IIa				Class IIb		Class IV
	HDAC1	HDAC2	HDAC3	HDAC8	HDAC4	HDAC5	HDAC7	HDAC9	HDAC6	HDAC10	HDAC11
Butyrate	712	300	543	106	>	>	>	>	>	1932	2771
Givinostat	100	400	40	150	1059	532	1000	541	35	150	200
Panobinostat	2	14	2	379	554	243	3113	1548	12	7	7
ITF-A	>	>	>	192	>	>	>	>	>	>	>
ITF-B	16	68	11	185	>	>	>	>	>	44	58

Data are presented as IC50 values. For butyrate, the values are in μM; > more than 10 mM. For the synthetic HDAC inhibitors, IC50 values are in nM. ITF-A; > more than 8000 nM. ITF-B; > more than 400 nM.

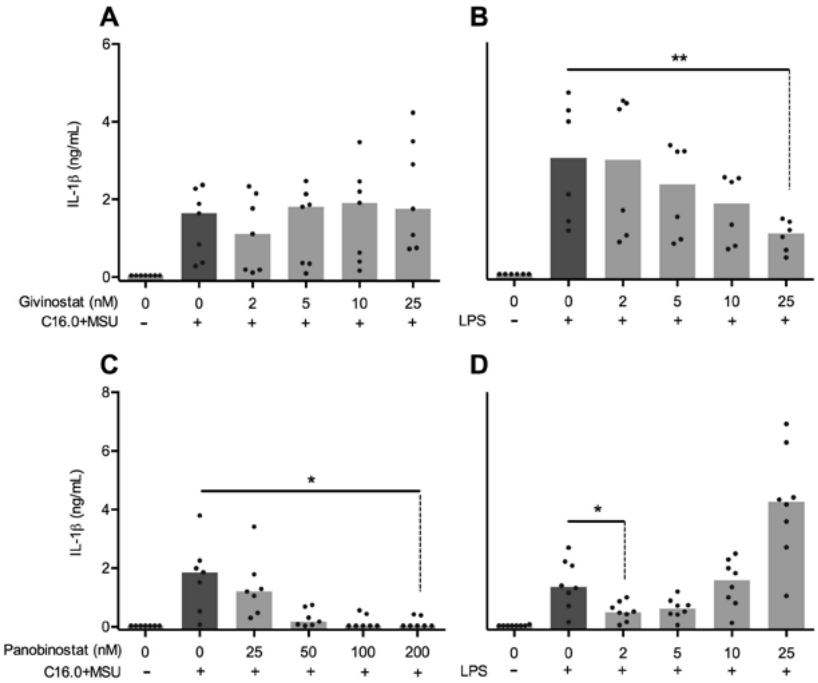


FIGURE 4. C16.0+MSU-induced IL-1β is inhibited by the pan-HDAC inhibitor Panobinostat, but not by Givinostat.

Freshly isolated PBMCs from healthy volunteers were pre-incubated for 1 hour with culture medium, Givinostat (A, B) or Panobinostat (C, D) and subsequently stimulated with C16.0+MSU (A, C) or LPS (B, D) for 24 hours. Release of IL- β in the cell culture supernatant was measured by ELISA. Data are shown as individual points and median. All panels include n=6.

Pan-HDAC inhibitor Panobinostat decreases C16.0+MSU-induced IL-1β production

The effects of butyrate were compared to two well-known pan-HDAC inhibitors, Givinostat and Panobinostat. Givinostat did not decrease C16.0+MSU-induced IL-1β, but LPS-induced IL-1β production was significantly decreased with 25 nM of Givinostat (Figure 4A and B). Panobinostat showed effects very similar to butyrate. It effectively decreased C16.0+MSU-induced IL-1β in a dose dependent manner. Conversely, it decreased LPS-induced IL-1β production only in the low concentration range (Figure 4C and D). Givinostat and Panobinostat do not affect PBMC viability after 24-hour culture (see online supplementary figure S3, A and B).

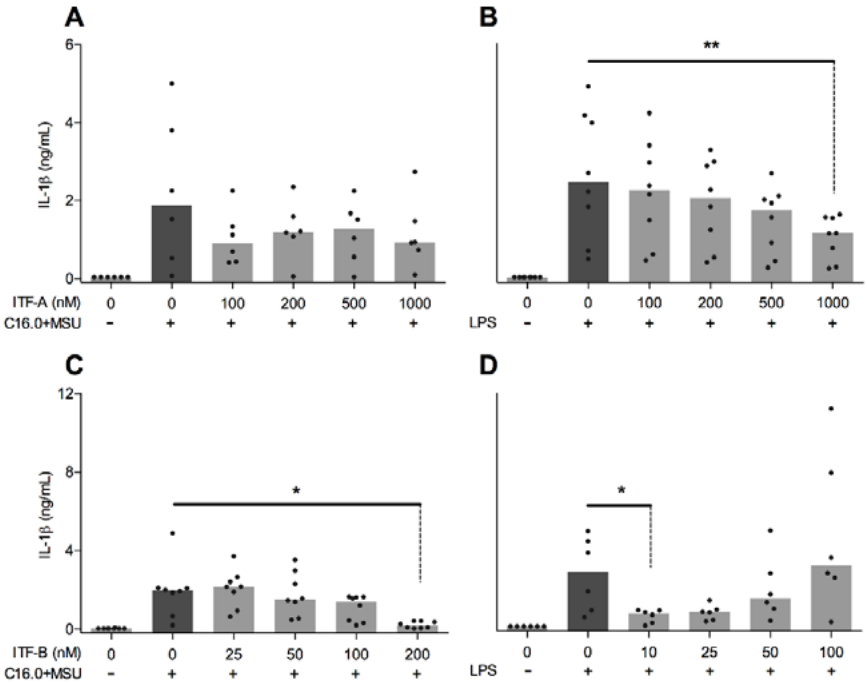


FIGURE 5. C16.0+MSU-induced IL-1β is inhibited by HDAC inhibitor ITF-B, but not by HDAC8 inhibition alone.

Freshly isolated PBMCs from healthy volunteers were pre-incubated for 1 hour with culture medium, HDAC8 inhibitor ITF-A (A, B) or HDAC inhibitor ITF-B (C, D) and subsequently stimulated with C16.0+MSU (A, C) or LPS (B, D) for 24 hours. Release of IL-1β in the cell culture supernatant was measured by ELISA. Data are shown as individual points and median. All panels include n=6.

HDAC inhibitor ITF-B decreases C16.0+MSU-induced IL-1β production

Since butyrate most strongly inhibits HDAC8, we studied the effects of a highly specific HDAC8 inhibitor, ITF-A (Table 1). Addition of ITF-A did not significantly decrease C16.0+MSU-induced IL-1β production (Figure 5A). However, it decreased LPS-induced IL-1β in a dose-dependent manner (Figure 5B). Next, we tested an HDAC inhibitor (ITF-B) that resembles butyrate in that it inhibits HDAC 1, 2, 3, 8, 10 and 11. Its effects on C16.0+MSU- and LPS-induced IL-1β are very similar to butyrate. ITF-B inhibits C16.0+MSU-induced IL-1β production in a dose-dependent manner. In contrast, LPS-induced IL-1β production is only inhibited by ITF-B at 10 nM, and in some donors even increases IL-1β production at higher concentrations. ITF-A and ITF-B do not induce cell death in human PBMCs in a 24-hour culture (see online supplementary figure S3, C and D).

DISCUSSION

In the present study, we have explored the capacity of butyrate and synthetic HDAC inhibitors to suppress *ex-vivo* cytokine production. To study MSU crystal-induced cytokine production, a priming stimulus is needed for transcription of pro-IL-1β. In all our experiments, we used a combination of C16.0 and MSU crystals. MSU crystals alone did not result in cytokine production, but C16.0+MSU induced a synergistic increase in IL-1β, IL-8 and IL-1Ra production. This is in line with previously reported synergy of MSU crystals with stearic acid.[9] Butyrate inhibited C16.0+MSU-induced cytokine production in a dose-dependent manner. Furthermore, we have shown that butyrate decreased both IL-1β mRNA and intracellular IL-1β. Based on this, we can conclude that butyrate exerts its effects at the level of transcription or translation and does not result from inhibition of activation or excretion of IL-1β. In contrast, LPS-induced IL-1β was significantly increased with 5 mM butyrate.

Butyrate is known to inhibit histone deacetylases. In agreement with published results[17], we show that butyrate inhibited class I HDACs specifically, with highest specificity for HDAC8. Additionally, the IC50 of butyrate with regard to suppression of C16.0+MSU-induced IL-1β is in the same range as the IC50 values for inhibition of the different class I HDACs, suggesting that this effect of butyrate is indeed mediated though class I HDAC inhibition.

Currently, 11 human HDACs and 7 sirtuins have been identified, which are classified according to their homology to different yeast HDACs.[18] The molecular mechanism by which HDAC inhibitors elicit anti-inflammatory effects remains unknown. Lysine acetylation modulates both histone and non-histone proteins, and affects a wide range of cellular processes.[19] The role of each individual HDAC in inflammation is still largely unknown. However, different HDACs may have opposing effects in inflammatory pathways, depending on the stimulus, cell type and site of lysine deacetylation.[20] For this reason, it is crucial to identify the effects of individual HDACs and to develop safe specific HDAC inhibitors, reducing the adverse

effects of inhibiting other HDACs.

Although butyrate effectively inhibits C16.0+MSU-induced cytokine production *ex vivo*, its use as possible treatment for gout has to be considered carefully. Butyrate concentrations used in our experiments are higher than its physiological concentration in peripheral blood, which lies around 4 μM.[21] Butyrate is naturally present mainly in milk products, but the majority of butyrate is produced endogenously via bacterial fermentation of indigestible fibres in the gut. Foods that are high in indigestible fibres are mainly whole grains and beans. To our knowledge, it is unknown to what extend a high fibre diet can increase human plasma butyrate levels. Although it is possible to enhance serum butyrate concentration in subjects by oral intake of the prodrug tributyrin,[22] no data are yet available for the validity of this approach. However, synthetic specific HDAC inhibitors are very potent at extremely low concentrations, making them attractive therapeutic agents for many different inflammatory diseases.

The pan-HDAC inhibitor Panobinostat[23] effectively decreased C16.0+MSU-induced IL-1β, whereas LPS-induced IL-1β was only decreased at 2 nM. These dose-dependent effects of Panobinostat closely resemble the effects of butyrate, suggesting a similar mechanism is involved. Addition of Givinostat, which is a well-known pan-HDAC inhibitor that was reported to have beneficial effects in several forms of arthritis *in vivo*,[15,16] decreased LPS-induced IL-1β at 25 nM. This is consistent with a previous study in human PBMCs.[24] However, Givinostat had no significant suppressive effect on C16.0+MSU-induced IL-1β.

The HDAC inhibitor ITF-B resembles butyrate not only in its specificity for HDACs, but also in its effects on LPS- and C16.0+MSU-induced IL-1β. At the concentrations used in our experiments, ITF-B has higher specificity for HDAC10 and -11 than butyrate. However, the resemblance between their effects on cytokine production is a clear indication that specific class I HDAC inhibition is the causative mechanism. ITF-A, a highly specific HDAC8 inhibitor, had no significant effect on C16.0+MSU-induced IL-1β production, suggesting that sole HDAC8 inhibition is insufficient to account for the cytokine-suppressing effect of butyrate. These findings with specific HDAC inhibitors indicate that the suppressive effects of butyrate are caused by class I HDAC inhibition, and in particular via inhibition of HDAC1, -2 and/or -3. This conclusion is supported by the observation that Panobinostat strongly inhibited IL-1β production at 200 nM. At this concentration, class IIa HDACs should be minimally affected by this inhibitor, while class I, IIb and IV should be well inhibited. Since butyrate and Panobinostat show a similar effect and the former inhibits HDAC10 and HDAC11 at high concentrations, inhibition of class I appears to be necessary for the inhibition of IL-1β induced by C16.0+MSU. Inhibition of HDAC10 and 11 might also play a role, but should be minor in the case of butyrate since it almost totally inhibited IL-1β at 1 mM, well below the IC50 for HDAC10 and 11 inhibition (Table 1).

The exact mechanism by which HDAC inhibitors suppress cytokine production is subject to much debate. An obvious candidate for the observed general cytokine suppression is NF-κB. NF-κB signalling can be regulated by acetylation at multiple levels,[25,26] but the functional effects of acetylation appear to be highly dependent on the protein and specific lysine residue that is targeted, the pro-inflammatory stimulus used and the cell type. HDAC1, -2 and -3 have all been reported to target NF-κB subunits. Both HDAC1 and HDAC2 have been described to suppress NF-κB signalling.[27-29] For HDAC3, both inhibitory[30] and stimulatory[31,32] effects on NF-κB signalling have been described. Next to direct deacetylation of NF-κB subunits, HDAC3 can also potentiate NF-κB activation through repression of nuclear receptors such as peroxisome proliferator-activated receptor γ[33], which has been implicated as a mechanism in the anti-inflammatory effects of butyrate.[34-36]

Opposing effects of HDACs within inflammatory signalling pathways make it difficult to pinpoint the exact mechanism of butyrate. After identification of the specific HDAC(s) involved, experiments with highly specific synthetic HDAC inhibitors should be performed to study mechanisms behind the cytokine-suppressing effect. Additionally, finding anti-inflammatory genes that are up-regulated by butyrate would warrant us to study the epigenetic profiles within these genes.

In conclusion, this study shows that butyrate inhibits C16.0+MSU-induced cytokine production at the mRNA level. This effect is most likely caused by inhibition of HDAC1, -2 and/or -3. However, these three HDACs might distinctly regulate cytokine production in LPS- and C16.0+MSU-stimulated PBMCs. Therefore, inhibition of all three HDACs may explain the different effects between the two stimuli. Although this study warrants further research on the function of each HDAC in gout, lysine acetylation is an important regulatory mechanism in inflammation and targeting specific HDACs could be a promising treatment option for gouty arthritis.

Acknowledgements

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Competing interest

None declared.

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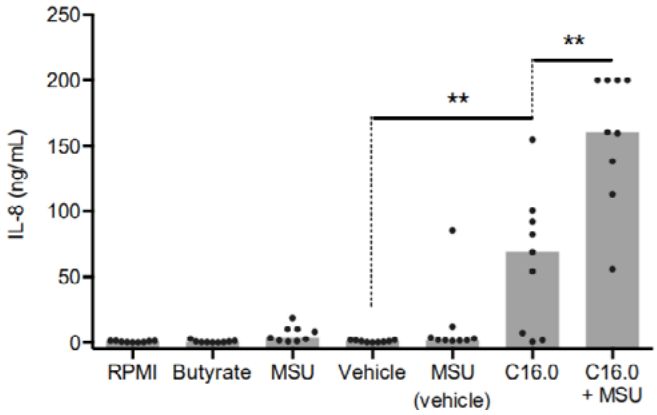
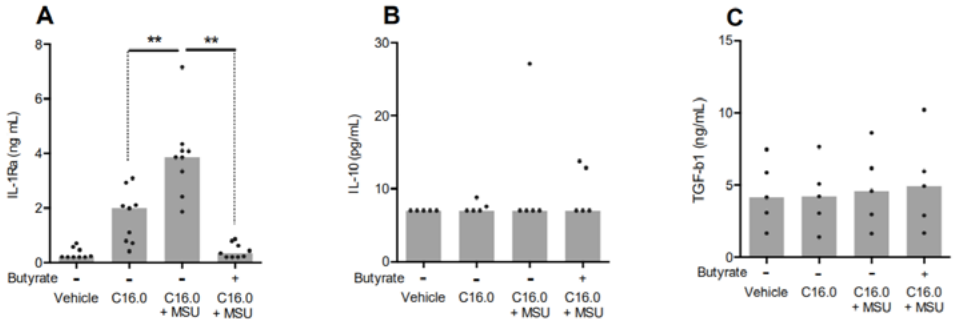
SUPPLEMENTARY INFORMATION



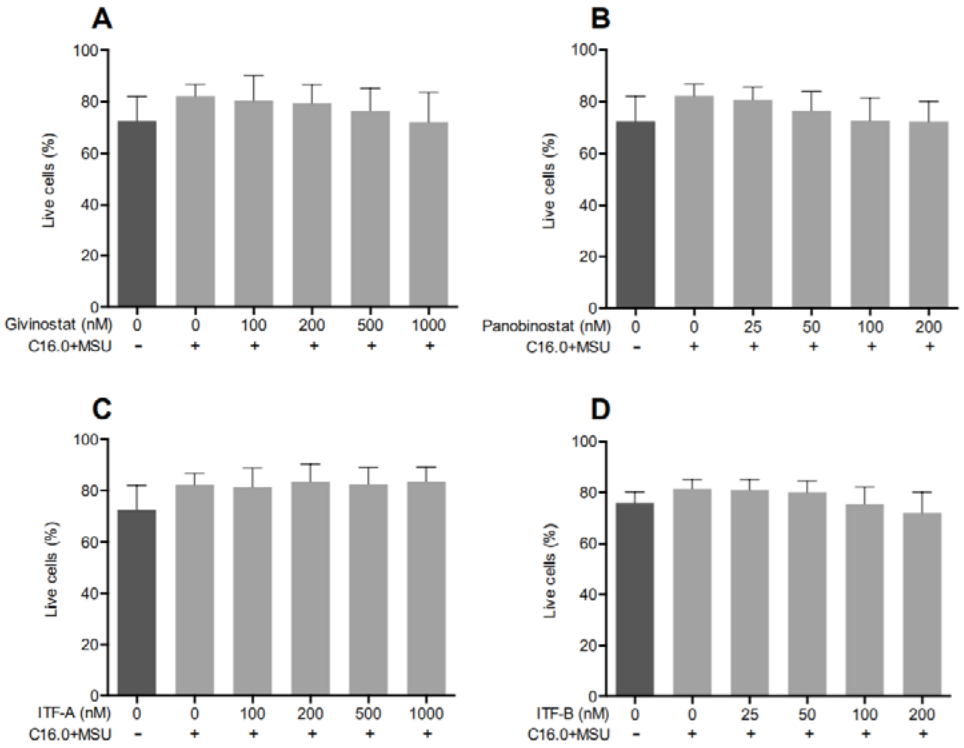
SUPPLEMENTARY FIGURE S1. The combination of C16.0 and MSU crystals produce a synergistic IL-8 response in human PBMCs.

Freshly isolated PBMCs from healthy volunteers were stimulated with 1 mM butyrate, 300 µg/mL MSU crystals, 50 µM C16.0 or a combination of C16.0+MSU for 24 hours. Release of IL-8 in the cell culture supernatants was measured by ELISA. Data are shown as individual points with a bar representing the median, **p<0,01, n=9.



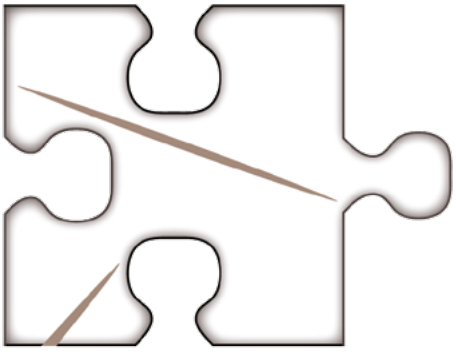
SUPPLEMENTARY FIGURE S2. Butyrate inhibits C16.0/MSU-induced IL-1Ra.

Freshly isolated PBMCs from healthy volunteers were pre-incubated for 1 hour with culture medium or 1 mM butyrate and subsequently stimulated with 50 µM C16.0 or a combination of 50 µM C16.0 and 300 µg/mL MSU crystals for 24 hours. Release of IL-1Ra, IL-10, and TGF-β1 in the cell culture supernatant was measured by ELISA. Data are shown as individual points with a bar representing the median, **p<0,01, n=9 in A and n=5 in B and C.



SUPPLEMENTARY FIGURE S3. HDAC inhibitors in combination with C16.0+MSU do not cause cell death in PBMCs after 24h culture.

Freshly isolated PBMCs from healthy volunteers were pre-incubated for 1 hour with culture medium or a synthetic HDAC inhibitor (Givinostat, Panobinostat, ITF-A or ITF-B) and subsequently stimulated with a combination of 50 µM C16.0 and 300 µg/mL MSU crystals for 24 hours. Cell death was assessed by flow cytometry. Cells were stained with Annexin V-FITC and presented as percentage of live cells (FITC-negative population). All conditions include n=4, except ITF-B 25 and 50 nM (n=2).



ALPHA-1-ANTI-TRYPSIN FC FUSION PROTEIN AMELIORATES GOUTY ARTHRITIS BY REDUCING RELEASE AND EXTRACELLULAR PROCESSING OF IL-1β, AND BY THE INDUCTION OF ENDOGENOUS IL-1RA

Joosten LA
Crişan TO
Azam T
Cleophas MC
Koenders MI
van de Veerdonk FL
Netea MG
Kim S
Dinarello CA

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Ann Rheum Dis. 2016 Jun;75(6):1219-27.

SUMMARY

Objectives: In the present study we generated a new protein, recombinant human alpha-1-anti-trypsin (AAT)-IgG1 Fc fusion protein (AAT-Fc), and evaluated its properties to suppress inflammation and IL-1β in a mouse model of gouty arthritis.

Methods: A combination of monosodium urate (MSU) crystals and the fatty acid C16.0 (MSU/C16.0) was injected intra-articularly into the knee to induce gouty arthritis. Joint swelling, synovial cytokine production and histopathology were determined after 4 hours. AAT-Fc was evaluated for inhibition of MSU/C16.0-induced IL-1β release from human blood monocytes and for inhibition of extracellular IL-1β precursor processing.

Results: AAT-Fc markedly suppressed MSU/C16.0-induced joint inflammation by 85-91% ($p<0.001$). *Ex vivo* production of IL-1β and IL-6 from cultured synovia were similarly reduced (63 and 65%, respectively). The efficacy of 2.0 mg/kg AAT-Fc in reducing inflammation was comparable to 80 mg/kg of plasma-derived AAT. Injection of AAT-Fc into mice increased circulating levels of endogenous IL-1Ra by 4-fold. We also observed that joint swelling was reduced by 80%, cellular infiltration by 95% and synovial production of IL-1β by 60% in transgenic mice expressing low levels of human AAT. *In vitro*, AAT-Fc reduced MSU/C16.0-induced release of IL-1β from human blood monocytes and inhibited proteinase-3-mediated extracellular processing of the IL-1β precursor into active IL-1β.

Conclusions: A single low dose of AAT-Fc is highly effective in reducing joint inflammation in this model of acute gouty arthritis. Considering the long-term safety of plasma-derived AAT use in humans, subcutaneous AAT-Fc emerges as a promising therapy for gout attacks.

INTRODUCTION

Several studies demonstrate that the inactive IL-1β precursor can be processed into an active cytokine independently of caspase-1 (reviewed in [1-3]). The proteinases that that are responsible for such caspase-1-independent conversion of IL-1β are primarily found in neutrophils, which are the main inflammatory cells in gouty arthritis. The neutrophil serine proteases proteinase-3 (PR3), neutrophil elastase and cathepsin G cleave the precursor within a few amino acids of the caspase-1 site at N-terminal 117 [1]. Dipeptidyl peptidase I (DPPI)-deficient mice are unable to activate neutrophil serin-proteases. However, treatment with a specific caspase-1 inhibitor provides protection against IL-1β-mediated cartilage damage caused by chronic destructive joint inflammation [2]. These data support the concept that targeting IL-1β release by dual inhibition of caspase-1 and serine proteinases would be of therapeutic value in neutrophil-rich inflammation.

The major natural inhibitor of serine proteases is alpha-1-anti-trypsin (AAT). Serum levels in healthy subjects range from 1-3 mg/mL, but rise during infections and inflammatory diseases. In addition to the inhibition of serine proteinases, AAT possesses a broad spectrum of anti-inflammatory and immune-modulatory properties [4, 5] independent of protease inhibition [6]. AAT inhibits caspase-1, -3 and -8; down-regulates TLR2 and TLR4 expression on immune cells; and is incorporated in lipid rafts, which inhibits cell activation and increases the expression of angiopoietin-like protein-4 [7-11]. In addition, peripheral blood mononuclear cells (PBMC) from human subjects deficient for AAT produce higher levels of inflammatory cytokines when stimulated *ex vivo* [12].

In the present study, we developed AAT-Fc, a new recombinant form of human AAT fused with the Fc domain of human IgG1, and evaluated its ability to suppress gouty arthritis, a classic IL-1β-mediated disease [13-15]. Based on several trials, IL-1β is clearly the causal cytokine in acute gout attacks. Reduced clinical manifestations and number of attacks have been demonstrated for the IL-1 receptor antagonist (IL-1Ra) anakinra, the IL-1 trap rilonacept and the human monoclonal anti-IL-1β canakinumab. In the present studies, experimental gouty arthritis was elicited by intra-articular (i.a.) instillation of MSU crystals mixed with palmitic acid (MSU/C16.0). Following the i.a. injection, there is joint swelling, influx of inflammatory cells and synovial cell cytokine production. We compared the effects of plasma-derived AAT to AAT -Fc on joint swelling, release of MSU/C16.0-induced IL-1β from human blood monocytes, PR3-dependent processing of the IL-1β precursor and the *in vitro* as well as *in vivo* induction of IL-1Ra. We also studied gouty arthritis in transgenic mice expressing human AAT. We conclude that AAT-Fc could be a safe and novel therapy for the treatment of MSU-mediated inflammation.

MATERIALS AND METHODS*

*A detailed Materials and Methods version is provided in the Supplementary information of the manuscript.

Animals

Male C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Human AAT transgenic mice (AAT-tg) were a kind gift from A. Churg [16]. Mice (either sex) were used at 10-12 weeks. The animal experiments were approved by the ethical committees of the University of Colorado (Denver, USA) and the Radboud University Medical Center (Nijmegen, The Netherlands).

MSU/C16.0-induced gouty arthritis

Joint inflammation was induced by i.a. injection of 300μg MSU crystals mixed with 200μM C16.0/BSA in 10μL PBS into the right knee joint of naive mice. Four hours after i.a. injection, macroscopic joint swelling was determined. Synovial tissue was isolated and either cultured for 2 hours in tissue culture medium at 37ºC or transferred directly into 200μL Triton X 100 (0.5% in PBS). In addition, knee joints were removed for histology. The MSU-induced peritonitis model (3mg MSU i.p.) was used to validate AAT-Fc treatment of the gouty arthritis model.

AAT treatment of gouty arthritis

Mice were injected intraperitoneally (i.p.) with 50μg AAT-Fc fusion protein 2h, 24h, 48h, or 72h prior to induction of gouty arthritis. Plasma-derived AAT (pd-AAT) (Prolastin C, Grifols, Barcelona, Spain) was purchased from the University of Colorado Pharmacy. Recombinant IL-1Ra (anakinra, SOBI, Stockholm, Sweden) was injected i.p. 2h before gouty arthritis induction. Ivlg (Gamunex, Grifols) or bovine serum albumin (BSA, Sigma-Aldrich, St-Louis, USA) was used as negative control in doses ranging from 50μg to 2mg.

RESULTS

Intra-articular versus systemic treatment of gouty arthritis with human AAT

To explore the anti-inflammatory efficacy of AAT in gouty arthritis, we investigated whether human AAT suppresses acute joint inflammation elicited by MSU/C16.0 when administered locally or systemically. As shown in Figure 1A/B, an i.a. dose of 2μg AAT-Fc alone did not induce joint inflammation or cell influx into the joint cavity. Next, we injected MSU/C16.0 i.a. together with 2μg AAT-Fc and observed a marked suppression of joint inflammation (Figure 1A/B). Local IL-1β and IL-6 production from synovial tissue extracts was also decreased (Supplemental Figure 1). We next investigated the ability of systemically administered AAT to suppress MSU/C16.0-induced intra-articular inflammation.

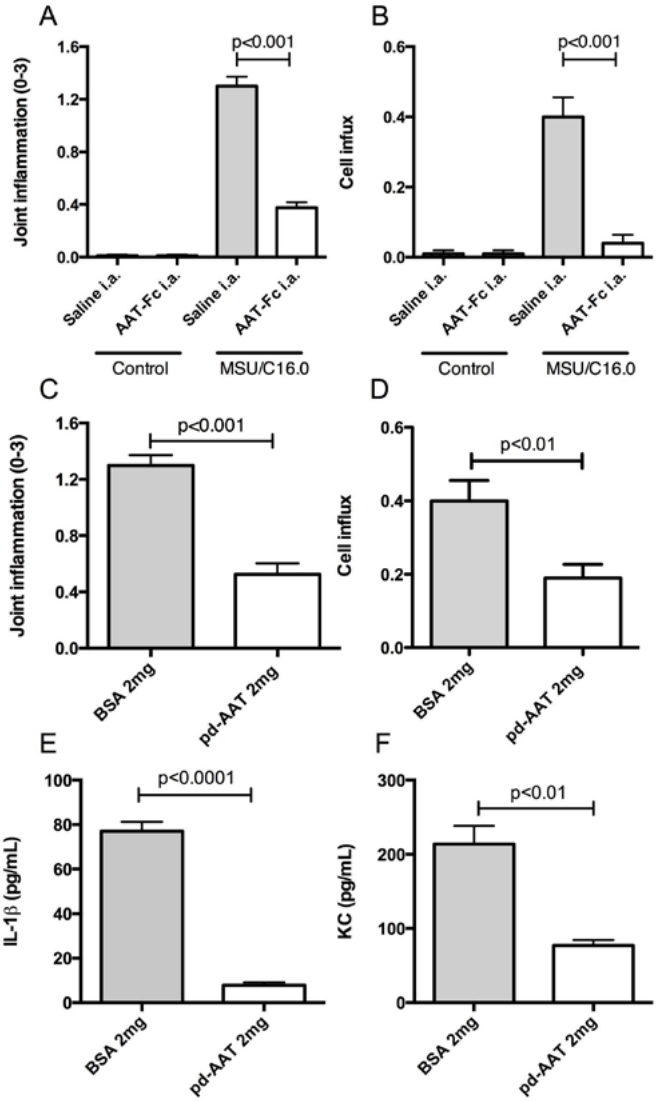

FIGURE 1. Intraarticularly (i.a.) application of AAT-Fc versus plasma derived AAT.

A. C56/Bl6 mice were i.a. injected with 6ul of either saline, AAT-Fc (2μg), MSU/C16.0 (300μg/200mM) or MSU/C16.0 (300μg/200mM) plus AAT-Fc (2μg). After 4h, joint inflammation was scored macroscopically after the skin was removed. 5 mice per group, 10 joints were injected, right and left. **B.** The number of inflammatory cells in the joint cavity and synovial lining were determined. 10 joints per groups were scored. **C.** Systemic treatment of plasma derived (pd)AAT. Mice were pre-treated for 1 hour with either 2mg BSA or 2mg pd-AAT. Thereafter gouty arthritis was induced by intraarticular injection of MSU/C16.0 (300μg/200mM). Joint inflammation was determined by macroscopic scoring. 5 mice per, 10 joints were injected. **D.** Cell influx. **E/F.** IL-1β and KC release of synovial tissue explants. Synovial tissue specimens were cultured for 2h in RPMI. Murine IL-1β and KC were determined by ELISA. Data are expressed as mean+/-SD. Mann-Whitney U-test was used for statistical analysis. The experiment was repeated once with similar results.

Human plasma-derived AAT was administered i.p. at a dose of 2mg per mouse (80mg/kg), which is comparable to the dose infused into humans treated weekly for AAT deficiency. Two hours later, MSU/C16.0 was injected i.a. After an additional four hours, the joints were examined and the degree of inflammation was scored. As shown in Figure 1C, there was significant reduction in joint inflammation. Consistent with these findings, the number of inflammatory cells in the joint cavity was also reduced (Figure 1D). In addition, both IL-1β and KC (murine homolog of IL-8) levels were decreased in AAT-treated mice compared to the vehicle control group (Figure 1E/F).

Comparison of plasma derived AAT and AAT-Fc fusion protein

We next compared plasma-derived AAT (Prolastin C) with recombinant AAT-Fc. Mice were given an i.p. injection of plasma-derived AAT (2mg) or AAT-Fc (50μg). After 2 hours, all mice received an i.a. injection of MSU/C16.0. Similar to the data shown in Figure 1C, 2mg plasma-derived AAT significantly reduced joint inflammation (Figure 2A, $p<0.001$). However, AAT-Fc at a dose of 50μg (40 times less) resulted in a similar reduction ($p<0.001$). In contrast, 50μg plasma-derived AAT had no effect (Figure 2A). The reduction in synovial extract IL-1β (Figure 2B) and IL-6 levels (Figure 2C) was comparable between mice treated with 2mg plasma-derived AAT and mice treated with 50μg AAT-Fc. Gouty arthritis attacks respond rapidly to treatment with the IL-1 receptor blocker anakinra [17-19]. Therefore, we also compared AAT-Fc treatment to treatment with anakinra. We used a high dose of 10mg anakinra (400mg/kg) in order to achieve total IL-1 receptor blockade. As depicted in Figure 2D, 50μg AAT-Fc reduced joint inflammation to a greater extent than total IL-1R blockade by anakinra. The reductions of synovial tissue levels of IL-1β (Figure 2E) and IL-6 (Figure 2F) were comparable for both treatments. To corroborate the above findings of AAT-Fc in a different MSU crystal-induced inflammation model, we applied AAT-Fc treatment on the MSU crystal-induced peritonitis model. Supplemental Figure 2A shows that AAT-Fc treatment had a similar suppressive effect as seen in the gouty arthritis model. Interestingly, again the anti-inflammatory effects of AAT-Fc treatment are comparable to high dose IL-1Ra treatment (Supplemental Figure 2B).

Prolonged protection of AAT-Fc fusion protein in gouty arthritis

A single injection of 50μg AAT-Fc 2h before the i.a. MSU/C16.0 injection results in a marked reduction in joint inflammation as well as cytokine production. AAT-Fc is comprised of two molecules of AAT (MW=55 kDa) plus the Fc domain of IgG (MW=172kDa). Therefore, AAT-Fc blood levels should be more constant compared to the monomeric plasma-derived AAT. For that reason, we administered 50μg AAT-Fc 72h prior to i.a. MSU/C16.0 injection. As shown in Figure 3A, AAT-Fc given i.p. 72h before MSU/C16.0 was not effective.

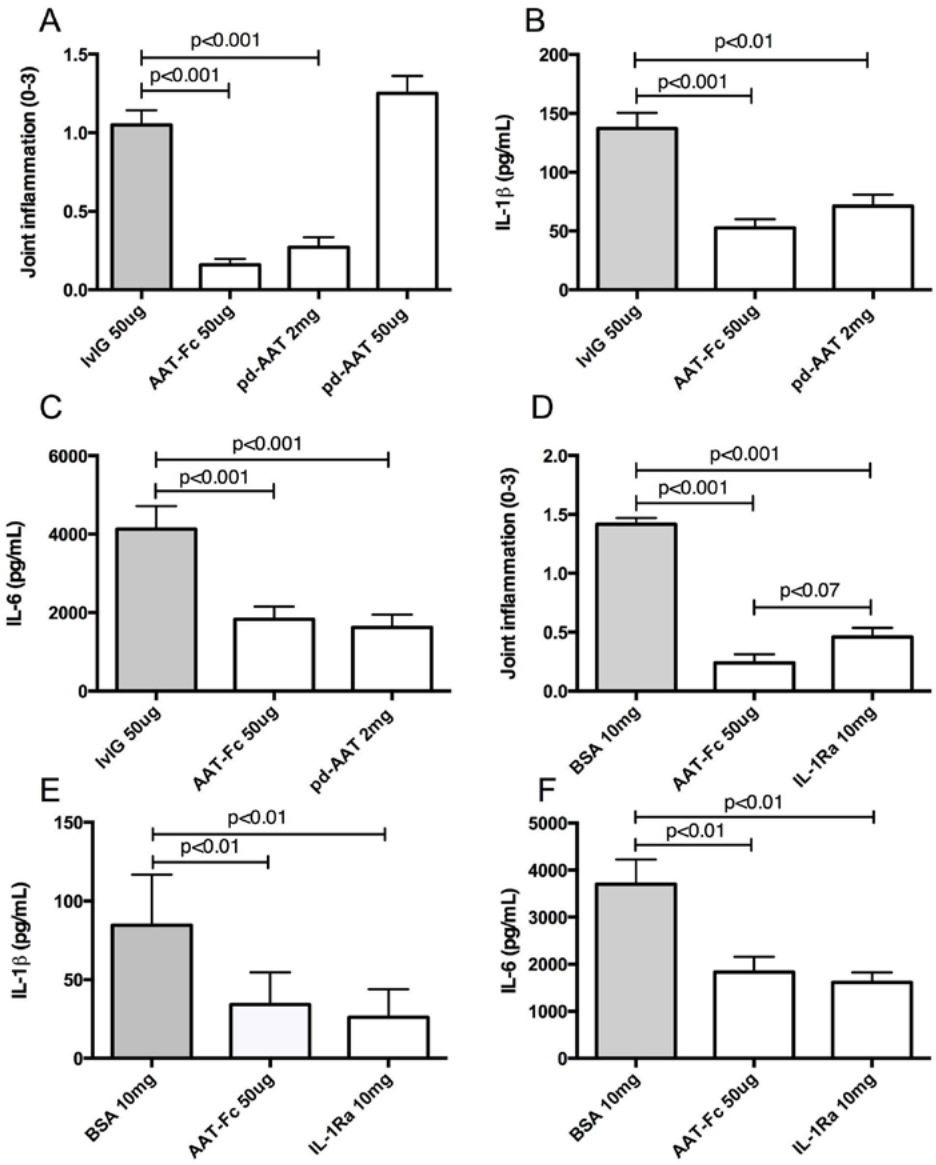

FIGURE 2. Systemic administration of AAT-Fc, pd-AAT and Anakin ra (IL-1ra).

Mice were injected intraperitoneally with either 50 µg Ivlg, 50 µg AAT-Fc, 50µg or 2mg pd-AAT one hour before gouty arthritis was induced by i.a. injection of MSU/C16.0 (300 µg /200mM). **A/D.** Joint inflammation determined at 4h after induction of gouty arthritis. N=5 mice per group, 10 knee joints were examined. **B/C/E/F.** Synovial tissue specimens were examined for IL-1β and IL-6 production. After isolation the explants were transferred into 200 µl Triton X (0.05% in PBS). Cytokines were measured by ELISA. N=5 explants per group. Data are expressed as mean+/-SD. Mann-Whitney U-test was used for statistical analysis. These experiments were repeated once with similar results.

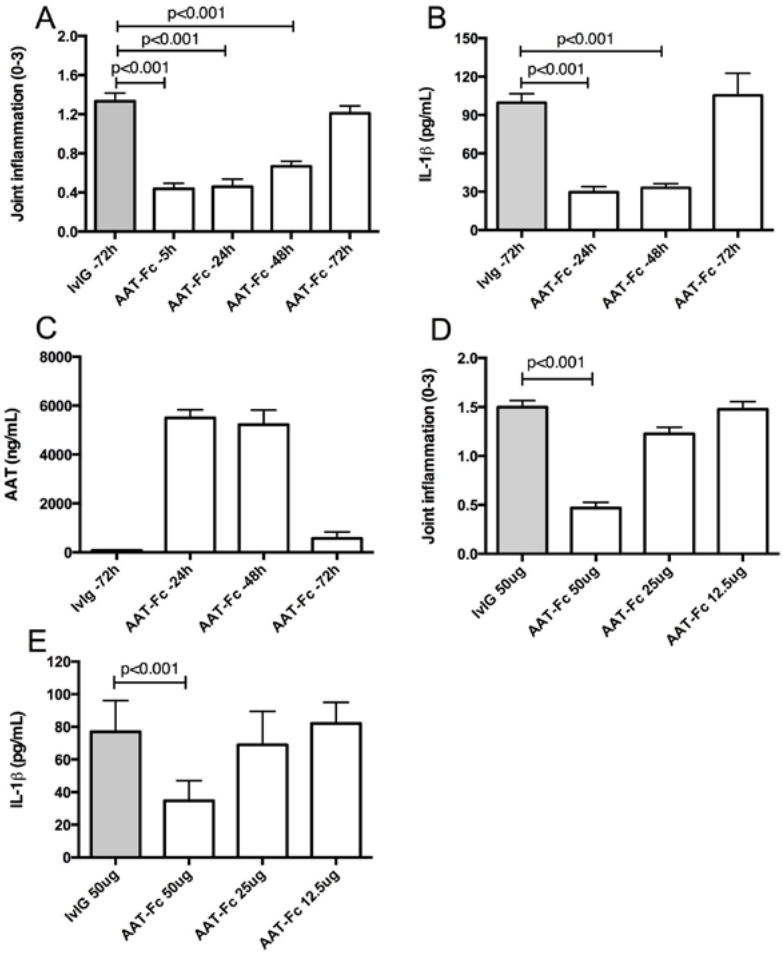

FIGURE 3. Time and dose dependent suppression of gouty arthritis by AAT-Fc.

A. C57/Bl6 mice were injected with 50μg of AAT-Fc or 50μg IvIg at several time point before induction of gouty arthritis. Joint swelling was scored 4h after injection of MSU/C16.0. **B.** IL-1β levels in synovial tissue specimens. **C.** Serum human AAT-Fc levels at different time points after i.p. injection of AAT-Fc. **D.** Dose-response of AAT-Fc. Mice were injected with of AAT-Fc or IvIg 1h before induction of gouty arthritis. **E.** Synovial tissue specimens were examined for IL-1β. For details see Figure 1 and Figure 2. Data are expressed as mean+/-SD. Mann-Whitney U-test was used for statistical analysis. These experiments were repeated once with similar results.

However, when 50μg AAT-Fc was injected 48h or 24h prior to induction of gouty arthritis, the reduction in joint inflammation was comparable to that observed when AAT-Fc was injected 2h prior to MSU/C16.0 (Figure 3A). The reduction in joint inflammation was consistent with lower levels of IL-1β released from the synovial specimens when AAT-Fc was administered either 48 or 24 hours before MSU/C16.0 (Figure 3B). We also measured the concentrations of human AAT in the circulation in mice that received AAT-Fc at different

time points. Figure 3C shows that the concentration of AAT-Fc declined after 72h, which is consistent with the lack of efficacy of AAT-Fc at that time point.

Dose-response of AAT-Fc

C57Bl/6 mice were injected systemically with 50, 25 or 12.5µg AAT-Fc 2 hours prior to the induction of gouty arthritis. Figure 3D reveals a clear dose-dependent effect. A dose of 50µg AAT-Fc provided optimal anti-inflammatory activity, which decreases progressively at 25 or 12.5µg AAT-Fc. Again, the dose-dependent reduction in joint inflammation (Figure 3D) was paralleled by a decrease in local IL-1β production (Figure 3E). Histological analysis revealed that 50µg AAT-Fc decreased the influx of inflammatory cells into the joint cavity of mice with gouty arthritis (Figure 4B-D), whereas no reduction in cell influx was observed in mice injected with doses of 25µg or 12.5µg AAT-Fc (data not shown). Figure 4A shows the reduction in joint swelling with the 50µg dose from Figure 3D.

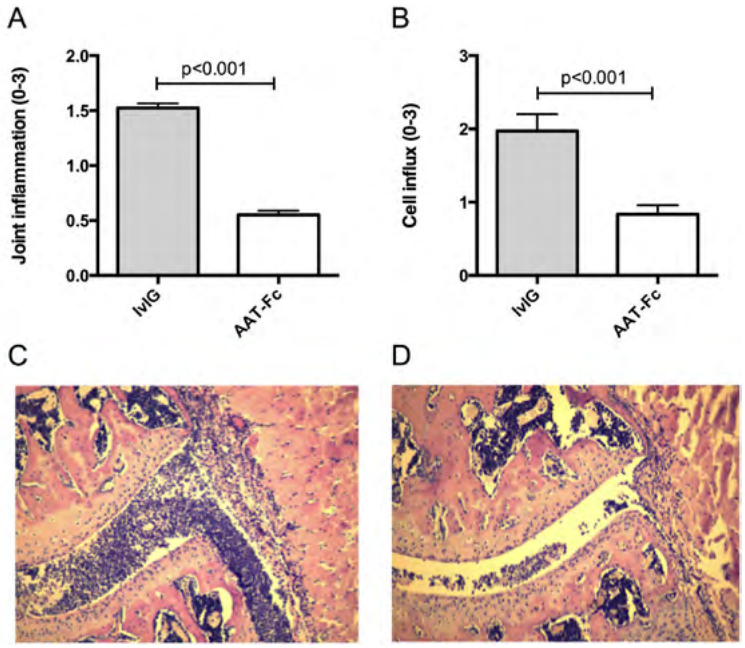

FIGURE 4. AAT-Fc suppresses joint inflammation.

A. Joint inflammation determined at 4h after induction of gouty arthritis. N=5 mice per group, 10 knee joints were examined. Mice were treated with 50µg of AAT-Fc or 50µg IvIg, 1h before induction of gouty arthritis. **B.** Cell influx. The number of inflammatory cells in the joint cavity, scored on a scale ranging from 0 to 3. 10 joints per group were examined. Data are expressed as mean+/-SD. Mann-Whitney U-test was used for statistical analysis. These experiments were repeated once with similar results. **C.** Histopathology of an inflamed knee joint of an IvIg treated mouse, 4h after induction of gouty arthritis. Note the severe infiltration of cells in the joint cavity. **D.** AAT-Fc treated mouse. H&E staining, original magnification.

AAT-Fc inhibits PR3-mediated processing of the IL-1β precursor into mature bioactive IL-1β

Serine proteinases, such as PR3, process the IL-1β precursor extracellularly into mature, active IL-1β [20]. Since plasma-derived AAT inhibits the enzymatic activity of PR3, we investigated whether recombinant AAT-Fc was able to inhibit PR3-mediated cleavage of the precursor into active IL-1β. A preparation of human IL-1β precursor [21] was incubated with PR3. After 20 minutes, the reaction mixture was added to the IL-1-responsive human A549 lung epithelial cell line to determine IL-1 bio-activity using the induction of IL-6 as a readout. As shown in Figure 5A, the IL-1β precursor at 100ng/mL did not induce IL-6 in the presence of either plasma-derived AAT or AAT-Fc. In contrast, the precursor incubated with PR3 induced a 5-fold increase in IL-6 (Figure 5A, right). Increasing concentrations of either plasma-derived AAT or AAT-Fc reduced the induction of IL-6. Either plasma-derived AAT or AAT-Fc alone without the IL-1β precursor had no effect on IL-6 production. The concentration of AAT-Fc that inhibit the generation of active IL-1β by PR3 was comparable to that of plasma derived AAT (Figure 5A, right).

AAT-Fc fusion protein inhibits IL-1β release from human CD14+ monocytes

To study the direct effect of AAT-Fc on the IL-1β release, we exposed freshly isolated $CD14^+$ human blood monocytes to AAT-Fc, followed by MSU/C16.0 stimulation. Figure 5B shows that $CD14^+$ monocytes release high levels (7,000 pg/mL) of IL-1β when exposed to MSU/C16, which is dose-dependently reduced in the presence of AAT-Fc. At 25μg/mL AAT-Fc, there was a 76% reduction ($p<0.01$) and at 12.5μg/mL, the reduction was 60% ($p<0.01$).

AAT-Fc induces endogenous IL-1Ra

The balance of IL-1 and IL-1Ra, the natural inhibitor of IL-1, affects the severity of inflammation. Studies in a mouse model of pancreatic islet transplantation demonstrated that plasma-derived AAT induced IL-1Ra gene expression [22]. AAT also augmented IL-1Ra production in LPS stimulated human PBMCs [6]. We next investigated whether AAT-Fc induced the production of endogenous IL-1Ra *in vitro* and *in vivo*. Figure 5C shows that negatively selected primary human blood monocytes produce high levels (12-fold over control) of IL-1Ra during 24h incubation in the presence of 25μg/ml AAT-Fc. The same concentration of plasma-derived AAT did not induce IL-1Ra (Figure 5C). We next measured the levels of circulating IL-1Ra in mice injected with AAT-Fc. Indeed, levels of resting state mouse IL-1Ra rose approximately 4-fold at 6h after i.p. injection with 50 μg AAT-Fc (Figure 5D). In addition, we observed that prior to i.a. MSU/C16.0 injection, mice treated with AAT-Fc exhibited elevated levels of serum IL-1Ra 6 hours later (Figure 5E).

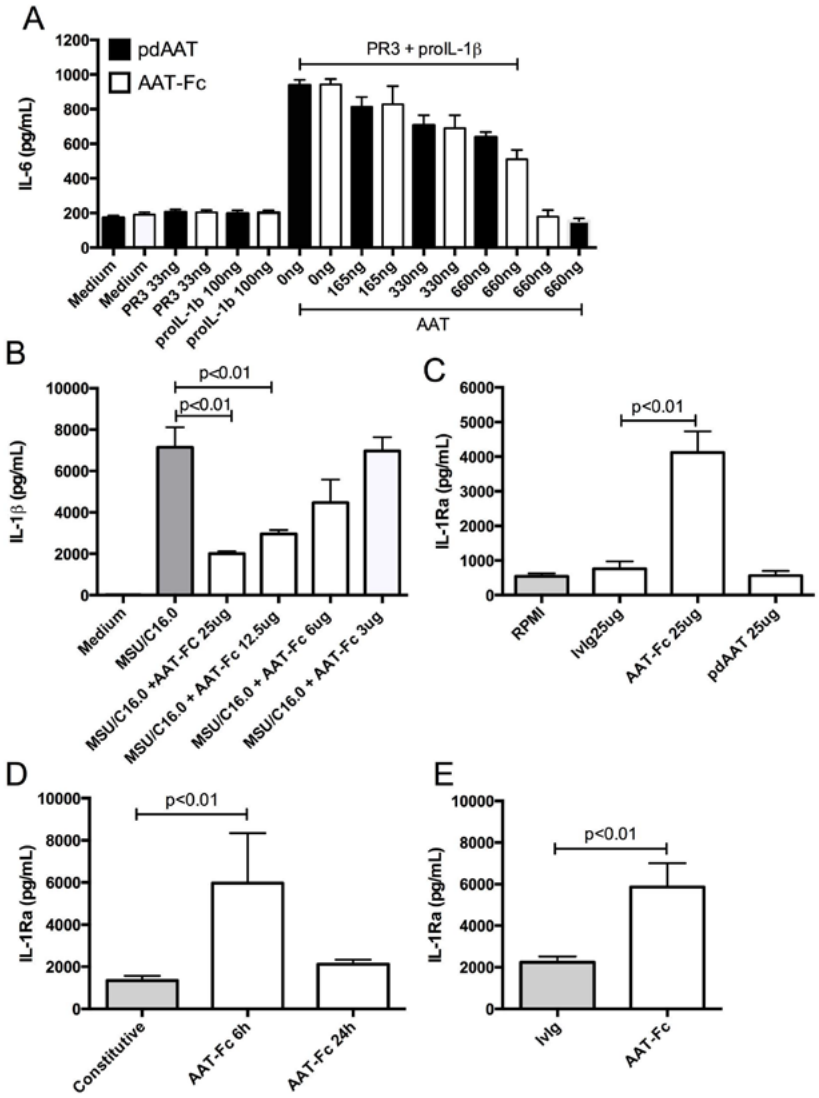

FIGURE 5. AAT-Fc inhibits PR3-mediated IL-1β processing, suppressed MSU/C16.0-induced IL-1*β*- and induces IL-1Ra- production.

A. Proteinase 3 and proIL-1β were incubated with or without either pdAAT or AAT-Fc in different concentrations. PR3 was pre-incubated for 30 minutes with pdAAT or AAT-Fc. IL-1β bioactivity was determined using the A549 cell line. **B.** Human PBMCs were incubated for 24h with MSU/C16.0 (300μg/ml; 200*μ*M) with or without AAT-Fc. IL-1*β* was determined by ELISA. N=6 donors. **C.** Negative selected human primary monocytes were incubated for 24h with IvIg, AAT-Fc and pdAAT. N=4 donors. IL-1Ra was measured with ELISA. **D.** Wild-type mice (n=5) were injected i.p. with 50μg AAT-Fc. Before AAT-Fc injection, after 6h and 24h blood was collected for IL-1Ra determination. **E.** Serum IL-1Ra levels in mice with gouty arthritis treated with AAT-Fc. N=10 mice per group. Data are expressed as mean+/-SEM. Mann-Whitney U-test was used for statistical analysis.

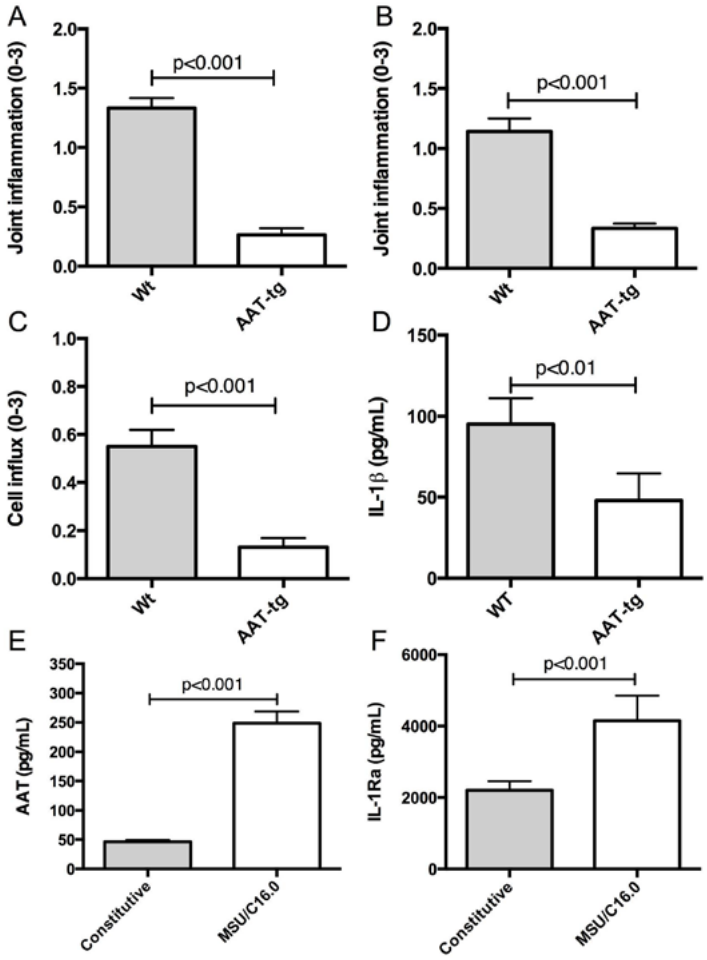

FIGURE 6. Suppression of gouty arthritis in human AAT-tg mice.

A/B. Gouty arthritis was induced in wild-type and human AAT-tg mice. N=5 mice per group, 10 joints were injected. Joint inflammation was determined after 4h. **C.** Influx of inflammatory cells in the joint cavity. N=10 joints per group. D. IL-1β levels in synovial tissue explants. N=10 per group. **E.** Human AAT levels in hAAT-tg mice. Constitutive and induced serum hAAT levels. MSU/C16.0 (3mg/2mM) was injected i.p., serum was collected after 6h. N=10 AAT-tg mice. **F.** Serum IL-1Ra levels in AAT-tg mice before and after injection i.p. with MSU/C16.0. Data are expressed as mean+/-SEM. Mann-Whitney U-test was used for statistical analysis.

Human AAT transgenic mice are protected from gouty arthritis.

Mice transgenic for human AAT (AAT-tg), which is driven by the surfactin C promoter for selective expression in type-2 lung epithelium [16], have low circulating levels of human AAT but are nevertheless protected in models of multiple sclerosis [23] and *Pseudomonas* pneumonia [24]. Here we demonstrated that human AAT-tg mice were also protected

against joint inflammation induced by local MSU/C16.0 injection. Figure 6A shows that 4 hours after i.a. injection of MSU/C16.0, there was moderate joint swelling in wild type C57Bl/6 mice, whereas AAT-tg mice showed 6-fold ($p<0.001$) less swelling. We could confirm these findings in a subsequent study (Figure 6B). Histological analysis revealed that the number of inflammatory cells was significantly reduced in AAT-tg mice compared to the wild-type mice (Figure 6C). The IL-1β production in synovial tissue explants was reduced by 50% compared to wild-type mice (Figure 6D). In line with these results, IL-6 and KC concentrations were also reduced (Supplemental Figure 2). Figure 6D reveals the constitutive level of circulating endogenous human AAT in AAT-tg mice. Following i.p. injection of MSU/C16.0, circulating AAT levels increased remarkably (Figure 6D) and serum IL-1Ra concentrations were increased further in the AAT-tg mice (Figure 6E).

DISCUSSION

We demonstrate that the novel AAT-Fc fusion protein markedly suppresses joint inflammation in murine acute gouty arthritis. The study also reveals that AAT-Fc inhibits the release of IL-1β by human blood monocytes elicited by the combination of MSU crystals plus the C16.0 fatty acid. In addition, the AAT-Fc fusion protein induced IL-1Ra, the natural inhibitor of IL-1, *in vitro* and in the circulation of mice. Related to the administration of AAT-Fc to mice, transgenic mice expressing human AAT are protected from MSU crystal-induced arthritis and also exhibit elevated endogenous levels of mouse IL-1Ra compared to wild-type mice. Together, the data provide the basis for AAT-Fc as a possible treatment option for acute gout attacks in humans. Furthermore, infusions of plasma-derived AAT have a 20 years history of safety in deficient patients [25, 26].

IL-1β is clearly the causal cytokine in the pathogenesis of acute gout attacks. Clinical trials using anakinra [17-19], the IL-1 trap (rilonacept) [27] or a neutralizing anti-IL-1β antibody (canakinumab) [14, 28] reveal a rapid and sustained reduction in the clinical manifestations as well as the number of acute attacks. Treatment with canakinumab was superior to the standard corticosteroid therapy in patients with refractory gout [14]. The present findings support the concept that AAT-Fc would be effective in humans since the fusion protein targets the production as well as the activity of IL-1β.

Not merely one mechanism accounts for the efficacy of AAT-Fc in this model of gouty arthritis. Gene expression, synthesis, processing and release of active IL-1β from mononuclear phagocytes are tightly regulated [29, 30]. Despite active caspase-1 in primary human blood monocytes [30], MSU crystals alone do not induce the synthesis nor processing of IL-1β [13], but require an additional signal, for example, lipopolyssacharide (LPS) or TLR2 ligands. Palmitic acid (C16.0) also provides a second signal [31]. Nevertheless, non-caspase-1 mediated cleavage of the IL-1β precursor into active IL-1β is known to occur [32]. Fantuzzi

and co-workers reported that the systemic inflammatory response elicited by subcutaneous turpentine was nearly the same in in caspase-1 deficient mice as in wild type mice [3]. Therefore, caspase-1 mediated cleavage of the IL-1β precursor bypassed particularly during neutrophil-dominated inflammation. Once the IL-1β precursor is released from dying cells, neutrophil-mediated processing takes place in the extracellular space. In an attempt to phagocytose urate crystals, cells release their contents, which would include the IL-1β precursor [33, 34]. Several neutrophil proteases such as elastase, proteinase 3 [20] and Granzyme B [35] can cleave the IL-1β precursor at sites close to the caspase-1 site as reviewed in [36].

AAT is the primary serum serine proteinase inhibitor [4, 5, 37-39] and in AAT deficient persons, the uncontrolled activity of serine proteinases, mostly neutrophil elastase, is regarded as causal in the pathogenesis of lung, liver and pancreatic chronic inflammation in these patients [25, 26, 40]. Since neutrophils are the dominant inflammatory cells in the joint space of acute gout, it is likely that neutrophil proteases bypass the inflammasome-caspase-1 pathway and cleave the IL-1β precursor extracellularly. Another possible proteinase that can process the inactive IL-1β precursor is mast cell chymase [41]. However, PR3 is highly effective in cleaving the IL-1β precursor into bioactive IL-1β [20]. In addition, AAT treatment results in remarkable downregulation of PR3 expression in mouse PBMCs [42]. Here we demonstrated that both plasma-derived AAT and AAT-Fc fusion protein inhibit PR3 conversion of the IL-1β precursor to an active cytokine (Figure 5A).

In addition to inhibition of PR3, the reduction in synovial IL-1β production by AAT-Fc may be due to inhibition of caspase-1, as reported for plasma-derived AAT in a model of acute myocardial infarction in the mouse [8] and renal ischemia[43]. However, another study indicates that plasma-derived AAT did not reduce caspase-1 activity in the THP-1 macrophage cell line or in cell free extracts, and had no effect whole human blood production of IL-1β [44]. Because IL-1 stimulates caspase-1 gene expression in human blood monocytes [45] and caspase-1 cleavage of the IL-18 precursor is IL-1β dependent [46], a reduction in IL-1β will also reduce the level of caspase-1 itself without directly affecting the enzymatic function of caspase-1.

Another mechanism by which AAT-Fc affects IL-1β production and activity is via a reduction in the expression of TLR. In humans with recent onset diabetes treated with 8 weekly infusion of AAT, IL-1β synthesis by circulating monocytes was reduced significantly upon stimulation with TLR agonists *ex vivo* [47]. AAT-Fc added directly to mouse insulin-producing islets suppresses the surface expression of TLR2 and TLR4 [6]

The ability of AAT-Fc to increase circulating levels of endogenous IL-1Ra also contributes to the efficacy of AAT-Fc in suppressing MSU/C16.0-mediated inflammation. In addition to the present study, elevated endogenous IL-1Ra by AAT was reported in a model of graft versus host disease in mice [42]. Acute gout in humans responds to a relatively low dose of anakinra

of 100mg, which results in a peak plasma level of less than 1 µg/mL and a short half-life. In AAT-tg mice have a low level of serum IL-1Ra, which may account for the resistance of these mice to acute MSU/C16.0.

Although the fusion of AAT to the Fc domain of IgG1 created a new molecule [48], fusion molecules of a naturally occurring serum proteins such as TNF receptors to the Fc domain IgG1 (etanercept) are used commonly as therapeutics in humans to increase plasma half-life [49]. One advantage of AAT-Fc over plasma-derived AAT is that injection of only 50 µg prevented MSU/C16.0-induced joint inflammation comparable to 2,000 µg of plasma-derived AAT (40-fold less). In humans, AAT-Fc could be injected subcutaneously compared to the need of an intravenous infusion of plasma-derived AAT. The greater potency of AAT-Fc over plasma-derived AAT may relate to the method of purification as discussed in [6]. The purification of plasma-derived AAT begins with cold ethanol precipitation (Cohn fractionation), which can oxidize the molecule followed by subsequent steps intended to maintain the elastase inhibition properties of AAT. In contrast, AAT-Fc is purified by a signal affinity step using Protein A chromatography [48].

Acknowledgements

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Competing interests

Charles A. Dinarello is Chief Scientific Officer of Omni Bio Pharmaceuticals and hold shares in this company. The AAT-Fc is patented by the University of Colorado and licensed by OMNI Bio Pharmaceutical. The US Patent number is: **8,633,305**.

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SUPPLEMENTAL INFORMATION

Materials and Methods

Preparation of MSU crystals

MSU crystals were prepared according to Seegmiller *et al.* [1]. Briefly, a 0.03M solution of MSU was prepared after diluting 1.0 g of uric acid (Sigma-Aldrich, St. Louis, MO) in 200 mL of sterile water containing 24g of NaOH. The pH was adjusted to 7.2 with HCl and subjected to 120 ºC for 6h to inactivate endotoxins. LPS contamination was controlled by LAL assay. The solution was left to cool at room temperature and stored at 4°C. The crystals were 5–25µm in length. To prepare MSU for in vitro and in vivo experiments, the fluid was removed; the crystals were weighed under sterile conditions and combined with palmitic acid (C16.0). A solution of 800µM C16.0, BSA 0.4% and ethanol 0.4% in RPMI was filter-sterilized (0.2µm) and thereafter mixed with uric acid crystals 1200µg/mL. This preparation was kept at 4ºC.

Generation of AAT-Fc fusion protein

Human pCAGGs-IgG1/AAT plasmids were co-transfected with the pSV-dihydrolfolate reductase (DHFR) vector (ATCC, Manassas, VA, USA) into the DHFR-deficient CHO cell line [2]. Stable clones were selected in medium containing G418 (500 µ/mL) and subsequently subjected to methotrexate selection for gene amplification. AAT-Fc was purified using protein A-conjugated beads that had been equilibrated with phosphate-buffered saline (PBS) containing 0.5 mol/L NaCl. AAT-Fc was examined for purity, LPS contamination and stored at -80 ºC until use. The preparation (second generation) used in the present study inhibited elastase using the method described in the Supplement of Jonigk, et al [3].

Monocytes isolations

Purified monocyte suspensions were obtained from PBMCs using MACS beads according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD14^+$ cells were isolated using CD14 microbeads followed by magnetic separation over selection columns. Negative selection of monocytes was performed after an initial monocyte-lymphocyte separation of PBMCs using high-density hyperosmotic Percoll solution, followed by MACS. Thereafter, monocytes were isolated by using the pan monocyte isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Monocyte suspensions were adjusted to $1x10^6$ cells/mL and experiments were performed using $1x10^5$ cells/well.

Joint inflammation measurement

Joint inflammation was measured by macroscopic scoring method [4, 5]. Macroscopic joint swelling was scored on a scale ranging from 0-3. After the skin was removed the knee joint

was scored, 0= no inflammation, 1= mild, 2= moderated and 3= severe inflammation. The scores start with 0.25, 0.50, 0.75, 1,0, up to 3.0. 0.25 is giving when the first signs of swelling and redness is seen. All values exceeding 0.25 are assigned as joint swelling. Joint swelling scoring was performed without knowledge of the experimental groups.

Cytokine determinations

Protein levels of murine IL-1β or IL-6 were measured in patellae washouts or synovial tissue extracts. Four hours after injection of MSU/C16.0, patellae were isolated from inflamed knee joints and cultured 2h at RT in RPMI 1640 medium containing 0.1% bovine serum albumin (200µL/patella). For total IL-1β levels, patellae with surrounding tissue were directly transferred to 200µL 0.05% Triton-X 100 in PBS. After repeated freeze-thawing, IL-1β was determined. Mouse cytokines, including mouse IL-1Ra were determined by ELISA from R&D Systems, Minneapolis, MN, USA. Human IL-1β and IL-6 was measured by ELISA (R&D Systems).

AAT determination

Levels of human AAT in mouse sera were determined using an AAT ELISA kit (Immunology Consultant Laboratory, Inc., Newberg, OR, USA). Blood was collected from mice and serum was diluted 20 times in ELISA diluent.

Histological analysis

Mice were sacrificed by cervical dislocation. Whole knee joints were removed and fixed in 4% formaldehyde for 7 days before decalcification in 5% formic acid and processing for paraffin embedding. Tissue sections (7µm) were stained with Haematoxylin/Eosin. Histopathological changes in the knee joints were scored in the patella/femur region on 5 semi-serial sections. Scoring was performed on decoded slides by two separate observers, using the following parameters: the amount of cells infiltrating the synovial lining and the joint cavity was scored from 0-3 [6].

Cleavage of the IL-1β precursor by PR3

100ng of recombinant human IL-1β precursor [7] was incubated for 2h with 33ng of PR3 at pH 7.2 (Athens Research & Technology, Athens, Georgia, USA). Thereafter, the bioactivity of the cleaved product was assessed by stimulation of IL-6 from human A549 cells. After 24h, IL-6 in the supernatants were determined by ELISA (R&D Systems). To inhibit PR3, the enzyme was pre-incubated for 30 minutes with AAT-Fc or plasma-derived AAT before mixed with the IL-1β precursor.

Statistical analysis

Differences between experimental groups were tested using the Mann-Whitney U-test. Data are expressed as mean ±SEM, unless stated elsewhere. Graphpad Prism 6.0 was used for statistical analysis.

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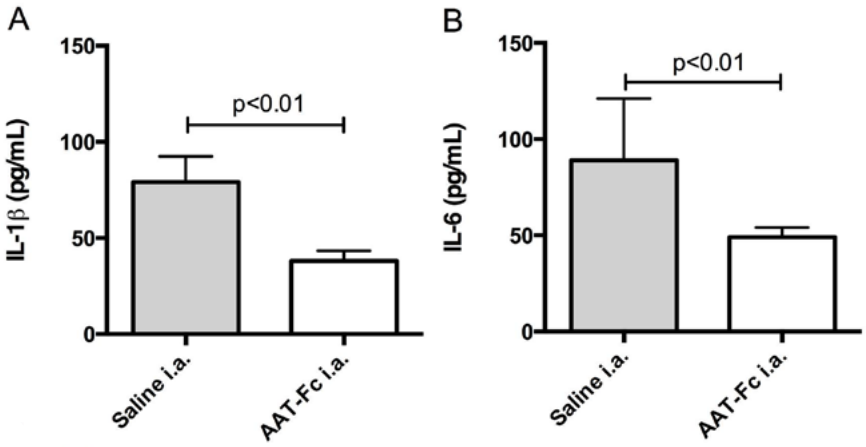
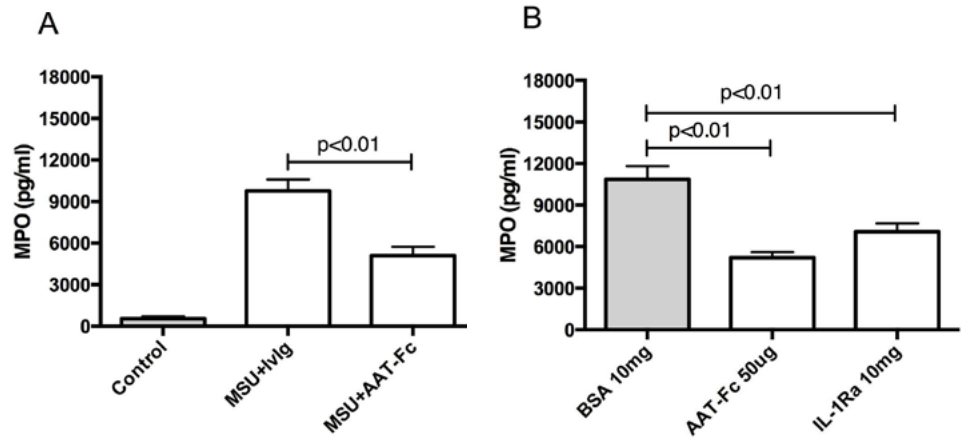
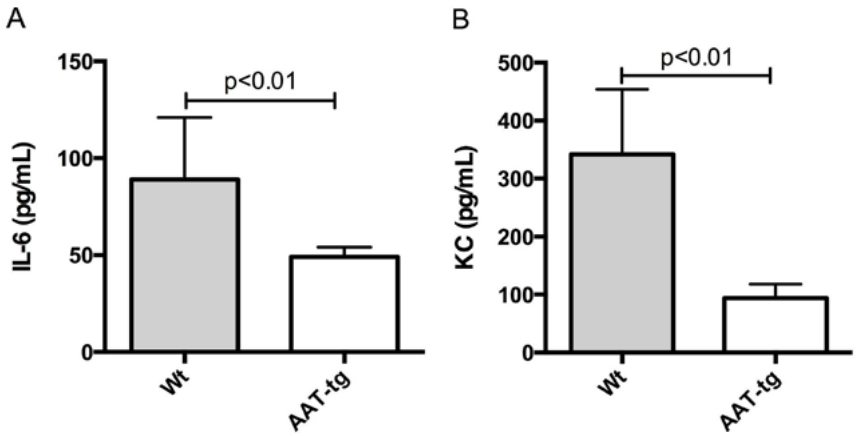
SUPPLEMENTAL FIGURES



SUPPLEMENTARY FIGURE 1. Intraarticularly (i.a.) application of AAT-Fc. C56/Bl6 mice were i.a. injected with 6ul of MSU/C16.0 (300µg/200µM) or MSU/C16.0 (300µg/200µM) plus AAT-Fc (2µg). After 4h, synovial tissue specimens were isolated and the cytokine release was determined. **A.** IL-1β production. **B.** IL-6 production. N= 5 specimens per group. Data are expressed as mean+/-SEM. Mann-Whitney U-test was used for statistical analysis.



SUPPLEMENTARY FIGURE 2. A. C57/Bl6 mice (n=5 per group) were i.p injected with 50µg Ivlg, 50µg AAT-Fc or saline (control). After 2h, 3mg (0.5ml of a solution of 6 mg/ml) MSU crystals were i.p. injected, only in the mice that received Ivlg or AAT-Fc previously. Four hours later, mice were sacrificed and 10ml of cold PBS was i.p. injected. Thereafter peritoneal fluid was isolated. Cell influx was determined by cell counting. MPO levels in the peritoneal fluid by Elisa. Mann Whitney U-test. Data are expressed as mean+/-SEM. **B.** Comparison AAT-Fc with IL-1Ra. BSA (10mg per mouse), AAT-Fc (50µg per mouse) or IL-1Ra (10mg per mouse) were injected i.p., 2h before MSU (3mg)crystals were injected. Thereafter peritoneal fluid was isolated. Cell influx was determined by cell counting. MPO levels in the peritoneal fluid by Elisa. Mann Whitney U-test. Data are expressed as mean+/-SEM.



SUPPLEMENTARY FIGURE 3. Wild-type (C56/Bl6) and AAT-tg mice were i.a. injected with 6ul of MSU/C16.0 (300µg/200µM). After 4h, synovial tissue specimens were isolated and the cytokine release was determined. **A.** IL-6 production. **B.** KC production. N= 5 specimens per group. Data are expressed as mean+/-SEM. Mann-Whitney U-test was used for statistical analysis.

INNATE IMMUNE MEMORY: IMPLICATIONS FOR HOST RESPONSES TO DAMAGE-ASSOCIATED MOLECULAR PATTERNS

Crișan TO
Netea MG
Joosten LA

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SUMMARY

Cells of the innate immune system build immunological memory via epigenetic reprogramming after stimulations with microbial ligands. This functional readjustment allows for enhanced nonspecific inflammatory responses upon secondary challenges, a process termed 'trained immunity'. The epigenomic blueprint of trained monocytes has been recently reported, which revealed several important immunologic and metabolic mechanisms that underlie these changes. Interestingly, similar long-term reprogramming of cytokine production has also been described to be induced by endogenous damage-associated molecular patterns (DAMPs). Here we present an overview of the novel data showing that endogenous alarm signals associated with tissue damage and sterile inflammation can induce trained immunity through epigenetic regulation of transcriptional programs. We describe new and old evidence of persistent effects of DAMPs in driving inflammation and enforce the concept that the influence of tissue-derived signals is critical in adjusting the magnitude and type of immune response built by the host. The better characterization of trained immunity for the persistence of inflammation induced by DAMPs would provide new possibilities for intervention in aging and autoinflammatory disorders.

INTRODUCTION

The common understanding of host innate immune responses is facing crucial changes. For decades, the consensus has been that the host immune response is based on the dichotomy of the innate and the adaptive responses, which are clearly distinct in terms of specificity and memory. Nevertheless, it is now established that innate immune cells, similarly to adaptive immune cells, do exhibit a degree of specificity through pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) on invading microbes [1]. In addition, by putting together pieces of evidence from different species, a new hypothesis has been proposed, stating that the adaptive immune system may not be unique in providing immunological memory [2]. Recent evidence supports the concept that indeed, cells of the innate immune system can also build a memory of past stimulations and alter their response upon new challenges. This enhanced state of immune activation of innate immune cells is now known as 'trained immunity' and its mechanisms and consequences for disease and therapy provide a fertile and exciting ground for new research [3].

This characteristic of the innate immune system is obviously very relevant in the context of host response during infectious diseases. Moreover, mechanistic links between trained immunity and the nonspecific effects of vaccines have already been postulated [4] and represent a promising link for the translation of these findings to clinical practice [5]. However, apart from microbial ligands generically named pathogen associated molecular patterns (PAMPs), the same PRR machinery has been shown to be used by non-infectious substances or particles coined by the term "danger signals" or damage-associated molecular patterns (DAMPs) [6]. These danger signals are endogenous in origin, are usually related to tissue destruction, and can elicit activation of the innate immune system determining low-grade chronic to severe acute sterile inflammation [7]. They also play established roles in the consolidation of adaptive immune responses [7]. In this review we focus on the hypothesis that DAMPs can induce trained innate immunity. We describe some of the known effects of danger signals on immune responses in general and elaborate on the hypothesis that DAMPs might also act as stimuli that induce long-term functional reprogramming in cells of the innate immune system.

Trained innate immunity

Since the concept of trained immunity has been proposed, several lines of evidence validated its importance for effective immune responses. In mice lacking functional T lymphocytes and B lymphocytes, a non-lethal infection with *Candida albicans* was shown to protect against a subsequent infection with a lethal dose of the same pathogen [8]. In purified human monocytes, in vitro pretreatment with *C. albicans* or the cell wall component

β-glucan was shown to increase long-term inflammatory responses to a second stimulation [8]. Similar findings in vitro and in vivo in mice were observed after training with the anti-tuberculosis BCG (Bacille Calmette-Guérin) vaccine, and this was also supported by data from healthy human volunteers vaccinated with BCG [9]. In both scenarios, this boosting effect on proinflammatory properties of the cells or systems studied, which was observed following the initial exposure to the stimulant, was dependent on circulating monocyte populations and occurred independently of B cells and T cells [8, 9]. This is in line with previous experimental studies showing that nude mice could be protected against *Schistosoma* by BCG vaccination [10], and where mice treated with BCG were protected against systemic candidiasis in a macrophage-dependent manner [11]. Similarly, innate immune mechanisms, such as the upregulation of polymorphonuclear cell counts, increased spleen macrophage candidicidal activity, and enhanced cytokine production (GM-CSF, IL-1 or TNF), were shown to be involved in conferring protection in mice against a lethal dose of *C. albicans* after exposure to a non-virulent strain [12-14]. Features of innate immune memory have also been described in a murine model of helminthic infestation in which the lung macrophages of mice challenged with *Nippostrongylus brasiliensis* develop an activated phenotype which is induced with the aid of neutrophils and consists in faster response to a second infection[15]. Moreover, macrophages retain this behavior against new challenge even when transplanted to a naïve host [15].

Another hallmark of trained immunity is the lack of specificity of the response. Adherent monocytes exposed for 24 hours to BCG, after 7 days of resting, were shown to produce increased levels of cytokines not only in response to *Mycobacterium tuberculosis* stimulation but also to *Candida* or *Staphylococcus* [9]. These nonspecific effects of BCG training are seen in epidemiological vaccination studies, in which the effects on overall mortality and morbidity are documented not only in humans vaccinated with BCG [16, 17] but also for other vaccines such as measles and DTP vaccines [18]. Apart from vaccines, natural exposure to viruses has also been shown to induce long-term non-specific effects, as described for latent infections with Epstein-Barr virus or cytomegalovirus, which were shown to afford protection in mice against subsequent *Listeria* or *Yersinia* challenge [19]. Interestingly, cord blood cells of newborns exposed in utero to hepatitis B virus from their chronically infected seropositive mothers exhibited trained immunity features consisting in higher IFNγ, IL12p40 and TNF production in response to stimulations with *P. aeruginosa*, *Uropathogenic E. coli*, *S. typhimurium*, *A. baumanii* and *L. monocytogenes* [20]. This suggests that trained cells give heterologous responses directed against a larger spectrum of microbes than the initial stimulant.

Last but not least, probably the most important mechanism(s) through which trained immunity is mediated is epigenetic regulation. Transcriptional and epigenetic profiling have revealed that genes coding for cytokines such as IL-6 or TNF are upregulated in monocytes

trained with β-glucan in a dectin-1 dependent manner [8] or trained with BCG in a NOD2 dependent manner [9]. Mechanistic investigations showed that this upregulation was accompanied by histone marks associated with open chromatin, such as trimethylation of lysine 4 residue of histone 3 (H3K4me3), H3K4me1 and H3K27Ac [8, 9, 21] (Figure 1). When the H3K4me3 epigenetic mark was assessed in individuals vaccinated with BCG, a stable increase in monocytes was observed even at 3 months after vaccination [9].

An important question in understanding trained immunity is the identity of the specific microbial ligand(s) inducing long-term effects in the host, and the identity of those which do not induce these effects. The possibility that a ligand decreases the inflammatory response upon restimulation has been shown for LPS in cells of the monocyte and macrophage lineage, in which high doses of LPS dampen cytokine responsiveness of monocytes to LPS itself [22], but also to other stimuli such as Pam3Cys [23]. A systematic approach aiming to test the effects of several PAMPs in vitro in adherent monocytes determined that the trained immunity or tolerance potential of various PRR ligands depends on the receptor and the ligand concentration studied [23], with other observations showing tolerance induced by TLR2 stimulation with Pam3Cys and training induced my MDP signaling via NOD2 (Figure 1). A very tight dependence of the inflammatory response on the dose of endotoxin has also been shown when comparing low dose LPS with super-low dose LPS in a murine sepsis model [24] or in THP1 cells [25]. This dose dependence consists in promoting a proinflammatory response by stimulations with LPS in the picograms/ml range while determining a refractory state upon nanograms/ml concentrations, and this appears to be mediated by differential signaling cascades downstream TLR4, that involve GSK3 and Akt phosphorylation [25]. More recently, it has been shown that LPS tolerance is associated with expression modules that are active in the monocyte while downregulating sets of genes associated to gene ontology terms "cellular ketone metabolic process" and "carboxylic acid metabolic process" that represent macrophage markers of differentiation [21]. Another example of differentially regulated gene that is expressed in tolerized cells compared to monocyte-derived macrophages is IRAK3 (interleukin-1 receptor-associated kinase 3), which negatively regulates TLR-induced responses and could play a role in the refractory state seen in immune tolerance [21]

When considering the high plasticity potential that is known to characterize the monocyte-macrophage lineage [26-28], the identification of gene regulation modules that determine the specific functional signature of cells after microbial insults could prove beneficial when targeting those specific cell types in disease. It is known that cells undergo normal differentiation via epigenetic programming, which in turn is brought about by various modifiers according to the specific program of that lineage. For example, monocytes differentiate into macrophages after migrating into tissues and develop a phenotype similar to that determined by one week culture in medium supplemented with human

serum which consists in downregulation of genes such as NF-kB subunits or inflammasome components [21]. However, it has also been described that certain r regions in the genome, remain latent under unstimulated conditions and are amenable to subsequent regulation provided contact with the relevant stimulus ensues [29]. One such example of this would be the identification of a panel of new enhancer regions that were induced after 4h of LPS treatment in differentiated mouse bone marrow macrophages although they were completely unmarked in basal conditions [29]. This behavior was also observed after stimulation with IFN-γ or IL-4, nevertheless, the enhancer panel was slightly different depending on the stimulus, providing a basis for the cellular plasticity of macrophages in response to different signals [29]. However, after washout of stimulus, enhancer responsiveness mostly disappeared in parallel with loss of histone acetylation, whereas H3K4me1 endured for days after washout, suggesting the possibility of a new and faster acetylation at the same position upon restimulation [29]. Recent evidence suggests that chromatin structure is modifiable in the context of infection (reviewed in [30]) and trained immunity adds value to this knowledge by providing evidence of adaptive features in macrophages through longer term effects of their first encounters with critical stimuli (reviewed in [26]).

In β-glucan trained cells, apart from the immune-related genes, major alterations have been observed in genes encoding glycolysis enzymes [31]. An interesting link between central metabolism and inflammation has increasingly received attention over the past years. A switch in the energetic behavior of the cell, shown to consist of upregulation of glycolysis and suppression of oxidative phosphorylation despite sufficient oxygen supply (known as the Warburg effect), was for decades considered a feature of cancer cells [32, 33]. Emerging data has highlighted this phenomenon as a hallmark of activated immune cells [34]. This metabolic switch seems to be a key process in trained immunity as well, induced via the Akt-mTOR-HIF1α pathway [31] (Figure 1).

Altogether, the concepts of training or tolerance describe processes in which certain PRR ligands determine not only immediate effects on the inflammatory properties of the cell, but also induce signals that are fine-tuned by the amount of ligand or type of receptor, and which modify the transcriptional program of the cell. This reprogramming is mediated by epigenetic marks, such as histone tail modifications, that will change the expression levels within various genomic regions. The combination of genes influenced by one particular training agent would foreseeably render its effects relevant for one or more potential future stimuli, depending on the genes involved. Therefore, one single stimulus could induce protective or detrimental effects in relatively distinct situations, as seen for vaccines and their nonspecific effects on the health of the vaccinated individual [16-18].

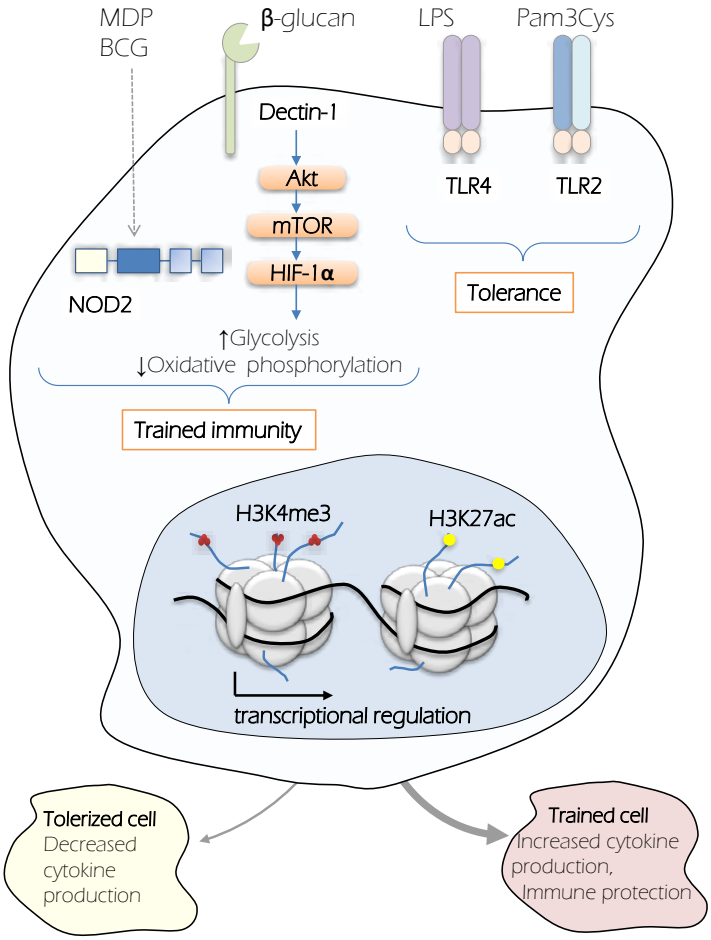

FIGURE 1. Schematic representation of trained immunity or tolerance induced by PAMPs.

Microbial PAMP ligands are recognized by PRRs on innate immune cells and can have long-term effects on the functional programme of the cell. For example, β-glucan from Candida has been shown to drive a metabolic shift in activated cells which is associated with enhanced gycolysis and diminished oxidative phosphorylation; this has been shown to occur via the Akt-mTOR- HIF-1α pathway. These metabolic changes are closely linked with an epigenetic reprofiling of the monocyte, mediated especially through histone modifications (e.g. H3K4 trimethylation, H3K27 acetylation), which increases cytokine production (IL-6, TNF), inflammation, and protection against subsequent infection ("Trained cell", bottom right). Similar training effects on the cytokine production of monocytes have been described for ligands of cytosolic PRRs such as MDP and BCG that involves signaling via NOD2. Conversely, dose-dependent stimulation of membraneous and endosomal PRRs determines an antiinflammatory phenotype, the prototype of which is dose-dependent, LPS-induced tolerance. This is mediated through similar histone modifications as in the case of training, but by a different pattern of gene expression than determines the cell differentiation to a tolerized phenotype; conserved expression of IRAK3 which is a negative regulator of TLR responses could be involved in this (explanations in text) . PAMP, pathogen associated molecular pattern; PRR, pattern recognition receptor; mTOR, mammalian target of rapamycin; HIF-1α, hypoxia induceble factor 1α; H3K4, histone3 lysine4; LPS, lipopolysaccharide; MDP, muramyl dipeptide; TLR, toll like receptor; NOD, nucleotide oligomerisation domain.

Although most of the work described this far was performed in monocytes, innate immune memory has been described in other cells as well. Evidence of long-term immune activation was also described for natural killer (NK) cells, another major innate immunity effector cell [35-37]. NK cells can, in certain conditions, proliferate in a manner similar to T cells or B cells, and then reside in lymphoid tissues until second stimulation [38]. Recent findings that directly address the capacity of NK cells to show features of trained immunity revealed that at 2 weeks and at 3 months after BCG vaccination, human NK cells can produce increased levels of proinflammatory cytokines (IL-1β, IL-6, TNF but not IFNγ)[39]. Mice deficient in NK cells displayed less protection against *C. albicans* in a murine model of BCG-induced training, showing that the innate immune training is partially dependent on NK cells, in addition to the monocytes [39]. Interestingly, recent evidence shows that NK cells can also engage in priming early developing monocytes during infection, playing a role in setting a functional program that is fine-tuned for stimuli that the monocyte has yet to encounter [40]. Seminal findings of Askenase et al [40] provide evidence that systemic IL-12 produced in the gut mucosal associated lymphoid tissue during oral infection with *T. gondii* in mice can stimulate NK cells in the bone marrow to activate and produce IFNγ. This role of NK cells determines priming of monocyte progenitors to develop regulatory features, expressing more anti-microbial factors such as guanylate binding protein 2 (GBP2), transcriptional regulators such as STAT1 or STAT2, and MHCII components, and this occurs before egress of monocytes from bone marrow to periphery. This is in line with the emerging role of innate lymphoid cells in inflammation [41] and suggests that NK cells are not only capable of building immunological memory themselves, but are also able to influence the bone marrow differentiation of other innate immune cells.

Another very relevant point that requires attention in the context of long-term innate immune effects is the mechanism of persistence of trained immunity. As in vitro studies point out, inflammatory phenotypes remain present in monocytes or macrophages even after removal of initial stimulus, showing that a memory of previous insult exists in peripheral or differentiated cells, as in the case of β-glucan training [8, 21, 31]. Nevertheless, in vivo scenarios imply turnover of blood immune cell populations and time-dependent depletion of the initially trained cells. In the context of the limited lifespan of the monocyte lineage, the observation that trained monocytes persist in the blood at three months post-BCG vaccination of healthy volunteers suggests that other mechanisms may take place to ensure maintenance of trained immunity. One possible explanation is that the initial stimulus persists in the host and continuously activates the cells. In the case of BCG, it is known that live inoculum can be found at the vaccination site for only 4 weeks in some volunteers [42], whereas persistence at other locations, such as the draining lymph nodes, is difficult to determine. While it cannot be excluded that live bacteria are still present, it is less likely that this can still mediate, in all volunteers, the training that is observed 3 months post-vaccination. In addition, in a murine model of *Candida*-induced protection[8],

no detectable levels of kidney fungal load or circulating cytokines were observed before reinfection, arguing against continuous stimulation of cells by the persistent training agent. Interestingly, another possibility that could explain the durable effects of training is epigenetic reprogramming at the level of progenitor cells. Emerging evidence now indicates that microbial stimuli can induce bone marrow reprogramming, one example being the IFNγ-dependent transcriptional regulation of monocyte progenitors [40]. In mice colonized with SFP (segmented filamentous bacteria), the bone-marrow derived dendritic cells (BMDCs) were shown to subsequently be protective against *Entamoeba histolytica* due to a cytokine profile characterized by elevated IL-23 and IL-17A [43]. Moreover, BMDCs retained this altered cytokine production and were able to protect against *Entamoeba histolytica* even when transplanted to SFP non-colonized hosts [43]. The potential of modified precursors to transmit their phenotype down their lineage was also demonstrated in mouse and human hematopoietic stem and progenitor cells (HSPC) treated with TLR2-agonist Pam3CSK4 which lead to the formation of derived macrophages with reduced cytokine production capacity (for TNF, IL-6 and IL-1β) and lower ROS production [44]. This phenotype was also shown to be transmitted by exposed cells in a paracrine manner to murine HSPCs that have not been exposed to Pam3CSK4 in an in vitro model of trans-well co-culture [44]. Finally, another possibility that may occur in tissues (though not in the circulation) may involve enduring epigenetic and functional reprograming of innate immune cells with long lifespans and capacity of self-renewing, such as macrophages.

While the beneficial effects of trained immunity may be numerous and worthwhile to enhance in the context of infection, potential deleterious effects, such as enhancing autoinflammatory processes, must also be considered. Whether the facilitated proinflammatory state in trained mononuclear cells also has detrimental consequences has started to be a point of study in the context of atherosclerosis, in which oxidized low-density lipoproteins (LDL) can reprogram monocytes and macrophages towards higher TNF, IL-6 and MCP-1 production and increased foam cell formation through epigenetic regulation involving H3K4me3 enrichment at promoter regions of cytokine genes [45]. Additionally, endogenous molecules are found to have stimulatory effects on the immune system, either acting on their own or in synergy with other ligands. We will focus here on the current indications that non-microbial ligands, such as the endogenous DAMPs, can also induce long-term effects on the innate immune response, resulting in a functional trained immunity program.

The danger hypothesis

Another dichotomy that functioned as paradigm in immunology is that the host immune system is built and programmed to recognize "self" and to react against "non-self". This "self" / "non-self" (SNS) model [46] was later modified to incorporate new concepts such as

the need for pattern recognition receptors to bind the "non-self" bacterial ligands giving rise to the "infectious non-self" (INS) model [47]. The INS model is widely accepted among scientists, it is the basis of immunologic response and it explains many observed immune processes. Nevertheless, there were still situations where the "non-infectious self" versus "infectious non-self" distinction alone could not entirely explain the phenomena that we see in real life, such as the impressive number of tolerated cell types and tissue-specific antigens towards which the organism does not build an immune reaction while preserving reactivity against foreign pathogens, or the presence of autoantibodies in the sera of healthy individuals without any symptoms while a small proportion of individuals develop autoimmune diseases [48]. In order to address this, a new theory emerged that was called "the danger model" [48, 49] which states that the host immune system is more sensitive to the damage brought to its tissues than to the foreignness of the stimuli it meets. In other words, the danger model states that the key players in the initiation of an immune reaction are not only the foreign stimulus and the immune cells, but also the tissues in which this encounter takes place, and the degree of destruction that a certain process causes in the tissues [48, 49]. Although the danger model has also been critiqued for not being able to explain all facets of the immune response on its own [50], it is now accepted among researchers that the interplay between PAMPs and DAMPs is crucial to the activation and the magnitude of the immune response.

According to the danger model, damage to the tissues determines the release of alarm signals or danger signals (DAMPs), which help the host to determine whether to build a reaction or not and, subsequently, what type of reaction that should be [6, 51]. At steady state, DAMPs perform physiological functions, but are released in the extracellular compartment or are relocated in the presence of stressors that cause injury to the tissues or during non-physiological cell death [52]. Altogether, DAMPs act like "inducers, sensors and mediators of stress" [52].

In the past 20 years since the model was described, an abundance of studies have identified various DAMPs and described their mechanisms of action (comprehensively reviewed in [52-54]), many of which are related to immunoscenescence [52, 55], cancer[52], autoimmune diseases [7] or autoinflammatory diseases [56]. In brief, DAMPs can be classified based on their biochemical nature into protein or non-protein signals. Protein DAMPs are mostly found in the intracellular compartment, where they are hidden from the receptors that recognize them. When these intracellular protein DAMPs are released as danger signals outside the cell, either by active secretion during stress or by passive translocation during the non-physiological death of the cell, their extracellular location leads to inflammatory reactions. Such DAMPs can be found in the nucleus or in the cytosol. An example of a nuclear DAMP is HMGB1 (high mobility group box 1), which is released by necrotic cells and has been shown to mediate inflammation by TNF induction in murine bone marrow

cells [57]. HMGB1 neutralization reduced inflammation in mice upon acetaminophen-induced liver necrosis [57]. Some examples of cytosolic DAMPs include S100 proteins [58], heat shock proteins [59], IL-1α [60], serum amyloid A [61], and β-amyloid [62]. For instance, in the S100 family of cytosolic proteins there are 21 structurally similar members which detect variations in calcium (Ca) concentrations and moderate the cellular responses to Ca fluctuations [63]. A large body of evidence describes the involvement of S100 proteins in cancer, where they have been shown to take part in processes such as growth, proliferation, metastasis, inflammation or angiogenesis (comprehensively reviewed in [63]). Recently, two S100 proteins (S100A8 and S100A9, also known as MRP8 and MRP14) have been shown to simulate TLR4 and to drive β-integrin activation in neutrophils [64], providing one mechanistic link between previous associations of these alarmins with inflammatory diseases which involve neutrophil recruitment (i.e. gout [65]). Cytosolic DAMPs are also represented by intracellular cytokines such as IL-1α, which was shown to be constitutively active and capable to signal via the IL-1 receptor without requiring cleavage [60] and which mediated neutrophil recruitment upon hypoxia and release from dying cells [66]. Non-protein DAMPs include purine derivatives (ATP[67] or uric acid[68]) and nucleic acids (DNA or RNA) [69]. Other endogenous signals that can mediate stress are oxidized LDL[70], saturated fatty acids [71] glucose or advanced glycation end products (AGEs)[72]. DAMPs such as heparan sulphate, that co-signals with HMGB1 via the receptor for advanced glycation end products (RAGE) [73], can also be generated in the extracellular matrix upon digestion of matrix components by proteases that are released in injury. A large set of DAMPs such as HMGB1, fatty acids, modified LDL or S100 proteins can signal both via PRRs as in the case of PAMPs (Toll-like receptors, Nod-like receptor, C-type lectin receptors, Retinoic acid Inducible Gene1-like receptors) or via receptors that are typically used by danger signals alone (Receptor for AGEs (RAGE), scavenger receptors (CD36, that binds modified LDL particles or fatty acids [74] or complement receptors [7, 75]). After DAMP recognition and initiation of signal, typically an innate immune response ensues which clears the infectious or sterile cause of the inflammation. In instances when non-self antigens are present, adaptive immune responses can also develop. Nevertheless, in other situations, low grade inflammation may persist for a longer period of time and this can be due to the incomplete clearance of the cause, to the high production of DAMPs [53-55], or to the enduring proinflammatory microenvironment that can subsequently prime neighboring cells in a paracrine manner [44]. We hypothesize that DAMPs could have a similar capacity to that observed for certain microbes or ligands, such as β-glucan or BCG, in training leukocytes or priming the immune response to remember previous injury and to maintain an activated state (Figure 2). This could be an important mechanism mediating the persistence of sterile inflammation and the susceptibility to metabolic and autoinflammatory conditions such as atherosclerosis, gout or diabetes mellitus, as will be discussed in the following sections.

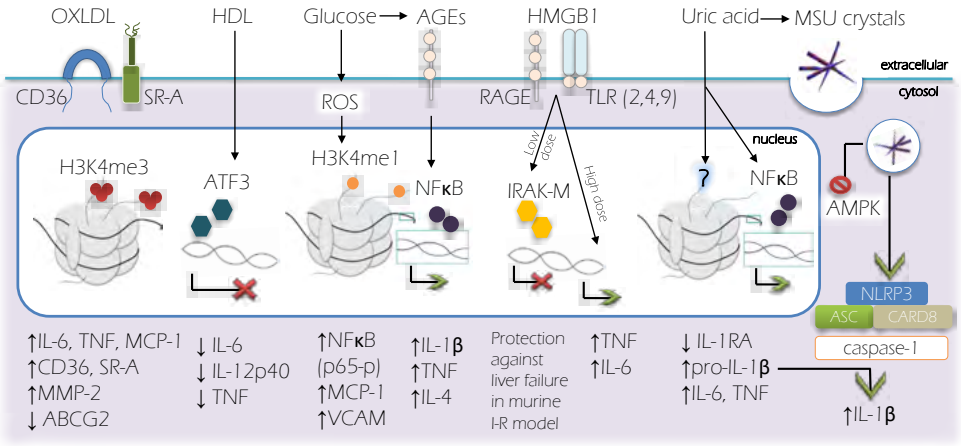

FIGURE 2. Molecular imprinting and trained immunity in relation to endogenous stimuli.

Damage to tissues is accompanied by the release of intracellular molecules or by the formation of alarmins in the extracellular environment. These molecules can signal via classical PRRs or other receptors and induce cytokine transcription, whereas others can activate inflammasomes (in this scheme, NLRP3) to produce bioactive cytokines (in this example, cleaved IL-1β). The proinflammatory effects of DAMP signaling have the role to rapidly clear aggressors, either infectious or sterile. In the case of chronic damage and persistent exposure to DAMPs, such as that observed with functional imprinting of innate immune cells can occur and this is likely to be mediated through epigenetic modifications. As a result, phenotypes of training or tolerance are likely to be developed by innate immune cells after exposure to DAMPs. oxLDL induces trained immunity in monocytes, manifested with higher cytokine production, chemokines, matrix metallo-proteinase 2 and scavenger receptor due to H3K4me3 enrichment at the promoter of these genes [78]. HDL induces an anti-inflammatory state in BMDM via activation of ATF3 which represses proinflammatory cytokines [83]. Hyperglycemia is associated with a wide range of epigenetic modifications such as DNA methylation (inhibitory mark) or histone modifications such as H3K4me1 which mediated NFκB activation, MCP1 and VCAM enhancement in human and bovine endothelial cells [100]. High glucose can determine de appearance of AGEs which can enhance IL-1, TNF and IL-4 in the macrophage differentiating monocyte [103]. HMGB1 has dual dose-dependent effects inducing tolerance at low doses in murine spleen cells, but protection against severe liver failure in mice with ischemia-reperfusion. Uric acid can prime monocytes to produce more pro-IL-1β while downregulating IL-1RA in a process that is likely to be methylation dependent [97]. Supersaturated uric acid also forms MSU crystals that play proinflammatory roles in the cell activating the inflammasome [89] or inhibiting AMPK which further determine proinflammatory cytokine production [94]. DAMP, damage associated molecular pattern; HMGB1, high-mobility group box 1; oxLDL, oxidized low density lipoprotein; AGE, advanced glycation end product,; RAGE, receptor for advanced glycation end products; CD36, cluster of differentiation 36; ROS, reactive oxygen species; NLRP3, NACHT, LRR and PYD domains-containing protein 3; ASC, apoptosis-associated speck-like protein containing a CARD; CARD8, caspase recruitment domain 8.

Without aiming to provide an extensive review on DAMP-induced inflammation, here we discuss some of the most studied DAMPs and describe how DAMPs induce priming or training of cells with which they interact. Furthermore, we elaborate on the postulation that innate immune memory may play a role in the pathogenesis of inflammatory diseases as consequences of DAMP-induced training of monocytes or macrophages.

Innate immune memory and DAMPs

Hypercholesterolemia, modified LDL cholesterol and HDL cholesterol

Atherosclerosis is the main cause of all cardiovascular diseases and modified low density lipoproteins (oxidized LDL or acetylated LDL) are known to be key inducer of atherosclerotic plaques[76]. In recent years the view on the pathogenesis of atherosclerosis has moved away from being considered a degenerative disease caused by the accumulation of lipids in the vessel layers, towards recognition of an important role played by inflammation. The inflammatory nature of atheroma plaque formation is recognized as a crucial feature, with modified oxidized LDL (oxLDL) serving as a DAMP and macrophage foam cells loaded with cholesterol droplets secreting chemokines that promote an inflammatory environment and recruit even more circulating monocytes to the atheroma site [70]. Interestingly, evidence from 1999 in mice showed that animals were more prone to develop atherosclerosis after BCG immunization [77]. Studies investigating trained immunity describe that macrophages previously trained by exposure to β-glucan exhibit transcriptional activation at several loci that encode cytokines or chemotactic proteins (such as IL-6, MCP-1, TNF, TGFβ, IL-18), but also at genes involved in metabolic processes important for atherogenesis (such as oxLDL uptake through CD36 or scavenger receptor A (SR-A), matrix degradation and plaque instability through cathepsin B, H, S, L, or matrix metallo protainase 9 or 2) [8]. This constellation of genes leads to the hypothesis that trained monocytes/macrophages could be proatherogenic, and that trained immunity may play an important role in the mechanism of plaque formation [45]. Subsequent experimental assessment has confirmed this hypothesis, showing that oxLDL itself can train primary human monocytes to secrete more proinflammatory cytokines (IL-6, IL-8, TNF, MCP-1), to express more PRRs and LDL receptors, and to increase the uptake of oxLDL, resulting in more foam cell formation [78]. This effect was not only present for the time of oxLDL exposure, but was visible after 7 days post stimulation in an in vitro model of trained immunity [78]. The transcriptional upregulation of proatherogenic genes *TNF, IL-6, MCP-1, IL-18, CD-36, SR-A and MMP2* was shown to coincide with enriched H3K4me3 at the gene promoters, confirming the epigenetic basis of this long-term effect [78]. This work was the first to demonstrate the capacity of an endogenous signal, oxLDL, to induce trained immunity, thereby providing a basis to understand the persistence of inflammation in atherosclerosis. More importantly, by describing the epigenetic background of this atherogenic phenotype, it allowed the possibility for new therapeutics that target the enzymes involved in inducing these epigenetic changes [78].

These findings add more evidence to the already established knowledge of epigenetic influences in atherosclerosis [79, 80]. Previous studies had shown that oxLDL can induce specific patterns of DNA methylation, another stable epigenetic mark at the level of DNA, in circulating monocytes and endothelial cells [81]. Moreover, evidence from mice fed a high cholesterol diet reveals DNA hypomethylation at promoter CpG regions of hematopietic

genes and increased monocyte counts [82]. Interestingly, bone marrow transfer from hypercholesterolemic mice to control mice led to persistence of the increased cell counts and the atherosclerotic potential [82], providing proof of metabolic imprinting at the level of progenitor cells.

In contrast to the numerous data supporting the idea that cholesterol and modified LDL particles drive cells to commit to a proinflammatory and atherogenic behavior, emerging indications show that high density lipoproteins (HDLs) can have opposite effects, reducing the inflammatory potential of primed cells by means of transcriptional repressor ATF3 (Activating Transcription Factor 3). In brief, ATF3 expression was upregulated in HDL-treated BMDM and CHIP sequencing revealed that ATF3 was enriched at promoter regions of cytokine genes that were produced in lower amount after HDL pre-treatment (such as IL-6, IL-12p40 or TNF), suggesting ATF3-dependent cytokine inhibition [83].

Monosodium urate crystals and uric acid

Monosodium urate (MSU) crystals and uric acid are DAMPs typically associated with gouty arthritis, which is another leading cause of morbidity in adult humans, predominantly in men. In addition to gout, recent studies suggest that MSU crystals and uric acid itself also play a role in signaling danger in common metabolic disorders of the adult [84], aging [55] or cancer [85]. It is known that the sequence of events involving urate-dependent signaling starts with cellular injury and death, causing intracellular purine metabolites such as uric acid to be released in the extracellular environment[86, 87]. Due to low solubility, high levels of uric acid eventually crystalize within tissues and the newly formed MSU crystals act as DAMPs on surrounding cells mounting inflammation [86, 87]. MSU has been shown to activate the NLRP3 inflammasome in THP1 cells [88] and to promote recruitment of ASC at the inflammasome formation site through polymerization of tubulin [89].

In gout, patients have abnormally high uric acid levels in the blood (hyperuricaemia) thereby being more prone to urate crystallization. The deposition of these crystals in the joint and subsequent triggering of inflammation comprises the typical manifestation of gouty arthritis, a very painful form of repeated spontaneous acute arthritic attacks that can sometimes progress to chronicity. Interestingly, in spite of the continuous presence of MSU crystals in the joints of patients, acute events occur episodically and vanish thereafter. This was scientifically explained in experiments which showed that MSU crystals alone are not sufficient to induce a full inflammatory reaction, but costimulatory signals such as LPS [90], Pam3Cys[91], long chain saturated fatty acids[71] or complement C5a [92] synergize with MSU in vitro and are presumably required to precipitate attacks in patients. This finding is in line with the two-step model of IL-1β production that has been proven several years ago and is currently accepted as a hallmark of IL-1β production [93]. This general model postulates that the initial induction of IL-1β takes place after PRR activation that induces

transcription and translation of pro-IL-1β precursor. Subsequently, the inactive precursor is cleaved by caspase-1 which is in turn is activated by the inflammasome [93].
More recently, MSU-dependent mechanisms of inhibition of molecular sensor AMPK (AMP-activated protein kinase) have been described [94], which add another proinflammatory feature to MSU crystals. Altogether, these data suggest that MSU is a candidate to induce long term effects in stimulated cells, especially because AMPK inhibits the mTOR pathway, which has been shown to play central role in trained immunity [31]. However, until now studies have focused on MSU as a trigger stimulus by way of rapidly eliciting inflammation, rather than by silent immune activation.
In contrast, this was not the case for soluble uric acid. Mononuclear cells of gout patients were observed to respond more prominently to TLR ligands than cells isolated from healthy controls [95, 96] and hyperuricaemia was assessed in relation to this differential response [97]. Interestingly, serum uric acid levels correlated with the amount of cytokine production in PBMCs of gout patients upon ex vivo stimulation [97]. In addition, in vitro priming of PBMCs or monocytes with soluble uric acid dose-dependently increased IL-1β and other proinflammatory cytokines, while uniquely downregulating IL-1 receptor antagonist (IL-1 RA) [97]. The enhanced cytokine production was persistent after the initial contact with uric acid [97]. Moreover, pharmacological inhibition of histone methyl transferases reversed uric acid priming effects and this provided evidence for longer term effects of hyperuricaemia, which are likely to be mediated by histone-related epigenetic marks [97].
Recently, hyperuricaemia was explored in relation to hypothalamic function [98] and it was revealed that uric acid induces NF-kB activation in hypothalamic cells and promotes inflammation and gliosis in a model of hyperuricaemia induced in rodents fed a high uric acid diet supplemented with oxonic acid (uricase inhibitor). This effect progressed to alter the hypothalamic functions in metabolic regulation (rapid induction followed by decline of neuroendocrine factor transcription, such as agouti related protein, proopiomelanocortin, thyrotropin-releasing hormone, or corticothropin releasing hormone), and rodents showed insulin resistance and dyslipidemia that was reversed by NFκB inhibition [98]. Of note, this effect was not seen in the case of short-term exposures to hyperuricaemia but was dependent on the persistence of the stimulus [98]. For uric acid, immune memory appears to be brought about through a mechanism of priming, consisting in continuous exposure for a prolonged period of time. Nevertheless, as mentioned previously [97], the monocytes still preserve a proinflammatory phenotype even after the removal of uric acid and the exact time dynamics of these effects have yet to be fully characterized.

Hyperglycemia and AGEs

Diabetes is another major metabolic disease associated with low-grade chronic inflammation and its hallmark is hyperglycemia. Abnormally high glucose levels are the culprit of a

sequence of events that culminate in irreversible vascular damage and subsequent organ dysfunction. Exposure of cells to high glucose environments in the context of diabetes has been shown to drive proinflammatory features in vascular endothelial cells, proliferation, and increased fibronectin expression that persists also when the cells are re-introduced to normoglycemic conditions [99]. This phenomenon has been termed "metabolic memory" or, more specifically, "the glycemic memory" [72]. An epigenetic basis for glucose-specific effects has been shown in bovine and human aortic endothelial cells, where increased ROS production and recruitment of Set7 methyltransferase enriches H3K4 monomethylation at the NFκB promoter under hyperglycemic conditions and these effects persist upon normalization of glucose levels [100]. This epigenetic modification of the NFκB promoter in turn mediates the upregulation of p65, MCP-1, and VCAM-1 [100]. However, less obvious effects were observed in PBMCs where high glucose media moderately increased monocyte-derived cytokines such as IL-β or IL-6 and had less effects in modifying IFNγ, IL-17 and IL-22 production [101]. In monocyte-derived macrophages cultured in hyperglycemic conditions, a dose-dependent increase in TNF, IL-6, IL-10 and IL-1RA was observed when stimulated with LPS or *M. tuberculosis* (MTB) H37Rv lysate, but not in a model of live MTB infection [101].

The proinflammatory environment in diabetes can be mediated by intermediary alarmins such as hyperglycemia-induced reactive oxygen species (ROS) [100] or irreversibly glycosylated proteins (generically called advanced glycation end products or AGEs) [99, 102]. AGEs signal via the receptor for AGEs (RAGE or AGER, Figure 2) and these have indeed been shown to mediate proinflammatory macrophage polarization (with enhanced IL-1β TNF and IL-6 production) in an NF-kB dependent manner [103]. Therefore, AGEs have the potential to induce long-lasting effects in the monocyte and make interesting candidates to assess in the context of trained immunity. Until now, much data have been gathered showing that epigenetic marks (histone modifications, DNA methylation and micro RNAs) are widely involved in the pathogenesis of diabetes at the microvascular level [99]. Deciphering the relationship between DAMPs and epigenetics is likely to shed more light into the functional reprogramming of vascular and circulating cells in diabetes and also on the way these cells become metabolically imprinted to drive disease onset.

HMGB1

Perhaps the most studied DAMP so far is the nuclear protein HMGB1, member of the high mobility group proteins. HMGB1 is a conserved protein in eukaryotic organisms, typically found in the nucleus where it is the most abundant non-histone protein associated with chromatin [104] and exerts roles as chromatin modifier and transcriptional regulator [105]. Extracellular HMGB1 is released during non-physiologic death [57] but can also be actively secreted (as reviewed in [106]) and was shown to mediate inflammatory reactions in a TLR4,

TLR2 or TLR9 and RAGE dependent signaling cascade (extensively reviewed in [106, 107]). The release of HMGB1 has been linked with a wide range of diseases such as sepsis [108, 109], autoimmune diseases [110], cardiovascular diseases [111], and many other diseases reviewed in [106, 107, 112] such as post-traumatic shock, ischemia-reperfusion injury or various types of cancers. Intriguingly, the HMGB1 protein has two domains that behave differently: box B specifically mediates the proinflammatory effects downstream HMGB1, while box A inhibits IL-6 and TNF secretion in THP1 cells in vitro and in a murine model of sepsis in vivo, and this effect is presumably due to competition with the binding of box B to the receptors, thereby tempering the signal [113]. Given the acknowledged role played by HMGB1 in sepsis, one study evaluated the long-term consequences of HMGB1 exposure on post-sepsis progression [114]. Interestingly, mouse splenocytes which had been pretreated with HMGB1 for 24 hours or 8 days responded with significantly higher TNF production when washed and restimulated with PAMPs such as Pam3Cys, LPS, CpG, or other DAMPs such as S100A12 [114]. This shows that HMGB1 can induce nonspecific priming of cytokine production in splenocytes, and that this induction persists even after HMGB1 has been eliminated from the environment [114]. As opposed to this, tolerizing effects were observed after low-dose HMGB1 priming in a murine model of liver ischemia-reperfusion injury, in which mice "preconditioned" with HMGB1 were protected from rampant inflammation and organ failure [115]. This latter effect was also mediated by TLR4 signaling but was associated with activation of the negative regulator IRAK-M [115]. It is unclear how HMGB1 exerts these seemingly opposing effects; it remains to be determined whether it is a question of dose (similar to LPS [24, 25, 116]) or by preferential binding of the different protein domains to the receptor. In addition, the molecular mechanism of these persistent effects of HMGB1 pre-exposure still needs to be assessed, with differential epigenetic regulation being an important possibility to take into account.

Conclusions and future prospects

Innate immunity is a sensitive system capable of integrating signals to induce adaptive host defense. It has developed unique receptors that allow recognition of molecular patterns that signal a threat to its homeostasis. These can be external infectious particles (PAMPs) or can be internal non-foreign particles that are normally hidden from PRRs but become exposed in times of stress. Although beneficial in its main purpose, excessive signaling via these receptors can become pathogenic. In situations when the inflammatory trigger persists and determines reminiscent inflammation, or when alarmins are abundant in the internal environment, chronic low-grade inflammation ensues and this is a hallmark of common metabolic diseases or aging.

Further down the line, exposure to PAMPs or DAMPs can elicit adaptive features in cells of the innate immune system, phenomenon known as trained immunity. Memory of past

signals allow for more potential to fight infectious intruders the second time an infection is encountered, and without involvement of the adaptive system. Knowing that most of the infections are cleared independently of the adaptive immune system, it becomes interesting to study how the training of the innate response would afford protection in the context of natural infection. Another way to exploit the beneficial effects of trained immunity is via the non-specific effects of vaccines on the host immune response [4, 117]. Moreover, in situations where immunomodulatory approaches are used to treat disease, like in the case of bladder cancer where some patients benefit from intra-vesical BCG instillations[118], the potential of the individual to develop trained immunity seems to be important for the success rate of the therapy [119].

In this review we have described emerging data showing that, in some instances, cells can become more prone to engage in autoinflammatory processes upon training. Moreover, very similar training or priming effects on the innate immune system have been described for some DAMPs themselves suggesting that tissue-derived alarmins can modulate the type and magnitude of the innate response and could potentially be involved in the persistence of inflammation or in immune tolerance. The potential for this type of responses to be imprinted also at the level of myelopoietic cells may constitute a further checkpoint in the long term training effects of DAMPs and it requires further study to unravel whether epigenetic regulation is indeed reset at the level of progenitor cells as well as in the periphery.

This effect of DAMPs as signals that induce priming or trained immunity is extremely important to be acknowledged and better characterized. Identifying endogenous molecules that can induce long term inflammatory effects would add significant knowledge to the general mechanisms of persistent inflammation or susceptibility to autoinflammatory episodes in some individuals. Even more importantly, this would also allow at least two types of intervention. One useful approach would be in the development of immunization strategies where endogenous enforcers of the immune response could work as vaccine adjuvants. Eventually, knowing the exact epigenetic mechanisms that regulate the memory of innate immune cells in response to DAMPs could facilitate attempts at reversing the functional imprinting in autoinflammatory diseases.

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

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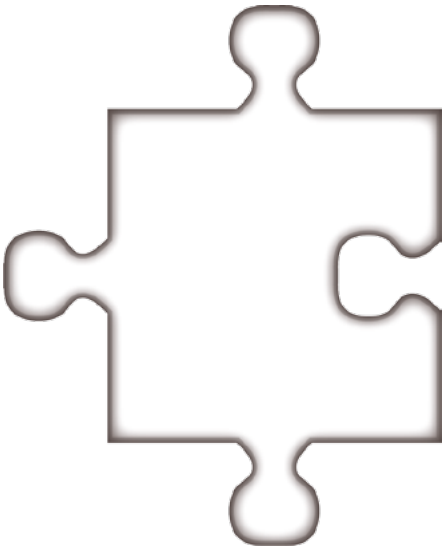

SUMMARY, GENERAL DISCUSSION AND PROSPECTS

11

Gout is a form of arthritis known since ancient times (1). Many kings and rulers were affected by this disorder, many famous personalities throughout history were also gout sufferers (2). This propensity for wellbeing social categories made gout known for centuries as the "disease of kings"(3). Nowadays, we know a lot more about the pathogenesis of gout, we have evidence of its high frequency regardless of social status (4), we have developed guidelines for better diagnosis and classification (5-7), and efforts are made to develop more specific and better tolerated therapies for gout (8, 9). This thesis represents a scientific endeavor to better understand some of the molecular mechanisms that enhance inflammation in some individuals compared to others, with a focus on gout and hyperuricemia.

In this thesis, we have focused on several aspects of inflammation in gout, as also summarized in Chapter 1, Figure1:

- To begin with, we have studied processes that have a role in the susceptibility to inflammation and gout. The process of autophagy and its involvement in cytokine production biology has been studied. We described that autophagy inhibition, in experimental conditions or through genetic factors, can be a susceptibility determinant for inflammatory diseases.
- Thereafter, we showed that, similarity to other autoinflammatory conditions, gout patients exhibit a higher cytokine production profile in response to *ex vivo* stimulations with gout-related stimuli. We hypothesized that this could be attributed to soluble uric acid exposure. We extensively studied the involvement of soluble uric acid in modifying the behavior of myeloid cells in response to MSU crystals in combination with TLR ligands. We showed that pathways are modulated to explain at least in part how uric acid could play a role in the natural history of gout: AKT is upregulated, autophagy is downregulated, reactive oxygen species production is inhibited, and epigenetic modifications could be induced by uric acid.
- Subsequently, we have targeted potential therapeutic candidates in order to assess if we could to limit uric acid crystal-induced inflammation. We described the beneficial roles of short chain fatty acid butyrate, class I histone deacetylase inhibitors, or the serine protease inhibitor alpha-1-antitrypsin (AAT).
- Finally, we highlighted the possible long-term consequences of exposure to sterile inflammatory stimuli, such as uric acid or MSU crystals but also other well-known metabolic stimuli. We reviewed the current literature on damage associated molecular pattern induced epigenetic reprogramming, and argued that sterile stimuli or metabolic deregulations might have persistent effects on cytokine production at the cellular level, with wide implications for inflammatory diseases.

This chapter summarizes the work we have presented in the previous sections, puts it into the current literature context, and discusses some of the future perspectives for research in the field. A schematic representation of the outlined findings is presented in Figure 1.

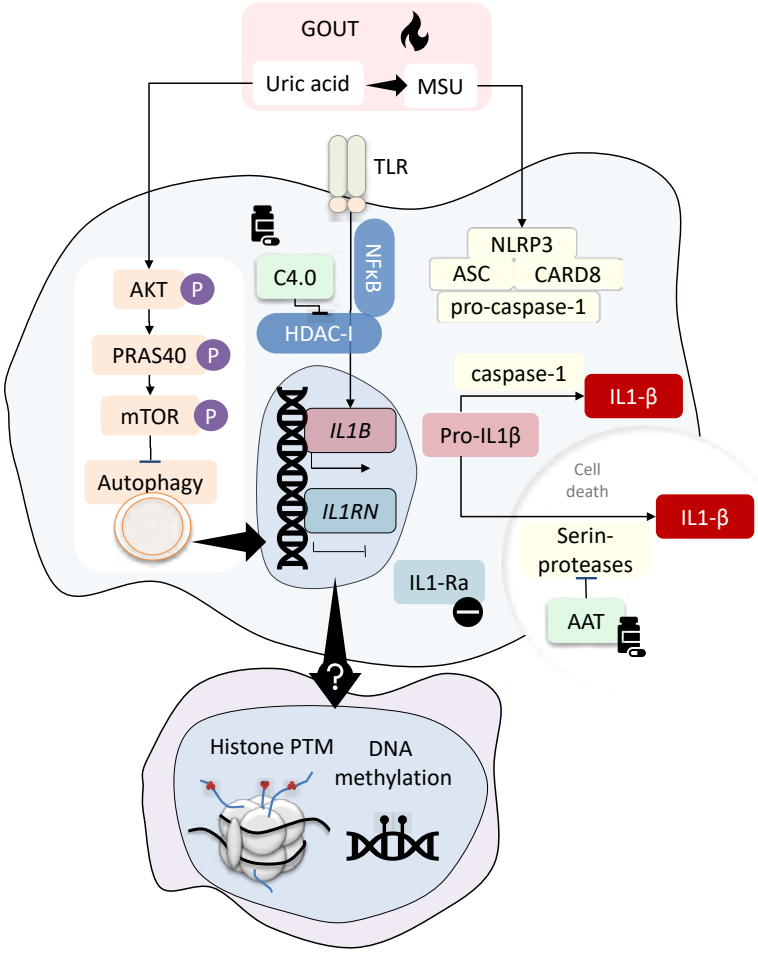

FIGURE 1. Schematic representation of data described in the thesis and outlined in this chapter.

Please refer to text for detailed explanations. MSU, monosodium urate crystals; AKT, protein kinase B; PRAS40, proline rich AKT substrate 40kDa; mTOR, mammalian target of rapamycin; TLR, toll like receptor; C4.0, butyrate; HDAC-I, histone deaceylases class I; NLRP3, NACHT, LRR and PYD domains-containing protein 3; ASC, apoptosis-associated speck-like protein containing a CARD; CARD8, caspase recruitment domain 8; IL1B, interleukin-1β gene; IL1RN, interleukin 1 receptor antagonist gene; IL-1β, interleukin-1β protein; IL-1Ra, interleukin 1 receptor antagonist protein; AAT, alpha-1-antitrypsin; PTM, posttranslational modifications.

In **Chapter 1** of the thesis, we provide a brief outline of the general concept of autoinflammation and the spectrum of conditions that are characterized by enhanced cytokine production mechanisms caused by genes or environment (10). A central role in autoinflammation is held by the overproduction of interleukin-1 (IL-1) and IL-1 blockade is therapeutic in this class of diseases (11). The central theme of this thesis is represented by hyperuricemia and gout, which has been further described in chapter 1 from a pathogenesis point of view, in the context of latest enhancements in recent years' research (12). Soluble uric acid is higher in primates than in other vertebrates, due to progressive loss of function mutations that culminated in the pseudogenization of the uricase gene, which rendered the gene completely non-functional (13). Uricase is an enzyme that metabolizes uric acid to the more soluble product allantoin. As this enzyme is absent in humans, our serum urate levels are enhanced to a normal range of 2-6 mg/dL. In the context of genetic variation and life style, serum urate levels may vary, and when they surpass the solubility level we define this condition as hyperuricemia. Hyperuricemia is known to be an associated metabolic factor in several diseases: cardiovascular, renal, metabolic syndrome (14). In gout, hyperuricemia is the sole necessary condition that leads to uric acid supersaturation and crystal deposition in tissues. The synovial localization of uric acid crystals determines inflammation at that site when secondary stimuli are present and act in synergy with the crystals to activate IL-1β (15). Next to hyperuricemia, we summarize that gout patients have a distinct gut microbiome that is less capable of producing the anti-inflammatory short-chain fatty acid butyrate and which might be a fruitful point for intervention in the future (16). We describe the involvement of the inflammasome protein platform and show the interactions of the NLRP3 inflammasome with other organelle dysfunction in the cell and autophagy (17). Finally, we highlight the importance of inflammasome-independent mechanisms that modulate IL-1β activation in gout (18). We describe the role of extracellular processing of precursor cytokines into active cytokines by neutrophil derived serine proteases, and how this can be controlled by serine protease inhibitors as potential new therapy in gout. Moreover, we review evidence that aggregated neutrophil extracellular traps and neutrophil ectosomes modulate the concentrations of inflammatory cytokines in gout playing a role in the resolution of inflammation (19). These data integrate a model for gout in which some aspects are widely known and accepted: the involvement of uric acid, the crystallization in tissues, the formation of active IL-1β. Nevertheless, it adds mechanistic questions for processes that are still in study and which can become new targets: the microbiome, inflammasome activation, phagosome deregulation processes, inflammasome-independent cytokine activation or neutrophil-mediated extracellular degradation of inflammatory cytokines.

In **Chapters 2 and 3** of this thesis, we study the process of autophagy and its general roles in inflammation, with an interest in the potential for targeting autophagy in inflammatory diseases. Autophagy is a cell housekeeping mechanism that has been genetically conserved throughout evolution. It consists in the formation of double membrane vesicles that engulf cytoplasmic cargo and deliver it to the lysosome for breakdown and recycling in times of nutrient deprivation(20). Later studies linked autophagy to innate immunity and pathogen-associated molecular pattern (PAMP) recognition (21) as well as MHC-II dependent activation of specific immunity (22). Moreover, SNPs in autophagy related genes *ATG16L1* and *IRGM* were associated with Crohn's disease (23). The study presented in **Chapter 2** is the first attempt to investigate the modulation of inflammatory cytokines by the process of autophagy in human myeloid cells. We have shown that disruption of autophagy has an important impact on inflammatory cytokine modulation, determining a remarkable and specific increase in IL-1β secretion. Interestingly, TNF production was diminished. We demonstrated that caspase-1 activation was not modified, all protein regulation being due to transcriptional changes. We have further investigated the involvement of MAPK pathways in the regulation of cytokine levels and obtained indications that p38 could be involved in TNF regulation in this setup. This study was further proven relevant by later reports that confirmed the basal inhibition of autophagy on IL-1 production in human cells, showing that autophagy is a mechanism that can target pro-IL-1β for degradation (24). While a lot of studies (mostly in murine cells) show an interaction between autophagy and inflammasome (25-27), this was the first to also show transcriptional regulation to be upregulated due to autophagy disruption.
Following up on the evidence that *ATG16L1* (Autophagy related 16 like 1) genetic variation is associated to Crohn's disease, in **Chapter 3** we further showed that carriers of the risk allele of the ATG16L1 T300A variant have modified ATG16L1 levels and show enhanced cytokine responses when cells are specifically triggered with muramyl dipeptide, a ligand of NOD2. In conclusion, genetic variation in autophagy related genes, in this example *ATG16L1,* is another associated factor to higher production of pro-inflammatory cytokines that could be a risk factor for inflammatory diseases.

In **Chapter 4,** we studied the behavior of cells isolated from gout patients compared to healthy controls, upon *ex vivo* stimulation with MSU crystals with or without TLR ligands. This report added another proof (15) that MSU crystals alone cannot induce IL-1β production. Moreover, it documented that cells of gout patients retain a proinflammatory pattern, secreting more IL-1β compared to cells of heathy volunteers, similarly to other studies describing higher cytokine production in autoinflammatory diseases patients (11).

We further pursued to explain this finding in **Chapters 5, 6 and 7** by hypothesizing that soluble uric acid could have a role to play in this higher cytokine production profile.
As mentioned earlier, the elevation of uric acid concentrations in humans has been associated to several other diseases than gout: metabolic syndrome, atherosclerosis, hypertension, type 2 diabetes, chronic kidney disease (14, 28-30), aging (31), or cancer (32), making uric acid an important link between the diseases of modern society associated with chronic low grade inflammation.
Moreover, reports from experimental studies have also provided evidence in the past that elevated uric acid concentrations have proinflammatory effects: mice with hyperuricemia have a higher cytokine production upon LPS challenge compared to control animals(33); NFκB was shown to be activated in mice injected intraperitoneally with uric acid in renal or pancreatic cells (34, 35); uric acid was revealed to induce NFκB activation in hypothalamic cells and promote inflammation and gliosis with possible consequences of altering the hypothalamic functions in metabolic regulation (36).
In our experiments described in **Chapter 5**, we have observed that cells originating from gout patients differ in their cytokine production capacity from control volunteers in a serum uric acid dependent manner. This correlation was intriguing, therefore we designed a protocol to mimic the uric acid initial exposure of cells by pre-treating cells with medium and uric acid (priming for 24 h). Thereafter, cells were washed and stimulated with TLR ligands in the presence or absence of MSU crystals (restimulation for 24 h). This protocol allowed us to test for cytokine responses in different cell suspensions, with variable levels of monocyte purity: PBMCs, Percoll enriched monocytes, negatively selected monocytes through magnetic bead separation. We have consistently been able to show that uric acid priming significantly, dose-dependently, enhances the transcription and protein levels of pro-inflammatory cytokines (IL-1β, IL-1α, IL-6, TNF) and decreases the production of anti-inflammatory cytokine IL-1Ra, but not IL-10.
This result was surprising, as IL-1 upregulation is usually accompanied by IL-1Ra upregulation, since they are closely linked in a modulatory feedback loop (37). IL-1Ra downregulation was already apparent after 24 h priming with uric acid alone, when IL-1β levels were not modified. Importantly, the specific downregulation of IL-1Ra is of particular interest and should be acknowledged even when the increase in IL-1β is not visible. In the more extreme cases of DIRA syndrome (Deficiency of IL-1 Receptor Antagonist) the clinical picture includes severe bone and joint tissue inflammation pinpointing to the significant vulnerability of IL-1Ra deficient patients to IL-1 mediated inflammation (38). Addition of anakinra *in vitro* reestablished the IL-1Ra and IL-1β levels after uric acid priming but did not have longer effects than the period of administration. Therefore, the priming effects of uric acid could be reversed by the addition of exogenous IL-1Ra but, mechanistically, other signaling molecules should be involved in order to explain why these changes in cytokine production occur and

persist even after uric acid washout. Through pharmacological inhibition, we have obtained indications that histone methyl transferase inhibition can block uric acid effects, leading us to hypothesize that histone modifications might mediate uric acid priming, as will be subsequently investigated.

In **Chapters 6**, we further studied the mechanism through which uric acid priming works in monocytes *in vitro*. We generated transcriptomic data through RNA-sequencing (RNAseq) in highly pure human monocytes treated for 24h with medium or uric acid. Known effects such as *IL1B* or *IL6* upregulation and *IL1RN* decrease in both RNA levels and protein levels could be confirmed. Pathway enrichment analysis was performed based on dynamic genes in the RNAseq dataset and functional experiments were performed to validate their involvement. An important candidate was the AKT pathway which was indicated by RNAseq findings through terms such as mTOR (upregulated genes) and downregulated FoxO transcription factors. We showed that AKT phosphorylation was enhanced upon uric acid treatment. Moreover, we described that PRAS40 phosphorylation was also induced in the presence of uric acid. PRAS40 is a molecule that exerts inhibitory roles on mTOR in non-phosphorylated state: Once phosphorylated, PRAS40 dissociates from the Raptor protein, releasing the inhibitory signal on mTOR (39). As mTOR activation is also a well-known autophagy inhibition mechanism (40), we have further assessed the levels of LC3-II autophagosome formation in response to uric acid. In our findings, we have further observed that autophagy levels are diminished in HeLa cells that overexpressed LC3 coupled with GFP. Therefore, we have made the link that, possibly due to mTOR stimulation, autophagy is inhibited by uric acid. The finding that basal autophagy levels are diminished, reminded us of the enhanced cytokine production observed in situations where autophagy was inhibited, as described in chapters 2 and 3, therefore showing that autophagy could be a possible new source for intervention in gout. While, based on previously cited reports, autophagy defects are a known cause for IL-1β overproduction, it is still not completely understood how IL-1Ra is uncoupled from IL-1β in uric acid priming. In our report and in other literature data (41) we have proven that pharmacological inhibition of autophagy can diminish IL-1Ra levels but further studies are necessary to show whether these two effects are mechanistically related. Interestingly, in our experimental setup, uric acid potently inhibited reactive oxygen species. Although there are reports that associate uric acid with inflammatory signaling through a ROS induction mechanism (42), we were not able to document this in our setup. Nevertheless, the scientific literature is still conflicting regarding this matter and uric acid appears to have dual roles in oxidative stress (43).

In **Chapter 7,** we focused on the causes of the persistence of uric acid effects. As described earlier, all cytokine data was obtained in a uric acid priming dependent manner, but produced only in response to second stimulation. At the time of stimulation, uric acid was no longer present in the culture medium, suggesting there should be a reminiscent

phenotype induced by the previous exposure. Following up on this, we describe that these cytokine variations are persistent even when a resting time is allowed between initial uric acid exposure and TLR stimulation. We hypothesized that a background for immunological memory might be present after uric acid priming and we explored whether uric acid treatment can induce epigenetic reprogramming in monocytes. Based on studies of our group linking metabolic stimuli to changes in histone marks at specific gene promoter regions, we tested two initial candidates through ChIP sequencing (H3K4me3 and H3K27ac). To our surprise, we could not find any genes significantly dynamic for these two marks in response to uric acid. Other histone marks or other protein posttranslational modifications could be involved in uric acid priming. Nevertheless, in a cohort of Maori ancestry, we were able to study DNA methylation levels and to correlate them with uric acid levels. Polynesian populations have genetic backgrounds that predispose them to hyperuricemia and gout (44). In this study, whole blood was assessed for DNA methylation in hyperuricemic versus normouricemic volunteers. Interestingly, several differentially methylated regions have been identified, among which some were functionally linked to IL-1 and IL-1Ra production (SMAD7, SOCS3). As pointed out, IL-Ra downregulation is one of the most intriguing findings caused by uric acid exposure, therefore potential candidates in this pathway are important to be investigated and validated in further experiments.

An important point for discussion in the context of hyperuricemia in both clinical and basic science settings is: how sure ca sure can we be that the effects we are studying are indeed due to abnormally high soluble uric acid concentrations and not to urate micro crystals?

For differences observed *in vivo* we cannot exclude that the presence of crystal deposits caused by hyperuricemia mediate the effects that we observe. For *in vitro* conditions, we have actively tried to identify if crystals start to form over the course of our experiments, but this showed negative data. There are a few cytokine production differences *in vitro* that discriminate between MSU crystals and soluble uric acid effects. In chapters 5-7 we have shown data that MSU crystals induce IL-1Ra both on their own and in synergy with other ligands. This shows a clearly different mechanism of action than soluble uric acid which significantly downregulates IL-1Ra. Seemingly reassuring is also the observation of the same cytokine pattern at lower uric acid concentrations (at which uric acid is 100% soluble), as presented in Chapter 5. Nevertheless, the possibilities that crystal deposition in asymptomatic hyperuricaemia can have systemic effects, by changing the inflammatory properties or the epigenetic state of immune cells, are a very interesting point for future studies.

Subsequently, two possible therapeutic interventions have been studied and presented in **Chapters 8 and 9.** Here, we used a combination of MSU crystals and long chain fatty acid palmitate (C16.0), to induce active IL-1β production and inflammation *in vitro* and

in vivo, respectively. In **Chapter 8**, we examined the capacity of short chain fatty acid butyrate(C4.0) to limit the cytokine production in PBMCs from healthy donors and crystal-proven gout patients. Butyrate was previously reported to be anti-inflammatory, mainly through inhibition of NFκB signaling (45, 46) and through inhibition of histone deacetylases (HDACs)(47). First, we showed that butyrate dose-dependently reduces MSU/C16.0 induced cytokines through a process mediated at transcriptional level. Furthermore, we performed assays to define the roles of other pharmacological HDAC inhibitors. We showed that different pan-HDAC inhibitors can have discordant roles on cytokine production in the same experimental setup, and same HDAC inhibitors can have opposite effects depending on the inflammatory stimulus. This indicates that HDAC inhibitor activity is extremely variable and specific knowledge of HDAC class, concentration and inflammatory stimulus is necessary for understanding the biological effects. In this study, we describe for the first time that butyrate inhibits class-I HDACs, and synthetic class-I HDAC inhibitors have very similar effects to those of butyrate. These findings are narrowing down the HDAC targets that could be treatment options for gouty arthritis. In **Chapter 9,** we refer to a murine model of gouty arthritis induced by intraarticular MSU/C16.0 injection where we describe extremely potent anti-inflammatory effects of recombinant human alpha-1-anti-trypsin (AAT)-IgG1 Fc fusion protein (AAT-Fc). AAT is one of the most abundant proteins in plasma and a crucial serine-protease inhibitor (48). The major source of serine-proteases are neutrophils, the main cell type that dominates gouty inflammation *in vivo*. In gouty arthritis, there is significant neutrophil influx into the joint. Upon cell death induced by sterile inflammation, neutrophils release proteases into the extracellular space. This has consequences for IL-1 mediated inflammation, as neutrophil serine-proteases can also cleave pro-IL-1 and, thus, promote inflammation (18). We have shown that administration of exogenous AAT (both plasma derived and the recombinant AAT-Fc fusion protein) have very strong properties for suppressing inflammation and interleukin (IL)-1β production in gouty arthritis. However, AAT-Fc has significantly more potent effects in reducing inflammation through several complementary effects in addition to protease inhibition, such as caspase-1 inhibition or IL-1Ra induction in monocytes. The potency of AAT-Fc enforces the concept that targeting inflammasome-independent IL-1β activation is beneficial in inflammatory diseases mediated by rich neutrophil influx. The new fusion protein also and provides a useful therapy tool with lower production costs and less invasive administration methods compared to the plasma derived AAT (49).

Finally, in **Chapter 10,** we have reviewed the literature and described current knowledge that the innate branch of the immune system possesses an epigenetically encoded memory of previous insults. The hypothesis that the immunological memory is not restricted to the adaptive branch of the immune system only, was originally presented in 2011 under the

name "trained immunity" (50) and was based on a large body of previous evidence that supported it. Trained immunity, as we have extensively described in this chapter, is now a widely studied process that is based on the epigenetic reprogramming of myeloid or lymphoid cells involved in innate immunity. Trained immunity has been mechanistically deciphered for several pathogenic stimuli and has relevance for susceptibility to infection, immunological tolerance or vaccine biology. In addition, here we have integrated old evidence and emerging data that sterile stimuli could also induce innate immune memory. The long-term effects of sterile stressors could be of great importance for a wide range of inflammatory diseases. For example the involvement of epigenetic memory is already well known and extensively studied through the concept of "glycemic memory" in diabetes mellitus (51). Trained immunity was already linked to atherosclerosis (52). We also argue that stimuli such as uric acid or MSU crystals could also drive a memory of the innate immune system that might be of high relevance to gout, but also to other diseases where hyperuricemia is involved. In conclusion, in this review we introduce the common knowledge on trained immunity as background for its putative implications in responses to damage associated molecular patterns and imply that this area has great implications for pathology and sterile inflammation.

PROSPECTS

In this thesis, we have attempted to address several questions that had the potential to explain some of the variance we observe in patients with hyperuricemia or gout. We have unraveled some very interesting findings in relation to uric acid mediated inflammation and vulnerability due to IL-1Ra decrease. We have seen that autophagy inhibition is now a mechanism that is involved in the upregulation of IL-1β also in gout, as well as in other autoinflammatory conditions. We have obtained indications of the AKT and PRAS40 phosphorylation in uric acid stimulated cells, leading to mTOR activation as signaling mechanism for uric acid priming. Moreover, we are currently investigating the chromatin dynamics in uric acid treated cells and the extent of the innate immune memory that could be induced in the context of gout and hyperuricemia. We have seen that histone deacetylase inhibitors are a potential therapy in gout, while exogenous AAT administration could prove significantly potent and safe therapy for limiting gouty arthritis.

However, a lot of scientific questions remain to be addressed in the future. When considering the effects of hyperuricemia, the supplementary data presented in Chapter 6 illustrates that uric acid can impact on many different biological processes for which hyperuricemia and DAMP mediated responses are of relevance. Not only inflammatory processes such those described so far are enriched, but also other unexplored processes such as type I interferon signaling pathway. It is questionable what kind of involvement this pathway has

in gout, but the signals for strong IRF (interferon regulatory factor) pathway involvement are observed, and future investigation is needed in this direction.

The concerning switch in IL-1β and IL-1Ra production in response to uric acid treatment will warrant more research in the near future. It is still not completely understood at which point is IL-1Ra uncoupled from IL-1β and whether one single pathway is involved in both cytokines' variation, or whether they are independently induced by different cascades. Now, we speculate that autophagy is one mechanism that could differentially regulate both cytokines, but the data we have is still not explanatory and more mechanistic research is needed to elucidate this.

Another important point of study is the validity of the IL-1/IL-1Ra shift in hyperuricemic patients *in vivo*. Our data presented in Chapter 5 described lack of IL-1Ra downregulation in plasma of gout patients with high uric acid levels. It is still not understood if this result is real or if other factors need to be integrated with the plasma measurements. It is known that gout patients will have signs of inflammation depending on disease status, the data will have confounding factors such as therapy and clinical parameter variation. Moreover, biologically, IL-1Ra will still be enhanced in inflammatory situations, thus, whether uric acid downregulates IL-1Ra compared to controls would be very difficult to assess in patients. Obtaining data on plasma cytokine variation in cohorts, integrating it with clinical data as well as with functional data, has the potential to address these issues and assess the clinical utility of our *in vitro* observations.

With the advent of big data analysis and genomic research, functional genomics approaches can be undertaken to also investigate how these phenotypic data are influenced by the genetic variation of the host. Moreover, genetic and genomics studies are crucial in finding out which are the genetic susceptibility factors associated to gouty inflammation, by controlling for the hyperuricemia related genetic risk. The need for genetic associated studies where hyperuricemic controls are used as reference is a promising new endeavor within the scientific community in the field of gout research. In light of the data presented in this thesis, targeted genetic research could also be undertaken for the processes that have been shown to be involved in gout related inflammation. For example, testing for autophagy gene variants, such as *ATG16L1* variants, among others, could be an interesting hypothesis for the genetic susceptibility to gout.

Next to genetic factors, however, environmental factors should also be taken into account when addressing gout susceptibility. As we have tried to argue, the evidence that innate immune memory plays a role in hyperuricemia is not entirely elucidated but is a very important new research question that should be addressed. The knowledge of epigenetic regulation in response to metabolic stimuli is not only important for the understanding of gout pathogenesis, but holds promise for therapy. Understanding the genetic susceptibility factors can help clinicians in prevent gout by acting on other modifiable risk factors. In

contrast, evidence of epigenetic changes in gout would constitute new resources for development of therapy, since epigenetic modifications are known to be reversible.
Another interesting point of focus that has scarcely been addressed is the potential for immune memory and persistent inflammation induced by MSU crystals. We do not expect that the *in vitro* findings described in this thesis are due to the crystalline form of urate, since we showed data that they induce different IL-1/IL-1Ra patterns. But for the data that we see *in vivo*, we cannot exclude that the systemic inflammatory effects are influenced also by MSU deposits.
Furthermore, if uric acid or MSU induce innate immune memory in gout, another major question involves the persistence of trained immunity *in vivo*. What is it that maintains the pool of cells that hold that primed/trained phenotype? Two possibilities could explain this: latent persistence with continuous activation of innate cells by the initial stimulus (most likely at the level of cell populations with long lifetimes and self-renewing capacity, such as tissue macrophages) or training at the level of progenitor cells. However, data for any of these mechanisms is scarce and explanatory additional studies are necessary.

The field of gout and hyperuricemia research is receiving increasing attention due to the burden of gout in terms of prevalence, in terms of bioeconomic consequences, but also because emerging research has attracted attention to some new proinflammatory mechanisms of potential for risk and therapy. Continuing these studies requires innovative strategies to integrate datasets and draw biologically relevant conclusions. If uric acid is proven to exert stable inflammatory effects non-restricted to MSU crystallization, than the field of hyperuricemia research is likely to impact on a broad range of uric acid associated inflammatory diseases in the future.

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Nederlandse samenvatting
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12

NEDERLANDSE SAMENVATTING

Jicht is een vorm van artritis die al sinds de oudheid bekend is. Veel koningen en heersers leden aan deze stoornis, en veel bekende persoonlijkheden door de geschiedenis heen waren ook jichtlijders. Door deze aanleg voor jicht bij de hogere sociale klassen stond jicht al sinds eeuwen bekend onder de naam „de ziekte van koningen" .Tegenwoordig weten we veel meer over de pathogenese van jicht. Jicht komt vaak voor, ongeacht maatschappelijke status. Er zijn richtlijnen ontwikkeld voor betere diagnose en classificatie, en er wordt geprobeerd om meer specifieke en betere tolerante therapieën voor jicht te ontwikkelen. Dit proefschrift vertegenwoordigt het wetenschappelijk streven de moleculaire mechanismen, die ontsteking veroorzaken bij jicht en hyperuricemie , beter te begrijpen.

In **Hoofdstuk 1** van dit proefschrift geven we een kort overzicht van het algemene begrip auto-inflammatie, (ontsteking gericht tegen het zelf) met een focus op hyperuricemie en jicht. In tegenstelling tot andere gewervelde dieren, is in primaten het oplosbare urinezuur verhoogd. Dit komt door verlies-van-functie mutaties in het uricase gen, waardoor het gen volledig niet functioneel is. Uricase is een enzym dat urine metaboliseert in oplosbaar allantoïne. Aangezien dit enzym afwezig is bij mensen, is ons serum uraatgehalte verhoogd tot 2-6 mg / dL. In samenhang met de genetische variatie en levensstijl kan het serumgehalte van urine zelfs nog hoger worden en wanneer het oplosbaarheidsniveau wordt overschreden treedt er hyperuricemie op. Hyperuricemie is bekend als een bijbehorende metabole factor bij verscheidene aandoeningen: cardiovasculaire, nierziekte en het metabole syndroom. Bij jicht leidt hyperuricemie tot oververzadiging van urinezuur en kristalafzetting in weefsels. De synoviale lokalisatie van urinezuurkristallen bepaalt de ontsteking in het gewricht, hetgeen wordt gemedieerd door het centrale cytokine (proinflammatoire signaalproteïne), interleukine-1 beta (IL-1β). Niettemin worden er ook andere processen bestudeerd die nieuwe spelers kunnen worden in jicht ontsteking, zoals beschreven in hoofdstuk 1: het microbioom, inflammasoom activatie, fagosome deregulatieprocessen, inflammasoom onafhankelijke cytokine activatie en neutrofielgemedieerde extracellulaire afbraak van inflammatoire cytokines.

In **Hoofdstuk 2 en 3** van dit proefschrift is het proces van autofagie en de betrokkenheid bij de cytokine productie bestudeerd. Autofagie is het mechanisme van de celhuishouding, dat door de evolutie genetisch bewaard gebleven is. Het bestaat uit de vorming van een soort blaasjes met dubbel membraan die cytoplasmatische lading opvangen en vervolgens voor afbraak aan het lysosoom leveren. Dit heeft tot doel voedingsstoffen te recyclen in tijden van ontbering. Latere studies koppelen autofagie aan aangeboren immuniteit, evenals aan de activatie van adaptieve immuniteit. We hebben beschreven dat remming van autofagie, in experimentele omstandigheden of door genetische factoren, bepalend kan zijn voor de gevoeligheid voor autoinflammatoire aandoeningen.

In **Hoofdstuk 4** hebben we het gedrag van cellen,(geïsoleerd van jichtpatiënten en gezonde controles), die gestimuleerd waren met MSU kristallen met of zonder TLR liganden, bestudeerd. Hieruit bleek eens te meer dat MSU-kristallen alleen, geen IL-1β-productie kunnen veroorzaken, omdat een tweede signaal cruciaal is voor jicht. Bovendien liet het zien dat cellen van jichtpatiënten meer IL-1β produceren in vergelijking met cellen van gezonde vrijwilligers.

In de **Hoofdstukken 5, 6 en 7** bespreken we deze bevindingen en de hypothese dat oplosbaar urinezuur een rol zou kunnen spelen in de hogere productie van cytokines.

In onze experimenten, beschreven in **Hoofdstuk 5**, hebben we geconstateerd dat de cellen van jichtpatiënten en gezonde controles verschillen in hun cytokine productiecapaciteit op een serum urinezuur afhankelijke manier. Deze correlatie was intrigerend, daarom hebben we een protocol ontworpen om de initiële blootstelling van cellen aan urinezuur na te bootsen, door de cellen voor te behandelen (priming) met medium en urinezuur (24 uur). Daarna werden de cellen gewassen en gestimuleerd met TLR liganden in aan- of afwezigheid van MSU kristallen (restimulatie gedurende 24 uur). We konden vaststellen dat priming met urinezuur, dosisafhankelijk, het transcriptie- en eiwitgehalte van pro-inflammatoire cytokinen (zoals IL-1β) verhoogt. Tegelijkertijd verlaagt urinezuur de productie van anti-inflammatoire cytokinen (zoals IL-1 receptor antagonist, IL-1Ra).

Dit resultaat was verrassend, aangezien IL-1 upregulatie gewoonlijk vergezeld gaat van IL-1Ra upregulatie via een modulerende feedback lus. In de meer extreme gevallen van het DIRA syndroom (Deficiëntie van IL-1 Receptor Antagonist) omvat het klinische beeld ernstige bot- en gewrichtsweefselontstekingen, die wijzen op de significante kwetsbaarheid van IL-1Ra-deficiënte patiënten voor IL-1 gemedieerde ontsteking.

In **Hoofdstuk 6** hebben we verder onderzoek gedaan naar het mechanisme van de werking van urinezuur priming in vitro. We hebben uitgebreid de betrokkenheid van oplosbaar urinezuur onderzocht in het modificeren van het gedrag van myeloïde cellen in reactie op MSU-kristallen in combinatie met TLR-liganden. We hebben aangetoond dat routes zijn gemoduleerd en hoe urinezuur een rol zou kunnen spelen in het ontstaan van jicht: AKT is upregulated, autofagie is downregulated, de productie van reactieve zuurstofsoorten wordt geremd, en epigenetische modificaties kunnen worden geïnduceerd door urinezuur . De bevinding dat de basale autofagieniveaus verlaagd zijn, herinnerden ons aan de verbeterde cytokineproductie, waargenomen in situaties waar autofagie werd geremd, zoals beschreven in hoofdstukken 2 en 3, waardoor autofagie mogelijk een nieuwe bron voor interventie in jicht zou kunnen zijn.

In **Hoofdstuk 7** richten we ons op de oorzaken van de aanhoudende urinezuureffecten. We behandelen de hypothese dat er een immunologisch geheugen aanwezig is na priming met urinezuur. Tevens zijn we nagegaan of urinezuurbehandeling epigenetische herprogrammering in monocyten kan veroorzaken. Gebaseerd op studies van onze groep,

die metabolische stimuli verbindt met veranderingen in histone kenmerken bij specifieke gen promotorregio's, hebben we twee eerste kandidaten getest (H3K4me3 en H3K27ac). Tot onze verrassing vonden we geen genen die significant dynamisch zijn voor deze twee kenmerken in reactie op urinezuur. Niettemin, in een cohort van Maori-afkomst, konden we DNA-methyleringsniveaus bij hyperuricemische versus normouricemische vrijwilligers bestuderen. Interessant genoeg bleken er verschillende differentieel gemethyleerde gebieden te kunnen worden geïdentificeerd, waaronder sommige functioneel gekoppeld aan IL-1 en IL-1Ra productie (SOCS3). Het is belangrijk dat potentiële kandidaten van deze route worden onderzocht en gevalideerd in verdere experimenten.

Vervolgens zijn in **Hoofdstuk 8 en 9** twee mogelijke therapeutische interventies bestudeerd. In **Hoofdstuk 8** lieten we zien dat het korte keten vetzuur butyraat dosisafhankelijk MSU / C16.0 geïnduceerde cytokinen verlaagt. Verder tonen we voor het eerst aan dat butyraat klasse I-histone-deacetylasen (HDAC's) remt, een groep enzymen met een rol in post-translationele modificaties van signaalproteïnen, waarvan aangetoond is dat ze anti-inflammatoire eigenschappen bezitten. In **Hoofdstuk 9** beschrijven we extreem krachtige anti-inflammatoire effecten van recombinant humaan alfa-1-anti-trypsine (AAT) -IgG1 Fc-fusie-eiwit (AAT-Fc) in een muizenmodel van jicht artritis. AAT is een van de meest voorkomende eiwitten in plasma en een cruciale serine-proteaseremmer. De belangrijkste bron van serine-proteasen zijn neutrofielen. Bij jicht artritis is er een significante neutrofiele instroom in het gewricht. Bij celdood, veroorzaakt door ontsteking, laten neutrofielen proteasen vrij in de extracellulaire ruimte. Dit heeft gevolgen voor IL-1 gemedieerde ontsteking, aangezien neutrofiele serine-proteasen ook pro-IL-1 kunnen klieven en aldus ontsteking bevorderen. We hebben aangetoond dat de toediening van exogene AAT-Fc zeer sterke eigenschappen heeft voor het onderdrukken van ontsteking en IL-1β-productie bij jicht artritis.

Ten slotte hebben we in **Hoofdstuk 10** de literatuur bekeken en de huidige kennis beschreven. Het aangeboren immuunsysteem bezit een epigenetisch gecodeerd geheugen na het doormaken van eerdere infecties („trained immunity"). Trained immunity is een proces dat gebaseerd is op de epigenetische herprogrammering van myeloïde of lymfoïde cellen die betrokken zijn bij de aangeboren immuniteit, hetgeen relevant is voor de gevoeligheid voor infectie, immunologische tolerantie of vaccinbiologie. Daarnaast hebben we hier oude bewijzen en nieuwe data geïntegreerd, ook steriele stimuli kunnen trained immunity induceren. De langetermijneffecten van steriele stressoren kunnen van groot belang zijn voor een breed scala aan ontstekingsziekten.

De intrigerende schakelaar in IL-1β en IL-1Ra productie in reactie op urinezuurbehandeling staat borg voor meer onderzoek in de toekomst. Het is nog steeds niet helemaal begrepen op welk punt IL-1Ra ontkoppeld wordt van IL-1β en of ze onafhankelijk worden geïnduceerd door verschillende cascades. Bovendien zijn genetische en genomische studies van cruciaal

belang om uit te vinden welke genetische gevoeligheidsfactoren geassocieerd zijn met jicht ontsteking, door het controleren op het hyperuricemie gerelateerde genetische risico. Het streven van de wetenschappelijke gemeenschap op het gebied van jichtonderzoek, naar genetische geassocieerde studies waarbij hyperuricemische controles als referentie worden gebruikt, zijn een veelbelovende nieuwe poging hiertoe.

Het gebied van hyperuricemie en jichtonderzoek krijgt steeds meer aandacht door de toenemende prevalentie en de bio-economische gevolgen, maar ook omdat nieuw bewijs de aandacht heeft gevestigd op nieuwe spelers in het ziekte- en therapierisico. Voortzetting van deze studies vraagt om innovatieve strategieën om verzamelingen gegevens te integreren en biologisch relevante conclusies te trekken. Als urinezuur in staat is stabiele ontstekingseffecten te veroorzaken, die niet beperkt zijn tot MSU-kristallisatie, zal het gebied van hyperuricemieonderzoek in de toekomst waarschijnlijk grote impact hebben op een breed scala aan geassocieerde ontstekingsziekten.

REZUMAT ÎN LIMBA ROMÂNĂ

Guta este o formă de artrită cunoscută din cele mai vechi timpuri. Mulți regi și conducători au fost afectați de această tulburare, multe personalități celebre din istorie au fost, de asemenea, suferinzi de gută. Această prevalență crescută în rândul persoanelor cu statut socio-economic ridicat a făcut ca guta să fie cunoscută de secole ca fiind "boala regilor". În zilele noastre cunoaștem mult mai multe despre patogeneza gutei, avem dovezi ale frecvenței sale ridicate, indiferent de statutul social, au fost elaborate ghiduri pentru o mai bună diagnosticare și clasificare, și se depun eforturi pentru a dezvolta terapii mai specifice și mai bine tolerate pentru gută. Această teză reprezintă un efort științific pentru a înțelege mai bine mecanismele moleculare care determină inflamația în gută și hiperuricemie.

În **Capitolul 1** al tezei, am prezentat o scurtă descriere a conceptului general de autoinflamație (inflamație îndreptată împotriva sinelui), cu accent pe hiperuricemie și gută. Acidul uric în stare solubilă are nivele mai crescute la primate în comparație cu alte vertebrate. Acest lucru se datorează unor mutații cu pierdere de funcție apărute în gena uricazei, ceea ce a făcut ca gena să fie complet nefuncțională. Uricaza este o enzimă care metabolizează acidul uric la alantoină solubilă. Deoarece această enzimă este absentă la oameni, nivelurile serice ale acidului uric sunt ridicate la un interval de 2-6 mg / dl. În contextul variației genetice și stilului de viață, concentrațiile serice ale acidului uric pot crește și mai mult, iar hiperuricemia apare atunci când nivelul de solubilitate este depășit. Hiperuricemia este cunoscută ca un factor metabolic asociat mai multor boli: cardiovasculare, renale, sindrom metabolic. În gută, hiperuricemia conduce la suprasaturația acidului uric și la depunerea cristalelor de urați în țesuturi. Localizarea sinovială a cristalelor de acid uric determină inflamația la nivelul articulațiilor, mediată de citokina interleukina-1 beta (IL-1β - proteină de semnalizare cu rol proinflamator). Cu toate acestea, sunt în studiu și alte procese potențial implicate în inflamația din gută, așa cum se subliniază în capitolul 1: microbiomul, activarea inflammasomului, procesele de dereglare a funcției fagozomilor, activarea citokinelor în mod independent de inflammasom, sau degradarea extracelulară a citokinelor inflamatorii mediată de neutrofile.

În **Capitolele 2 și 3** ale acestui manuscris, a fost studiată autofagia și implicarea sa în biologia producției de citokine. Autofagia este un mecanism de întreținere celulară care a fost conservat genetic pe parcursul evoluției. Aceasta constă în formarea de vezicule cu membrană dublă care înglobează încărcătura citoplasmatică și o livrează la lizozom pentru degradare. Acest proces are ca scop reciclarea nutrienților în momente de lipsă a acestora în mediul extern. Ulterior, alte studii au creeat o legătură între autofagie și imunitatea înnăscută, precum și cu activarea imunității adaptive. Prin studiile noastre, am descris că inhibarea autofagiei, în condiții experimentale sau prin factori genetici, poate fi un determinant de susceptibilitate pentru bolile autoinflamatorii.

În **Capitolul 4**, am studiat comportamentul celulelor izolate de la pacienți cu gută în comparație cu celulele izolate de la martori sănătoși, în urma stimulării cu cristale de urați. Acest studiu a adus o nouă dovadă că doar cristalele de urați nu pot induce producția IL-1β deoarece un al doilea semnal este necesar pentru gută. Mai mult, am arătat că celulele pacienților cu gută secretă mai multă IL-1β în comparație cu celulele voluntarilor sănătoși.
Am urmărit în continuare să explicăm această constatare în **Capitolele 5, 6 și 7** prin ipoteza că acidul uric solubil ar putea avea un rol de jucat în acest profil de producție crescută de citokine.
În experimentele descrise în **Capitolul 5**, am observat că celulele provenite de la pacienții cu gută diferă în ceea ce privește capacitatea lor de producție de citokine într-o manieră dependentă de acidul uric. Această corelație a fost intrigantă, prin urmare, am urmat un protocol *in vitro* care să imite hiperuricemia prin tratarea prealabilă a celulelor timp de 24 de ore cu mediu de cultură care conținea acid uric. Ulterior, celulele au fost spălate și stimulate cu liganzi TLR în prezența sau absența cristalelor MSU (restimulare timp de 24 ore). Am reușit să documentăm faptul că acidul uric crește nivelurile de expresie ale citokinelor proinflamatorii (cum ar fi IL-1 β). În același timp, acidul uric scade producția de citokine antiinflamatorii (cum ar fi antagonistul receptorului pentru IL-1, IL-1Ra).
Acest rezultat a fost surprinzător, deoarece reglarea în sus a IL-1 este de obicei însoțită de reglarea în sus și a IL-1Ra printr-o buclă de feedback. În cazurile mai extreme ale sindromului DIRA (deficiența antagonistului receptorului pentru IL-1), imaginea clinică include o inflamație severă a țesutului osos și a articulațiilor, ceea ce identifică vulnerabilitatea semnificativă a pacienților cu deficit de IL-1Ra la inflamația mediată de IL-1.
În **Capitolul 6**, am studiat extensiv implicarea acidului uric solubil în modificarea comportamentului celulelor mieloide ca răspuns la cristalele de urați în combinație cu liganzii TLR. Am arătat că, cel puțin parțial, căile de semnalizare proinflamatorii sunt modulate de către acidul uric și că aceasta poate juca un rol în istoria naturală a gutei: AKT este reglat în sus, autofagia este reglată în jos, producția speciilor reactive de oxigen este inhibată, iar modificările epigenetice pot fi induse de acidul uric. Constatarea că nivelurile autofagiei bazale sunt diminuate, ne-a amintit de creșterea producției de citokine observată în situațiile în care autofagia a fost inhibată (așa cum este descris în capitolele 2 și 3). Astfel, autofagia ar putea fi o posibilă sursă nouă de intervenție în gută.
În **Capitolul 7**, ne-am axat pe cauzele persistenței efectelor acidului uric. Am emis ipoteza că un fond de memorie imunologică ar putea fi prezent după expunerea la acid uric și am analizat dacă tratamentul cu acid uric poate induce reprogramarea epigenetică la nivelul monocitelor. Pe baza studiilor efectuate de grupul nostru care leagă stimulii metabolici de modificări histonice la nivelul regiunilor promotor ale genelor candidat, am testat două mărci inițiale (H3K4me3 și H3K27ac). Spre surprinderea noastră, nu am putut găsi nici o zonă dinamică în mod semnificativ pentru aceste două mărci în urma tratamentului cu acid uric. Cu toate acestea, într-o cohortă de origine Maori, am putut studia nivelurile de metilare

a ADN-ului în cadrul unei cohorte de pacienți cu hiperuricemie *versus* normouricemie. Interesant, au fost identificate câteva regiuni metilate diferențial, dintre care unele sunt conectate cu producția de IL-1 și IL-1Ra (ex. SOCS3). Potențialii candidați în această cale sunt importanți pentru a fi investigați și validați în experimente următoare.

Ulterior, două studii vizând posibile ținte terapeutice au fost efectuate și prezentate în **Capitolele 8 și 9**. În **Capitolul 8** am arătat că butiratul (acid gras cu catenă scurtă de atomi de carbon) reduce citokinele induse de MSU și C16.0 (combinație între cristalele de acid uric și palmitat, acid gras saturat cu 16 atomi de carbon). Mai mult, am arătat pentru prima dată că butiratul inhibă deacetilazele de clasă I (HDAC), un grup de enzime cu roluri în modificările post-translaționale ale proteinelor, care s-au dovedit a avea proprietăți antiinflamatorii. În **Capitolul 9**, descriem efectele antiinflamatorii extrem de puternice ale proteinei de fuziune alfa-1-anti-tripsina umană (AAT) – cuplată cu factorul cristalizabil al imunoglobulinei de tip IgG1 (AAT-Fc), obținută prin tehnologia ADN recombinant. AAT este una dintre cele mai abundente proteine din plasmă și un inhibitor important al serin-proteazelor. Sursa majoră a serin-proteazelor sunt neutrofilele. În artrita gutoasă, în articulație există un influx semnificativ de neutrofile. La moartea celulelor induse de inflamație, neutrofilele eliberează proteaze în spațiul extracelular. Acest lucru are consecințe asupra inflamației mediate de IL-1, deoarece serin-proteazele neutrofilelor pot, de asemenea, să cliveze pro-IL-1 și, astfel, să promoveze inflamația. Am demonstrat că administrarea de AAT-Fc exogenă are proprietăți foarte puternice pentru suprimarea inflamației și a producției de interleukină-1 în artrita gutoasă la șoricei de laborator.

În cele din urmă, în **Capitolul 10,** am analizat literatura de specialitate și am descris cunoștințele actuale despre răspunsul imun înnăscut și memoria codificată epigenetic a acestuia ("trained immunity", imunitate antrenată). Imunitatea antrenată este un proces bazat pe reprogramarea epigenetică a celulelor mieloide sau limfoide implicate în imunitatea înnăscută, prin care acestea dezvoltă o memorie a insultelor anterioare, process cu relevanță pentru sensibilitatea la infecție, toleranța imunologică sau vaccinare. În plus, în acest capitol am integrat dovezi vechi și date noi conform cărora stimulii sterili ar putea, de asemenea, să inducă memoria răspunsului imun înnăscut. Efectele pe termen lung ale agresorilor sterili ar putea avea o importanță deosebită pentru o gamă largă de boli inflamatorii.

Disocierea dintre producția IL-1β și IL-1Ra ca răspuns la expunerea la acid uric justifică nevoia pentru mai multă cercetare în viitor. Încă nu este complet înțeles în ce punct IL-1Ra este decuplată de calea de semnalizare a IL-1-β sau dacă acestea sunt induse independent de cascade diferite de semnalizare. Mai mult, studiile genetice și genomice sunt esențiale pentru a afla care sunt factorii genetici de susceptibilitate asociați inflamației din gută, prin controlul riscului genetic asociat hiperuricemiei. Studiile de asociere genetică, în care controalele hiperuricemice sunt utilizate ca referință, reprezintă un efort promițător în cadrul comunității științifice din acest domeniu de cercetare.

Cercetarea în gută și hiperuricemie capătă o atenție sporită din cauza impactului acestei boli în termeni de prevalență, în termeni de consecințe bioeconomice, dar și pentru că dovezile noi au atras atenția asupra unor noi factori implicați în riscul și terapia gutei. Continuarea acestor studii necesită strategii inovatoare pentru a integra seturi de date și pentru a trage concluzii relevante din punct de vedere biologic. Dacă se demonstrează că acidul uric exercită efecte inflamatorii stabile, fără restricție la forma cristalizată, atunci domeniul de cercetare al hiperuricemiei este probabil să aibă un impact în viitor asupra unei game largi de boli inflamatorii care asociază nivele elevate ale acidului uric.

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CURRICULUM VITAE

Tania Crișan was born on the 21st of July 1987 in Târgu Mureș, Romania. She attended the "Avram Iancu" high school in Cluj-Napoca with the main subjects Mathematics – Computer science/English and participated in several Biology Olympiads. In 2006 she started Medical School at the "Iuliu Hațieganu" University of Medicine and Pharmacy in Cluj-Napoca and she graduated in 2012. Since 2008 she also performed research in the Medical Genetics Department under the supervision of dr. Radu Popp and Prof. Dr. Ioan V. Pop. During her studies she performed clinical internships in Angers, France, and research internships in Nijmegen, the Netherlands, where she learnt the first steps in immunology research at the Experimental Internal Medicine Laboratory, Radboud UMC. In January 2013 she started working on her PhD project at the same department under the supervision of Prof. Dr. Leo Joosten and Prof. Dr. Mihai Netea. During her PhD she had the chance to present her work at numerous conferences and to get trained at various courses in the Netherlands and abroad. In 2016 she finished her doctoral contract and returned to Cluj, Romania, to pursue her Medical Genetics residency and her academic carrier. Now, she is continuing her research work in the field of gout and hyperuricemia under the guidance of Leo Joosten at the University in Cluj. She also teaches Medical Genetics at the "Iuliu Hațieganu" University of Medicine and Pharmacy. In 2015, Tania married Mircea Crișan.

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