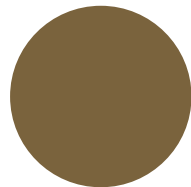




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Immunometabolic pathways in the regulation of trained immunity



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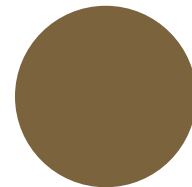
PHD
BASIC AND APPLIED BIOLOGY

Immunometabolic pathways in the regulation of trained immunity

Anaís V. Ferreira

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2022

PhD Thesis Co-Supervision between
the University of Porto and Radboud University Nijmegen



IMMUNOMETABOLIC PATHWAYS IN THE REGULATION OF TRAINED IMMUNITY

Anaísa V Ferreira

Colophon: The research presented in this thesis was performed at the Department of Internal Medicine of the Radboud University Medical Center, within the Radboud Center for Infectious Diseases and the Radboud Institute for Molecular Life Sciences.

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ANAÍSA VALIDO FERREIRA

IMMUNOMETABOLIC PATHWAYS IN THE REGULATION OF TRAINED IMMUNITY

Dissertation to obtain the degree of Doctor of Philosophy at Radboud University Nijmegen and the degree of Doctor in Basic and Applied Biology at University of Porto, Faculty Abel Salazar Institute of Biomedical Sciences

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“The difficult we do right away; the impossible takes a little longer”

- Mario Capecchi

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ABSTRACT

The innate immune system displays long-term functional alterations that promote heightened responses, a feature known as trained immunity, or innate immune memory. The mechanisms that induce and control trained immunity programs have been the focus of research for the last years, nevertheless, our understanding of the metabolic networks that govern trained immunity is incomplete. In the studies presented in this thesis, we explored the contribution of distinct metabolic pathways in trained immunity induced by the Bacille Calmette-Guérin (BCG) vaccine or β -glucan.

Reactive oxygen species (ROS) promote the clearance of pathogens and mediate signal transduction. Here, we show that the ROS scavenger molecule glutathione contributes to the increased responsiveness of trained macrophages. Trained macrophages have increased glutathione content and pharmacological modulation of glutathione levels reduced pro-inflammatory cytokine production. Also, in a cohort of BCG vaccinated individuals, basal plasma glutathione concentration was positively associated with pro-inflammatory cytokine production. In parallel, different studies have suggested the importance of lipids for the induction of trained immunity. We observed that monocyte enrichment in lipid mediators is crucial for the induction of trained immunity by BCG. Specifically, lipoxygenases derived mediators were sufficient to increase macrophage responsiveness. In accordance, monocytes derived from BCG vaccinated individuals are enriched in 12-lipoxygenase products.

Trained immunity is conferred by metabolic reprogramming, of not only peripheral innate immune cells, but also hematopoietic stem cells (HSCs). The spleen, as a site of extramedullary hematopoiesis and a reservoir for immature monocytes, could contribute to systemic trained immunity. We evaluated the peripheral immunity features of β -glucan trained splenectomized mice and observed that spleen removal prevented neutrophilia. Thus, pointing to the limited role of the spleen in the induction and maintenance of trained immunity *in vivo*.

Knowledge on the mechanisms that regulate trained immunity programs contribute for its potential therapeutic role. Here, we report that dimethyl itaconate (DMI), a derivative of the immunomodulatory metabolite itaconate, induces trained immunity. Mechanistically, DMI

alters monocyte redox state and glutathione concentrations, similarly to what we have also observed for other trained immunity inducers.

Overall, the present thesis provides relevant insights into the regulation of trained immunity. Systemically, we showed that the spleen is crucial for the increase of circulating neutrophils, while at a cellular level, we demonstrated the relevance of glutathione and lipid mediator pathways. We also identified DMI as a new inducer of trained immunity that could be contemplated in the future as a therapeutic tool to increase innate immune functions.

CHAPTER 1

GENERAL INTRODUCTION, AIM AND OUTLINE OF THE THESIS

GENERAL INTRODUCTION

1. The immune system remembers

We are constantly exposed to organisms that may cause disease depending on their pathogenicity and our defense mechanisms. The immune system is divided into two interacting parts differing on speed and specificity of the response. The innate immune system confers immediate defense with reduced specificity while adaptive immunity responses are highly specific and take several days to develop. Immune memory is attributed to the adaptive immune system, in which responses to a particular pathogen are developed more rapidly with each successive exposure [1]. This feature of the adaptive immune system is the foundation for the use of vaccines, which induce a pathogen-specific immune response and provide long-lasting protection against infection [2].

However, although invertebrates and plants lack an adaptive immune system, they exhibit greater resistance to subsequent infections. Plants can mount a defense against pathogens and acquire memory, whereby the first immune response confers long-term protection against a wide range of pathogens [3]. This systemic acquired resistance (SAR) illustrates the ability of an innate immune system to display memory characteristics. In fact, there is a growing body of evidence in human and murine models that allocates features of long-term memory to the innate immune system [4]. This phenomenon has been termed trained immunity, a *de facto* innate immune memory, whereby innate immune cells encounter an inflammatory challenge and later respond in a heightened manner to a secondary event.

The highly administered vaccine Bacille Calmette-Guérin (BCG) against *Mycobacterium tuberculosis*, like other live attenuated vaccines, was suggested by different epidemiological studies to confer protection against infections other than tuberculosis [5–7]. The protective role of BCG was best illustrated by several randomized controlled trials, in which neonates vaccinated with BCG exhibited lower all-cause mortality, primarily through the prevention of sepsis and respiratory tract infections [8]. Indeed, whole blood stimulation assays showed that BCG-vaccinated infants have increased production of cytokines upon heterologous challenge [9]. Distinct studies indicate the increase of effector functions of innate immune cells after BCG vaccination. BCG vaccinated individuals display neutrophils with an increased antimicrobial capacity [10] and peripheral blood mononuclear cells and natural killer (NK) cells that produce higher levels of innate pro-inflammatory cytokines upon

heterologous stimulation [11,12]. Human challenge studies show that BCG vaccination reduces yellow fever viremia [13] and malaria parasitemia [14]. Both models highlighted the correlation between protection and pro-inflammatory responses, with enhanced monocyte activation and IL-1 β production in the BCG vaccinated group. In addition to BCG, other agents also induce innate immune memory programs. *C. albicans*, or the fungal cell wall component β -glucan, enhance monocytic proinflammatory cytokines production after an unrelated secondary stimulation [15]. Sterile endogenous stimuli also induce trained immunity. Oxidized low-density lipoprotein (oxLDL) induces a long-lasting proinflammatory phenotype in monocytes, which has been associated with atherosclerosis and foam cell formation [16] and myeloid cells of mice fed a western-type diet exhibit a long-lasting trained immunity phenotype [17]. Recently, other inducers of trained immunity have been identified, namely extracellular labile heme was shown as an inducer of trained immunity, increasing mouse survival in a polymicrobial sepsis model [18].

Trained immunity stimuli induce epigenetic changes that modulate gene acceptability of the transcriptional machinery and thus confer long-term potential for increased gene transcription [19–22]. However, the long-lasting protection of trained immunity cannot be secured by monocytes, which survive in circulation approximately 1.5 to 7 days [23]. Induction of trained immunity skews hematopoietic stem cells (HSCs) towards myelopoiesis, generating trained differentiated cells [24–26]. A human BCG vaccination study demonstrated that HSCs of BCG vaccinated individuals are transcriptionally distinct from non-vaccinated individuals [27]. Indeed, HSCs of trained mice conserve epigenetic marks for at least 12 weeks and conferred increased expression of associated genes upon restimulation [28]. Epigenetic modifications can be associated with not only the maintenance of trained immunity features but also the transmission of those traits between generations. In plants, transgenerational SAR depends on epigenetic histone and DNA modifications [29]. Recently the transference of trained immunity across generations was shown in mice [30]. The progeny of male mice previously infected with *C. albicans* were protected against heterologous infections. This protection was encoded in sperm DNA of the infected parental mice, which showed differences in DNA methylation associated with immune gene loci. Alterations to the epigenetic landscape are interwoven with cellular metabolic changes. Metabolites act as signaling molecules, cofactors and substrates that modulate protein activity and ultimately contribute to cell function.

2. Metabolic regulation of trained immunity

[REVIEW PAPER]

THE ROLE OF CELL METABOLISM IN INNATE IMMUNE MEMORY

Anaísa V. Ferreira, Jorge Domínguez-Andrés, Mihai G. Netea

Journal Innate Immunity. 2022;14:42–50

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Abstract

Immunological memory is classically attributed to adaptive immune responses, but recent studies have shown that challenged innate immune cells can display long-term functional changes that increase nonspecific responsiveness to subsequent infections. This phenomenon, coined *trained immunity* or *innate immune memory*, is based on the epigenetic reprogramming and the rewiring of intracellular metabolic pathways. Here, we review the different metabolic pathways that are modulated in trained immunity. Glycolysis, oxidative phosphorylation, the TCA cycle, amino acid and lipid metabolism are interplaying pathways that are crucial for the establishment of innate immune memory. Unraveling this metabolic wiring allows for a better understanding of innate immune contribution to health and disease. These insights may open avenues for the development of future therapies that aim to harness or dampen the power of the innate immune response.

Introduction

Immunological memory has been classically attributed solely to the adaptive immune system. Once activated, B and T cells proliferate and confer specific long-lasting memory and thus award protection. Although invertebrates and plants lack an adaptive immune system, it is well documented that they exhibit greater resistance to subsequent infections. Specifically, systemic acquired resistance (SAR) has been described in plants whereby the first immune response confers long-term protection against a wide range of pathogens [31]. In addition, epidemiological studies point that vaccines, notably in live attenuated forms, such as measles, polio oral vaccine or *Bacillus Calmette-Guérin* (BCG), elicit non-specific effects that reduce mortality due to protection against non-targeted diseases [32,33]. Presently, there is a growing body of evidence in human and murine models that support the concept that the innate immune system also has features of long-term memory, whereby innate immune cells encounter an inflammatory challenge and later respond in a heightened manner to a secondary event. This phenomenon has been termed *innate immune memory* or *trained immunity* and does not rely on a particular stimulant thus conferring protection against heterologous pathogens (**Figure 1**) [34,35]. Mice inoculated with the fungus *Candida albicans* became resistant to a secondary lethal infection. This protection was still observed in *Rag1*-deficient mice, demonstrating that the phenomenon is independent of T and B cell function [15].

Innate immune memory has been described primarily in myeloid cells, such as monocytes, macrophages, and possibly dendritic and mast cells in response to a variety of challenges. Lymphoid cells such as natural killer (NK) cells or innate lymphoid cells (ILCs) have also been recently shown to display long-term memory characteristics [36–38]. Monocytes or macrophages exposed to *C. albicans*, or the fungal cell wall component β -glucan, show enhanced production of proinflammatory cytokines upon a unrelated secondary stimulation such as lipopolysaccharides (LPS) or *Mycobacterium tuberculosis* [15]. Sterile endogenous stimuli can also induce trained immunity. Oxidized low-density lipoprotein (oxLDL) induces a long-lasting proinflammatory phenotype in monocytes, which has been associated with atherosclerosis and foam cell formation [16]. However, a long-lasting protection cannot be secured by short-lived cells. Recent mouse studies have shown that BCG and β -glucan skew hematopoietic stem cells (HSCs) towards myelopoiesis, generating trained

monocytes and ultimately conferring partial protection against pulmonary tuberculosis and chemotherapy-induced myeloablation respectively [24,25]. Also, HSCs harvested from mice injected with LPS led to a decrease of mortality of the transplanted mice to *Pseudomonas aeruginosa*. This protection was still conferred by re-transplantation of the LPS exposed HSCs into a secondary host [28].

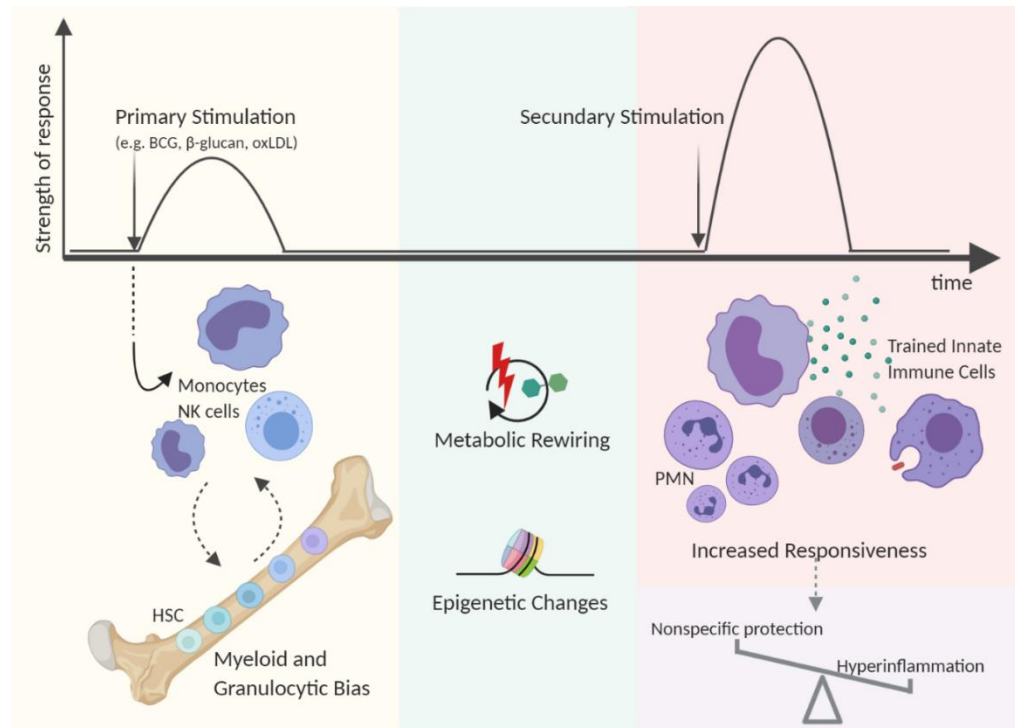


Figure 1 Innate immune memory. Pathogens or non-microbial ligands activate the innate immune system and induce metabolic rewiring and epigenetic changes. This modulation also leads to a myeloid and granulocytic bias of the hematopoietic stem cell compartment, promoting the production and release of trained innate immune cells, which exhibit increased responsiveness to posterior heterologous restimulation. Thus, trained immunity contributes to long-term non-specific protection conferred by vaccines, while promoting hyperinflammatory phenotypes. (PMN, polymorphonuclear leukocytes) [96].

This central impact of trained immunity at the level of bone marrow progenitors was also recently explored in a human BCG vaccination study, where it was demonstrated that the composition of HSCs did not change after vaccination. Nonetheless, BCG vaccination was shown to induce transcriptional changes in HSCs while concomitantly increasing responsiveness of innate immune cells to unrelated challenges [27]. In accordance, a cohort of BCG vaccinated newborn infants exhibited an increase in neutrophil counts in comparison to non-vaccinated children [27]. In addition to hematopoietic stem cells, other

stem cells may exhibit features of memory. Epithelial stem cells are progenitors of differentiated epithelial cells that form a physical barrier against external agents. Mouse models of skin sterile inflammation and fungal infection were used to show that epithelial stem cells persist after the skin returns to homeostasis and later contribute to enhanced epidermal wound healing of the previously inflamed skin [39].

Innate immune memory, in contrast to the adaptive immune memory, is not conferred neither by genomic rearrangements nor by clonal diversity and selection. Instead, the different inducers of trained immunity trigger epigenetic changes in the host cell. Trained cells harbor stable chromatin marks in gene regulatory regions that seemingly have no effect on steady-state gene expression. These chromatin adaptations modulate its accessibility to the transcriptional machinery and confer a long-term increased gene transcription in response to a secondary trigger. A second hallmark of trained cells is the rewiring of metabolic pathways. Cellular metabolism has not only been correlated to cell function, but also is an important regulator of epigenetic changes. Metabolites act as signaling molecules, cofactors and substrates that modulate the activity of chromatin modifying enzymes. This close interplay between metabolism and epigenetic reprogramming in trained immunity urges further exploration to understand the mechanisms underlying the different cellular metabolic pathways implicated in innate immune memory. The present review will discuss current findings that put metabolic pathways such as glycolysis, oxidative respiration and the TCA cycle at the center of the induction of trained immunity (**Figure 2**). Furthermore, we will discuss future therapeutic strategies that may arise from the modulation of innate immune pathways aimed at the dampening of the trained phenotype present in pro-inflammatory diseases or at inducing the increased responsiveness in immunosuppressed conditions.

The immunometabolic pathways involved in trained immunity

Glycolysis and oxidative respiration

Cells are extremely plastic and can adjust their metabolism according to their bioenergetic needs that change to adapt to microenvironmental changes. Glucose breakdown rapidly

produces adenosine triphosphate (ATP) and pyruvate. Pyruvate can then be used in the TCA cycle or be further oxidized to lactate. Glycolysis is often increased in activated T cells [40] and pro-inflammatory macrophages [41]. In accordance, trained macrophages have an increased glucose consumption and release high levels of the glycolysis end-product lactate [22]. This pathway is not an efficient ATP producer (only two ATP molecules are produced from one molecule of glucose) but can be quickly stimulated and amplified. The enhanced glycolytic rate is enabled by the increased expression of glycolytic enzymes which is sustained through epigenetic changes [42]. Glycolysis increase is common to different training stimuli such as β -glucan, BCG and oxLDL, and has been described not only in the monocyte-macrophage populations, but also in hematopoietic stem cells of β -glucan-exposed mice [25]. This hallmark of trained immunity is mediated by the AKT- mTOR- HIF1 α pathway [42,43]. *In vitro* inhibition of this pathway decreases the enhanced responsiveness of trained cells. Also, myeloid cell specific HIF1 α knockout mice exposed to β -glucan were more sensitive to an *S. aureus* infection than their trained wild-type controls [43,44]. In addition, humans treated with metformin, a known inhibitor of mTOR, presented decreased responsiveness to restimulation after BCG ex vivo training [42].

In contrast, oxidative respiration relies on the Krebs cycle and mitochondrial machinery to generate ATP, which occurs more efficiently (in total 36 molecules of ATP from one molecule of glucose) but at a slower rate than glycolysis. In OXPHOS, molecules of the electron donor nicotinamide adenine dinucleotide (NADH) are oxidized and donate electrons to the electron transport chain (ETC). There, electrons are sequentially relayed through the ETC complexes resulting in the reduction of oxygen and the pumping of protons across the inner membrane, generating water and a proton gradient. The shuttled protons then drive ATP synthesis generated by F₀F₁-ATP synthase. A pioneering study first described that β -glucan-induced memory in human monocytes leads to a metabolic rewiring with an increase of aerobic glycolysis and a dampened OXPHOS pathway [43]. However, further studies with lower doses of β -glucan or with BCG as a inducer of innate immune memory show that, concomitantly to an increase in glycolysis, these cells consume higher levels of oxygen [42,45,46]. This highly energetic state, where glycolytic and OXPHOS pathways are both potentiated, was further observed in human monocytes exposed to the sterile innate immune memory inducer oxLDL [47]. In addition, a recent study has described suggestive associations between common single nucleotide polymorphisms in genes

related to OXPHOS and variation in the degree of responsiveness of β -glucan trained monocytes [46]. These data collectively suggest that the metabolic shift towards aerobic glycolysis and the classically described Warburg effect is not common to the establishment of innate immune memory. Instead, enhanced OXPHOS appears as a regulator of the induction of the trained phenotype.

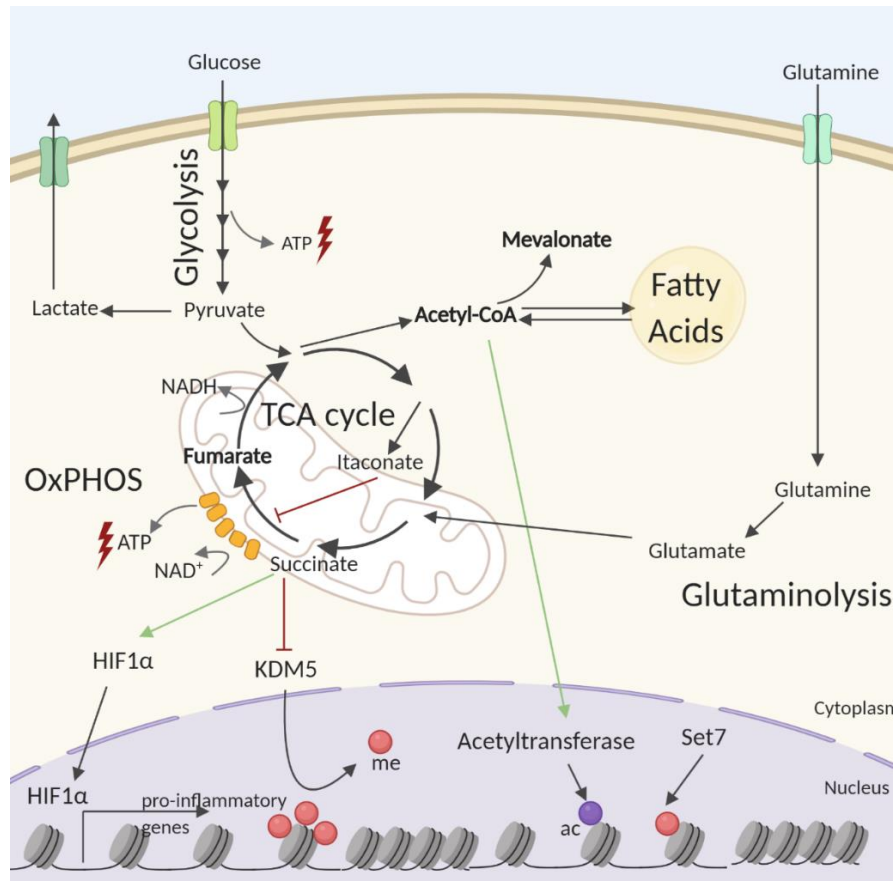


Figure 2 Trained immunity is supported by a metabolic rewiring. Trained cells exhibit an upregulation of glycolysis, tricarboxylic acid (TCA), oxidative phosphorylation (OXPHOS), glutaminolysis and fatty acid metabolism. OXPHOS and glycolysis provide adenosine triphosphate (ATP) while TCA cycle intermediates modulate the function of different proteins. Succinate inhibits the degradation of the transcription factor hypoxia-inducible factor 1- α , which in turn promotes the transcription of various pro-inflammatory and glycolytic genes. Succinate also inhibits the enzymatic activity of histone demethylase lysine-specific demethylase 5 (KDM5) while acetyl-CoA is an acetyl donor for histone acetyltransferases. This promotes epigenetic changes that facilitate the transcription of pro-inflammatory genes triggered by a posterior stimulation. (NAD, Nicotinamide adenine dinucleotide; me, methyl; ac, acetyl.) [96]

Interestingly, the enhanced electron-hungry OXPHOS pathway might contribute to the higher NAD(P)⁺/NAD(P)H ratio observed in trained cells [43][44], which may influence mitochondrial metabolism. Mitochondrial ATP production relies on NADH, which is

produced by the TCA cycle and fatty acid β -oxidation. Curiously, monocytes exposed to oxLDL exhibited increased mitochondrial ROS production, that was ablated upon mTOR inhibition [44]. These findings might suggest a relevant role for mitochondria function and metabolism in the induction of trained immunity that warrants further exploration.

TCA cycle and amino acid metabolism

In addition to providing for the energetic needs of the cell, metabolic rewiring also modulates the metabolic substrates available. Different metabolites have been attributed signaling functions by inhibiting the activity of enzymes or triggering protein post-translational modifications. Specifically, the TCA cycle integrates multiple catabolic and anabolic pathways. In short, glycolysis supplies pyruvate that is metabolized into acetyl-CoA, the fuels of the TCA cycle. Acetyl-CoA can also be derived from the β -oxidation of fatty acids, while the TCA cycle intermediate citrate can be used as a precursor for lipid biosynthesis. An alternative carbon source is provided by glutaminolysis, which replenishes the TCA cycle by the conversion of glutamate into α -ketoglutarate (α -KG). Also, the oxidation of succinate to fumarate by the enzyme succinate dehydrogenase (SDH), known as complex II of the ETC, shuttles an electron to power ATP production by the OXPHOS pathway [48,49].

The induction of innate immune memory hinges on the accumulation of TCA cycle intermediate metabolites, which may result from the interplay between glycolysis and glutaminolysis [22]. The transcription factor HIF1 α , activated in trained cells, blocks the conversion of pyruvate to acetyl-CoA by inhibiting pyruvate dehydrogenase [50]. Conversely, glutamine metabolism is increased and supplements the TCA cycle with α -KG derived from glutamate. Glutaminolysis inhibition impaired the prototypical increased responsiveness of β -glucan trained macrophages [22]. In addition to glutamine, trained cells show an enhanced consumption of the amino acid aspartate, however its role in the induction of trained immunity remains to be clarified. Methionine consumption is also increased in β -glucan exposed monocytes [22]. It is intriguing to consider that methionine is the precursor of S-adenosylmethionine (SAM), a methyl donor critical for DNA and histone methylation which plays a central role in chromatic dynamics and gene expression patterns [51].

Metabolic rewiring is crucial for long-term gene expression and epigenetic changes in innate immune memory. Trained monocytes amass increased levels of succinate and fumarate, which are competitive inhibitors of multiple α -KG dependent dioxygenases (α -KGDD) [52]. The α -KGDD family of enzymes include histone and DNA demethylases such as the Jumonji (Jmj)-KDM5 family which actively remove lysine trimethylation, such as H3K4me3 and H3K27me3 marks [53]. The cell-permeable monomethyl fumarate was shown to increase the responsiveness of human monocytes, enrich the activating mark H3K4me3 deposition at the promoter regions of the pro-inflammatory genes *TNFA* and *IL6*, while also inhibiting the activity of KDM5 [22]. Succinate and fumarate also have the relevant role of stabilizing HIF1 α by the inhibition of prolyl hydroxylases, which constitutively promote the degradation of HIF1 α . The increased levels of the transcription factor HIF1 α also promote IL-1 β transcription and upregulation of glycolysis [54]. HIF1 α induces the expression of histone demethylases [55], adding another possible indirect way through which the ratios of succinate, fumarate and α -KG could influence epigenome remodeling.

SDH, or complex II, oxidizes succinate into fumarate and shuttles an electron along the ETC. β -glucan trained monocytes show increased expression of different SDH subunits [56]. Also, genetic human studies have identified suggestive SNPs in SDH subunit coding genes associated with changes in responsiveness [46,56]. The regulation of transcription of SDH genes has been shown to be modulated by the lysine methyltransferase Set7. *In vitro* pharmacological inhibition of Set7 or the use of *SETD7* knock-out mice led to a decrease of the β -glucan induced upregulation of SDHB transcripts [46]. Interestingly, another modulator of SDH activity also regulates the trained immunity phenotype: itaconate is derived from the TCA cycle and produced by macrophages in pro-inflammatory conditions [57]. Considered as a break mechanism to the pro-inflammatory response, itaconate is attributed anti-inflammatory and antioxidant properties. It activates the master regulator of the antioxidant response, the transcription factor Nrf2 [58], and is an inhibitor of SDH by occupying the binding pocket of succinate [59]. Notably, the *in vitro* β -glucan trained phenotype is hindered by concomitant exposure to the membrane permeable derivative dimethyl itaconate, possibly due to SDH inhibition [56]. This break in the TCA cycle could in turn compromise the fumarate accumulation necessary for epigenetic remodeling. Itaconate has also been described to alkylate thiol groups and interact with the ROS scavenger glutathione [60], the glycolytic enzymes lactate dehydrogenase (LDH) [58],

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [61], and fructose-bisphosphate aldolase [62]. Interestingly, LDH and GAPDH are also possible targets for succinylation [54]. However, whether such mechanisms play a role in innate immune memory remains to be elucidated. The discovery of itaconate as a relevant immunomodulator in the context of mammalian responses is recent [57,63,64], and exciting new studies are exploring the intricate role of this metabolite in diverse inflammatory contexts [65–67].

Lipid metabolism

Cells obtain fatty acids from *de novo* lipogenesis and exogenous uptake. Glycolysis, glutaminolysis and the TCA cycle contribute to produce citrate, which in turn is converted to acetyl-CoA and gives rise to palmitate. Palmitate can be further elongated and desaturated to generate a multitude of lipid species, which can be subsequently stored as lipid droplets. These organelles contribute to the production of inflammatory mediators that regulate immune responses [68]. The stored fatty acids can be degraded to restore acetyl-CoA levels through β -oxidation. Interestingly, *in vitro* exogenous supplementation of acetyl-CoA induced a pro-inflammatory phenotype in human monocytes [69]. Acetyl-CoA serves as an acetyl donor that may favor histone acetylation and fuels the cholesterol pathway and fatty acid biosynthesis. Mevalonate, a metabolite of the cholesterol pathway, has been shown to regulate trained immunity [70]. Indeed, statins, which inhibit the rate-limiting enzyme that produces mevalonate, decreased the responsiveness induced by β -glucan, BCG and oxLDL [70]. Moreover, mutations in mevalonate kinase, which lead to mevalonate accumulation, trigger an autoinflammatory clinical phenotype, the hyper immunoglobulin D syndrome. These patients show a constitutive trained immunity profile which could contribute to their clinical picture of sterile inflammation attacks [70]. Central trained immunity also relies on the mevalonate pathway as shown by the accumulation of various species of cholesterol esters in HSCs. Inhibition of the enzyme upstream of mevalonate in a mouse model of β -glucan induced innate immune memory reduced HSC expansion bias towards myelopoiesis [25]. Interestingly, systemic increase of cholesterol and inflammatory mediators also triggered innate immune memory in an atherosclerosis mouse model. Monocytic cells isolated from mice exposed to high-fat diet for a limited period showed increased responsiveness to ex vivo stimulation, even after the animals returned to a chow

diet. Concomitantly the hematopoietic cell compartment proliferated and was biased towards granulocyte monocyte precursor cells, in a NLRP3 inflammasome dependent manner [17].

Fatty acid synthesis also appears to play a role in the induction of innate immune memory. Hematopoietic progenitors of β -glucan-trained mice were enriched for lipids with shorter and more saturated acyl chains with a decrease of alkyl-ether-linked phosphatidylcholines and phosphatidylethanolamines [25]. In accordance, HSC of mice trained with LPS and exposed to a second injection of LPS exhibited increased expression of genes related to fatty acid metabolism and oxidative phosphorylation [28]. The hormone aldosterone has also been recently described to induce trained immunity *in vitro* and epigenetically regulate the promoter regions of genes of the fatty acid synthesis. Moreover, the increased innate immune responsiveness induced by aldosterone was ablated by inhibition of this pathway [71].

The nuclear liver X receptor (LXR) is a transcription factor that coordinates gene expression linked to cholesterol and fatty acid metabolism. Pharmacological inhibition of LXR decreased the responsiveness of BCG-trained macrophages [69]. Moreover, LXR activation alone could replicate the macrophage trained phenotype, which was also dependent on the mevalonate pathway. Other transcription factors such as sterol regulatory element binding protein (SREBP) and peroxisome proliferator-activated receptors (PPARs) are regulators of lipid metabolism, however their involvement in the induction of innate immune memory warrants further exploration [72].

Perspectives for Therapies

Trained immunity is considered an evolutionary adaptation that allows for long-lasting broad-spectrum resistance to pathogens, increasing the hosts capacity to mount an immunological response. Conversely, this responsive immune state can become maladaptive and exacerbate chronic inflammatory conditions if inappropriately activated by endogenous ligands. Characterizing the specific changes that distinct innate immune cells subsets undergo in different diseases, dependent on stimuli and microenvironments, could aid in enhancing or curbing the effects of trained immunity.

Inhibition of innate immune memory could be beneficial for inflammatory conditions found in atherosclerosis [16], organ transplants [73], central nervous system inflammation [74], lupus erythematosus [75], and chronic diseases associated with the Western-type lifestyle [17,76]. On the other hand, the enhanced responsiveness of trained cells can be harnessed to improve an impaired host defense, such as in patients suffering from immunodeficiencies, immunoparalysis [19,77], immunosenescence [78] or cancer [25]. Tumor-associated macrophages are often immunosuppressive and allow tumor progression. In this line, BCG is often used as an immunotherapy in the treatment of bladder cancer [79]. Although immune system activation allows for elimination of cancer cells, chronic inflammation may also promote neoplastic transformation and tumor progression [80]. Consequently, it is of importance to explore therapies that induce trained immunity in a durable manner but that can also be inhibited once clinical benefit is achieved. Neurodegenerative diseases are associated with chronic inflammation and may be exacerbated by peripheral infections, and possibly by trained immunity. Innate immune memory triggered on a genetic mouse model of Alzheimer by LPS led to an increase of amyloid plaque formation and associated neuritic damage [81]. Also associated with age is immunosenescence, the gradual deterioration of the immune system associated with age [82]. Therapies that combine the induction of adaptive and innate immune memory could ameliorate the immunosenescence. In this regard, BCG vaccination decreased the incidence of new respiratory infections in elderly individuals in a recent randomized clinical trial [83]. At the same time, BCG vaccination administration prior to other vaccines was able to boost antibody responses [13,84]. In addition, BCG vaccination has been hypothesized as a preventive measure against the newly emerged SARS-CoV2 virus infection and multiple randomized controlled trial are currently testing this potential [85].

Innate immune memory could be inhibited or potentiated at different levels, namely by engaging specific receptors or modulating cellular metabolism and epigenome. Fungal ligands rely on the dectin-1 pathway [86], the metabolite mevalonate signals through the insulin growth factor 1 receptor [70], and N-acetyl muramic acid has been shown to induce a NOD2-dependent trained immunity phenotype [11]. Also, clinical trials have shown the therapeutic value of anti-IL-1 β for rheumatoid arthritis, gout, type 2 diabetes and cardiovascular disease [87,88], possibly through the inhibition of trained immunity mechanisms. Inhibition of glycolysis and mTOR as well as the mevalonate pathway could

contribute to a decrease in trained immunity, as is seen *in vitro* with the use of rapamycin [22][43] and fluvastatin [70], respectively. However, epigenetic rewiring ensures the longevity of innate immune memory leading to persistence disease. As such, modulating the epigenetic landscape with inhibition of histone or DNA methyltransferases is an attractive alternative [89]. As mentioned above, different inhibitors of innate immune memory pathways have been tested *in vitro*, but *in vivo* usage encompasses new challenges. Adequate targeting to myeloid and hematopoietic stem cells is crucial as it decreases off-target effects and enhances bioavailability while decreasing toxicity. The use of functionalized nanoparticles could aid in targeting and in the delivery of desired compounds. In a mouse model of heart transplant, the trained phenotype of the infiltrating cells was reduced by administration of a nanoparticle that targeted myeloid cells for inhibition of mTOR [73]. In addition to pharmacological modulators, the recently developed CRISPR/dCas9 could also be exploited in the future. dCas9 blocks transcription of a targeted gene by decreasing its accessibility or can also be modified to facilitate the recruitment of transcriptional activators and thus promote the transcription of a specific gene [90]. This technology is currently under development, but it might prove a viable therapeutic option in the future, for gene expression regulation in general, and specifically in the field of innate immune responses.

Conclusion

Innate immune memory relies on several metabolic pathways that provide the metabolites and energy necessary for epigenetic changes. Glycolysis, oxidative phosphorylation, glutaminolysis, the TCA cycle and cholesterol metabolism have been shown to be at the center of this enhanced responsiveness. Other mechanisms, such as mitochondrial respiration, redox and lipid metabolism may be possible players in the induction of trained immunity and warrant future exploration. However, further technological advancements in the field of metabolomics and epigenetics are necessary to promote the identification of further relevant pathways and the interplay between them. Ultimately, innate immune memory has been attributed a contributing role for the pathogenesis of autoinflammatory and autoimmune diseases. In contrast, an impaired innate immune system might be stimulated by inducers of trained immunity and regain responsiveness. This dichotomy

makes trained immunity an attractive therapeutic target. However, the development of future effective and safe therapies will first call for an increased effort towards thorough understanding of not only the molecular mechanisms regulating innate immunity, but also of the contribution of non-immune and microenvironmental players.

Conflict of Interest Statement. MGN is a scientific founder of TTxD.

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AIM AND SCOPE OF THE THESIS

Innate immune memory relies on interconnected metabolic and epigenetic changes which support enhanced effector functions. The investigation of these processes has been the focus of this growing field, leading to the identification of key metabolic pathways, and epigenetic players. Despite great efforts, many possible contributors to the induction, establishment and maintenance of trained immunity remain unknown. In this work we aimed to explore the underlying mechanisms that support trained immunity, with a strong focus on contributing metabolic pathways (**Figure 3**).

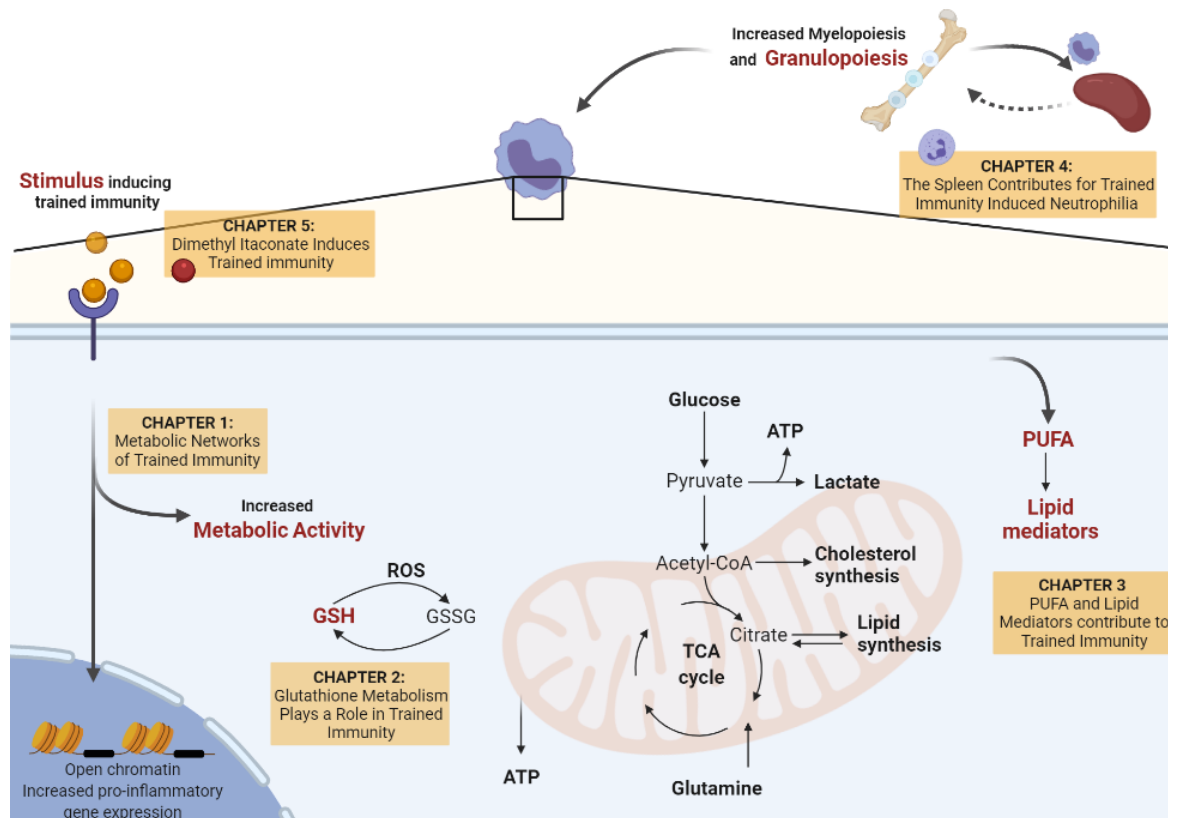


Figure 3. Schematic overview of this thesis's chapters [96]

Firstly, in **Chapter 1**, we discuss the current findings that put metabolic pathways at the center of the induction of trained immunity. There we provide an extensive review that highlights the reliance of trained immunity on the overall enhancement of metabolic activity. This high metabolic activity results in an increase in the production of ROS. Accordingly, **Chapter 2** explores the involvement of the ROS scavenging molecule glutathione in trained

immunity. We resorted to an established *in vitro* model of trained immunity of primary human monocytes [91] and took advantage of a cohort of healthy individuals [92], in which we analyzed the association between generic variants in genes coding for glutathione metabolism and responsiveness of *ex vivo* and *in vivo* trained peripheral blood mononuclear cells (PBMCs). We also found correlations between the baseline levels of circulating metabolites involved in glutathione metabolism and the cytokine production capacity of PBMCs isolated from BCG-vaccinated individuals.

As discussed in **Chapter 1**, other metabolic pathways remain to be fully explored. Namely, lipid metabolism is understudied in the context of trained immunity, but some insights point to the possible relevance of these pathways [70]. **Chapter 3** dissects the lipidome of β -glucan or BCG exposed monocytes. We showed, through pharmacological approaches and genetic associations, that long-chain fatty acid desaturation and the production of lipoxygenases derived lipid mediators are crucial for BCG enhanced monocyte responses. These findings were corroborated in monocytes derived from healthy individuals vaccinated with BCG.

Interestingly, due to the short half-life of monocytes in the circulation, the long-lasting protection secured by innate immune memory is assured centrally, with alterations in the hematopoietic immune cell progenitors which are skewed towards myelopoiesis and granulopoiesis [18, 19, 86]. Since the spleen is a reservoir of undifferentiated monocytes and is considered the prime organ for extramedullary hematopoiesis [87, 88], **Chapter 4** investigates the role of the spleen in the establishment of trained immunity. We resorted to the well-established β -glucan trained immunity mouse model, showing that spleen removal prevents neutrophilia. The levels of other immune cell types, and the cytokine production capacity were not modulated, pointing to a limited role of the spleen in systemic trained immunity.

This thesis focuses on trained immunity programs induced by β -glucan and BCG, in which metabolites such as fumarate and mevalonate are enriched. Importantly, the exogenous presence of these molecules is sufficient to induce immune memory [22][70]. Itaconate is a metabolite recently described as immunomodulatory and we briefly discussed the current literature on its immune effects in **Chapter 5.1**. These insights may contribute for future use of sterile molecules in the induction of trained immunity to therapeutically potentiate innate immune functions. Accordingly, **Chapter 5.2**, examines dimethyl itaconate (DMI), a

derivative of the endogenous metabolite itaconate, as a potential inducer of trained immunity. We show that DMI triggers trained immunity, increasing metabolic activity, affecting epigenetic changes, and enhancing cytokine production of monocytes, while also boosting mouse survival to a *Staphylococcus aureus* infection.

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CHAPTER 2

[RESEARCH PAPER]

GLUTATHIONE METABOLISM CONTRIBUTES TO THE INDUCTION OF TRAINED IMMUNITY

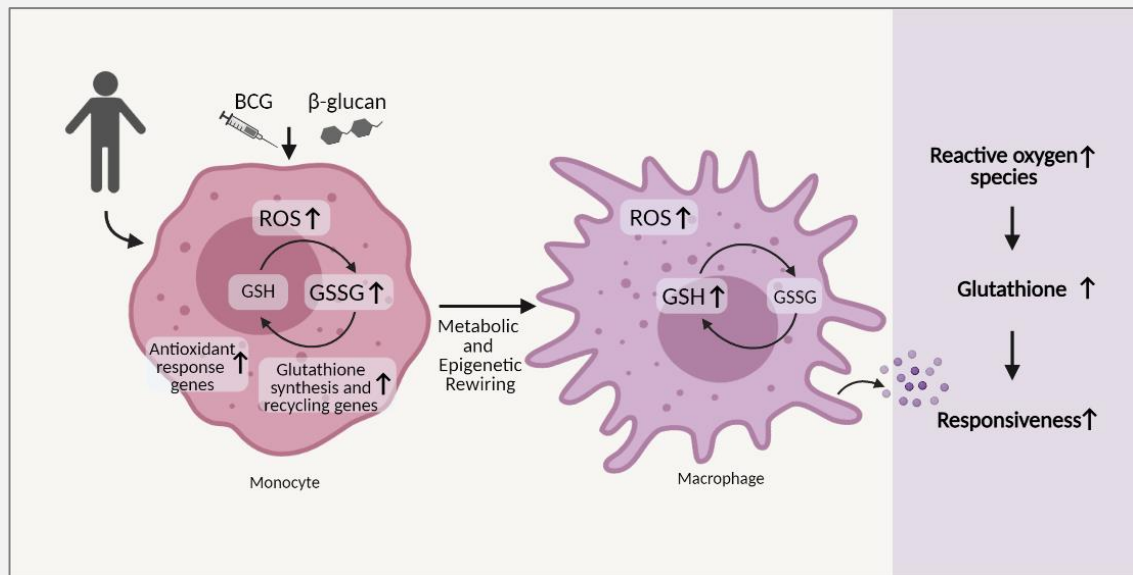
Anaisa V. Ferreira, Valerie A.C.M. Koeken, Vasiliki Matzaraki, Sarantos Kostidis, Juan Carlos Alarcon-Barrera, L. Charlotte J. de Bree, Simone J.C.F.M. Moorlag, Vera P. Mourits, Boris Novakovic, Martin Giera, Mihai G. Netea, Jorge Domínguez-Andrés

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Abstract

The innate immune system displays heterologous memory characteristics, which are characterized by stronger responses to a secondary challenge. This phenomenon termed trained immunity relies on epigenetic and metabolic rewiring of innate immune cells. As reactive oxygen species (ROS) production has been associated with the trained immunity phenotype, we hypothesized that the increased ROS levels and the main intracellular redox molecule glutathione play a role in the induction of trained immunity. Here we show that pharmacological inhibition of ROS in an *in vitro* model of trained immunity did not influence cell responsiveness; the modulation of glutathione levels reduced pro-inflammatory cytokine production in human monocytes. Single nucleotide polymorphisms (SNPs) in genes involved in glutathione metabolism were found to be associated with changes in pro-inflammatory cytokine production capacity upon trained immunity. Also, plasma glutathione concentrations were positively associated with *ex vivo* IL-1 β production, a biomarker of trained immunity, produced by monocytes of BCG-vaccinated individuals. In conclusion, glutathione metabolism is involved in the induction of trained immunity, and future studies are warranted to explore its functional consequences in human diseases.



Introduction

Until recently, adaptive immunity was thought to be the only component of host defense characterized by the capacity to retain memory, tailoring T and B cell responses to a specific antigen. However, a growing body of recent research has also attributed characteristics of heterologous memory to the innate immune system, a process termed trained immunity [1]. Monocytes exposed for a short period of time to *Candida albicans*, the fungal cell wall component β -glucan, or even sterile stimuli as oxidized low-density lipoprotein (oxLDL), show enhanced production of pro-inflammatory cytokines upon an unrelated secondary stimulation long after the initial stimulus has been removed [2, 3]. In addition, epidemiological studies have shown that the *Bacillus Calmette-Guérin* (BCG) vaccination against tuberculosis boosts the antimicrobial function of innate immune cells, subsequently inducing non-specific protection to unrelated infections and decreasing all-cause mortality [4].

Trained immunity is rooted in epigenetic changes that modulate the accessibility of genes of the pro-inflammatory response for the transcriptional machinery of the cell. Epigenetic rewiring in trained innate immune cells is accompanied by metabolic changes that promote pathways such as glycolysis, oxidative phosphorylation, and the cholesterol biosynthesis [5, 6]. Metabolites derived from these pathways are signaling molecules and cofactors that can in turn modulate the activity of chromatin modifying enzymes. Glutathione (GSH) is the main antioxidant preserving the intracellular redox balance and has been suggested to contribute to epigenetic changes [7]. In addition, stimuli that induce trained immunity, such as BCG and oxLDL increase the production of reactive oxygen species (ROS) of macrophages upon secondary stimulation [8-10]. We hypothesized that the increased metabolic activity of trained macrophages would also increase ROS levels and modulate GSH, which in turn would influence the strength of trained immunity responses

Materials and Methods

Isolation of human peripheral blood mononuclear cells (PBMC) and monocytes. Buffy coats from healthy donors were obtained after written informed consent (Sanquin Blood Bank, Nijmegen, The Netherlands). Ethical approval was obtained from the CMO Arnhem-Nijmegen (NL32 357.091.10). Isolation was performed by differential density centrifugation over Ficoll-Paque (GE Healthcare, Chalfont St Giles, UK). Subsequently, isolation of monocytes was performed with a hyper-osmotic Percoll (Sigma-Aldrich, St Louis, MO, USA) density gradient centrifugation and washed once with pyrogen-free cold phosphate buffered saline (PBS). Cells were resuspended and later cultured in RPMI 1640 Dutch modified medium (Invitrogen, Waltham, MA, USA) supplemented with 5 µg/mL gentamicin (Centraform, Etten-Leur, the Netherlands), 2 mM Glutamax (Gibco, Waltham, MA, USA), and 1 mM pyruvate (Gibco). To ensure maximal purity, Percoll-isolated monocytes were left to adhere to polystyrene flat bottom plates (Corning, Sigma-Aldrich, New York, NY, USA) for 1 h at 37 °C 5% CO₂ and then washed once with warm PBS.

***In vitro* trained immunity model.** Adherent monocytes were cultured as previously described [11]. Briefly, 10⁵ monocytes were seeded on flat-bottom 96-well plates (Greiner, Kremsmünster, Austria) and incubated with 1 µg/mL β-glucan or 5 µg/mL BCG vaccine (InterVax, Toronto, ON, Canada) in RPMI with 10% human pooled serum. β-1,3-(D)-glucan (β-glucan) from *Candida albicans* was kindly provided by Professor David Williams (College of Medicine, Johnson City, TN, USA). When indicated, cells were preincubated with 0.5µM diphenyleneiodonium (DPI), 50 µM α-tocopherol (AT) (Sigma-Aldrich), 0.5 µM ascorbic acid (AA), 1 mM N-acetyl cysteine (NAC) (Sigma-Aldrich), or 100 µM DL-buthionine sulfoximine (BSO) (Sigma-Aldrich) for 1 h prior to β-glucan addition. After 24 h, cells were washed once with warm PBS and left to differentiate in RPMI supplemented with 10% pooled human serum for 5 days. Medium was refreshed at day 3 of culture. At day 6, macrophages were restimulated with 10 ng/mL *Escherichia coli* lipopolysaccharide (LPS; serotype 055:B5, Sigma-Aldrich) for an additional 24 h.

Reactive oxygen species (ROS) quantification. Monocytes/macrophages were cultured for 2 h, 24 h, or 6 days, washed, detached with cold PBS, and incubated with 5 µM H₂DCFDA (Life Technologies, Waltham, MA, USA) in PBS for 30 min at 37 °C. Cells were analyzed by flow cytometry (CytoFlex, Beckman Coulter, Brea, CA, USA). Data analysis

was performed using the Kaluza 2.1 software. Cell aggregates were gated out based on the forward scatter (FSC)-height versus FSC-area plot and the monocyte/macrophage population was gated according to FSC and side scatter (SSC). Data are presented as mean $\pm$ SEM and analyzed with Friedman's test followed by Dunn's multiple comparisons test. A p value below 0.05 was considered statistically significant, as indicated by asterisks ($*p < 0.05$).

RNA sequencing data of human monocytes stimulated *in vitro*. Gene expression analysis of monocytes exposed *in vitro* to β -glucan was performed using previously published RNA-seq data [12]. Briefly, PBMCs were isolated by centrifugation in Ficoll-Paque (GE Healthcare). Monocytes were purified using negative selection with beads for CD3⁺ (T cells), CD19⁺ (B cells) and CD56⁺ (NK cells) positive cells (Miltenyi Biotec). Cells were cultured following the described *in vitro* trained immunity model and exposed to 5 μ g/mL β -glucan. Monocytes were collected at 4h and 24h after exposure. Genes related to antioxidant response and glutathione metabolism were extracted from the list of significantly dynamic genes ($p < 0.05$, FC > 2.5 , RPKM > 1) in the *in vitro* monocyte-to-macrophage trained immunity model. Data is presented as the $\log_{10}$ of the ratio between monocytes treated with β -glucan and non-stimulated monocytes.

Glutathione quantification. Quantitative analysis of intracellular reduced (GSH) and oxidized (GSSG) glutathione was performed using nuclear magnetic resonance (NMR) spectroscopy as previously described [13]. Briefly, growth medium was removed and cultured cells were washed with warm PBS (37 °C) and quenched with liquid nitrogen to arrest metabolism. The cells were subsequently scraped off the plates and extracted using a cold (−80 °C) solution of methanol/chloroform/water, 8.1:0.9:1 (vol/vol/vol). The extracts were kept frozen on dry ice for at least 30 min and were subsequently centrifuged for 20 min at 18,000 $\times g$ at −4 °C. The protein pellet was kept for protein quantification while the supernatants containing the intracellular metabolites were dried under a gentle stream of nitrogen and NMR samples were prepared by dissolving the dried material with 0.22 mL of 0.15 M phosphate buffer (pH 7.4) in deuterated water containing 0.05 mM trimethylsilyl propionic-d₄-sodium salt as internal standard for NMR referencing and quantification. An 1D ¹H NMR spectrum was collected for each sample on a 14.1 T (600 MHz for ¹H) Bruker Avance II NMR, using the *noesygppr1d* pulse sequence (Topspin v3.0, Bruker Biospin Ltd, Karlsruhe, Germany). All spectra were processed and imported in Chenomx NMR suite 8.4

(Chenomx NMR suite, v8.0, Edmonton, AB, Canada) for the quantification of glutathione. All concentrations were normalized to the total protein mass of each sample. The latter was quantified by dissolving the protein pellet in buffer (1:1, 1% SDS: 8M Urea (0.25M Tris pH:8)) with sonication. The protein concentration was then measured using Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to its manual.

***In vitro* and *in vivo* trained immunity models for genetic and metabolic analysis.** In the *in vitro* model, adherent monocytes were incubated either with culture medium only as a negative control, 2 µg/mL of β-1,3-(D)-glucan or 5 µg/mL BCG (300BCG cohort: BCG-Bulgaria strain, Intervax, Canada), for 24 hours at 37°C in the presence of 10% pooled human serum. On day 6, cells were re-stimulated for 24 hours with either 200 µL RPMI or LPS 10 ng/mL (serotype 055: B5; Sigma-Aldrich). In the *in vivo* model, healthy adults were vaccinated with a standard dose of 0.1 mL BCG intradermally in the left upper arm, and blood was collected before and 14 and 90 days after vaccination. In each visit, 5×10⁵ PBMCs were stimulated with heat-killed *Staphylococcus aureus* (10⁶ CFU/mL) or left unstimulated at 37°C with 5% CO₂. Secreted cytokines were measured in both models after 24h of stimulation, and the fold increase cytokine responsiveness induced by the training stimuli was used as a measure for trained immunity [14].

Genetic analysis. Genetic analysis was performed using a cohort of healthy individuals of Western European descent from the Human Functional Genomics Project (<http://www.humanfunctionalgenomics.org>). The 300BCG cohort consists out of 325 adults from the Netherlands (44% males and 56% females, age range 18 – 71 year). The study was approved by the local ethics committee CMO region Arnhem- Nijmegen, NL58553.091.16. DNA samples of individuals (n = 325) were genotyped using the commercially available SNP chip, Infinium Global Screening Array MD v1.0 from Illumina. Opticall 0.7.0 with default settings was used for genotype calling [15]. Samples with a call rate ≤ 0.99 were excluded, as were variants with a Hardy-Weinberg equilibrium (HWE) ≤ 0.0001, and minor allele frequency (MAF) ≤ 0.001. Strands of variants were aligned and identified against the 1000 Genome reference panel using Genotype Harmonizer [16]. We then imputed the samples on the Michigan imputation server using the human reference consortium (HRC r1.1 2016) as a reference panel, and we filtered out genetic variants with an R² < 0.3 for imputation quality, and MAF < 5% [17], resulting in approximately 4 million single-nucleotide polymorphisms (SNPs) for follow-up QTL mapping. Both genotype and

cytokine data on *in vitro* trained immunity responses was obtained for a total of 267 individuals from the 300BCG cohort. Three samples were excluded due to medication use (of which one was identified as a genetic outlier), and one sample due to onset of type 1 diabetes during the study. First, the fold change of cytokine production between trained and non-trained cells was taken as a measurement for the magnitude of the trained immunity response. Following quality check for cytokine distribution and after excluding genetic outliers, we mapped the log-transformed fold changes of cytokine production to genotype data using a linear regression model with age and sex as covariates to correct the distributions of fold change of cytokine production. We used a cutoff of $p < 9,99 \times 10^{-3}$ to identify suggestive quantitative trait loci (QTL) associations affecting trained immunity responses. Using the *in vivo* trained immunity model, both genotype and cytokine data was obtained for a total of 296 individuals. In addition to the outliers described for the *in vitro* QTL mapping, 18 evening vaccinated individuals were excluded, resulting in 278 samples before QTL mapping. First, raw cytokine levels were log-transformed and ratios of cytokine production between the visits were taken as the fold change of cytokine production. The fold change of cytokine production was mapped to genotype data using a linear regression model with age and sex as covariates. We used a cutoff of $p < 9,99 \times 10^{-3}$ to identify suggestive QTL associations affecting trained immunity responses. R-package Matrix-eQTL was used for cytokine QTL mapping [18].

Metabolomics analysis. Metabolite levels of individuals from the 300BCG cohort were measured before BCG vaccination. The metabolic features were measured and annotated by the General Metabolics (Zurich, Switzerland) using flow injection time-of-flight mass (flow-injection TOF-M) spectrometry[19]. Non-targeted metabolites were annotated according to human metabolites database (HMDB). All metabolomic measurements were performed in duplicates, and the average value was calculated for each sample per metabolite. Spearman correlation analysis was performed on metabolites and *ex vivo* cytokine fold changes in relation to before vaccination responses. To test the relation between glutathione levels and the SNPs of interest identified in the genetic analysis, a linear regression analysis with age and sex included as covariates was performed for each individual SNP.

Cytokine quantification and analysis. Cytokine production was determined using commercial ELISA kits for IL-1 β , IL-6 and TNF α (R&D Systems, Minneapolis, MN, USA)

following the instructions of the manufacturer. Data are presented as mean $\pm$ SEM and analyzed with two-way repeated measures ANOVA followed by Sidak's multiple comparisons test. A p value below 0.05 was considered statistically significant, as indicated by asterisks ($*p < 0.05$).

Results

β -glucan- and BCG-induced trained immunity trigger a sustained reactive oxygen species production

Increased ROS release has been previously described as a feature of the trained immunity phenotype [20]. Monocytes exposed to β -glucan or BCG for 2h and 24h exhibit increased ROS. Interestingly, this increase was sustained and present after 5 days of resting in culture media (**Figure. 1A**). Enhanced ROS levels are accompanied by transcriptional changes of different antioxidant genes in monocytes exposed to β -glucan for 24h (**Figure. 1B**). Mitochondrial superoxide dismutase *SOD2* and different thiol containing molecules are upregulated, namely thioredoxin-dependent peroxide reductases 1-6 (*PRDX 1-6*) and sulfiredoxin 1 (*SRXN1*). The expression of thioredoxin (*TXN*) and thioredoxin reductase (*TXNRD*), relevant thiol containing ROS scavengers, are also augmented.

However, neither the inhibition of the major ROS producer NADPH oxidase with diphenyleneiodonium (DPI), nor the addition of the antioxidant molecules α -tocopherol (AT) and ascorbic acid (AA) prior to incubation with β -glucan, modulated the increased macrophage responsiveness. In macrophages exposed to β -glucan, TNF α and IL-6 secretion 24h after LPS restimulation were not modified by this pharmacological approach (**Figure. 1C,D**). However, exposure to DPI alone increased IL-6 production upon LPS stimulation. (**Figure. 1C**). Pretreatment with the general ROS scavenger N-acetyl cysteine (NAC) caused a decrease in TNF α and IL-6 production when compared to cells exposed to β -glucan alone (**Figure. 1D**). Taken together, we show that increased ROS production plays a minor role in the increased cytokine production of β -glucan-induced trained immunity. In addition, trained immunity sustained increased ROS levels could be allied to the modulation of the cellular antioxidant glutathione, since NAC is not only a ROS scavenger but can also act as a precursor for glutathione synthesis.

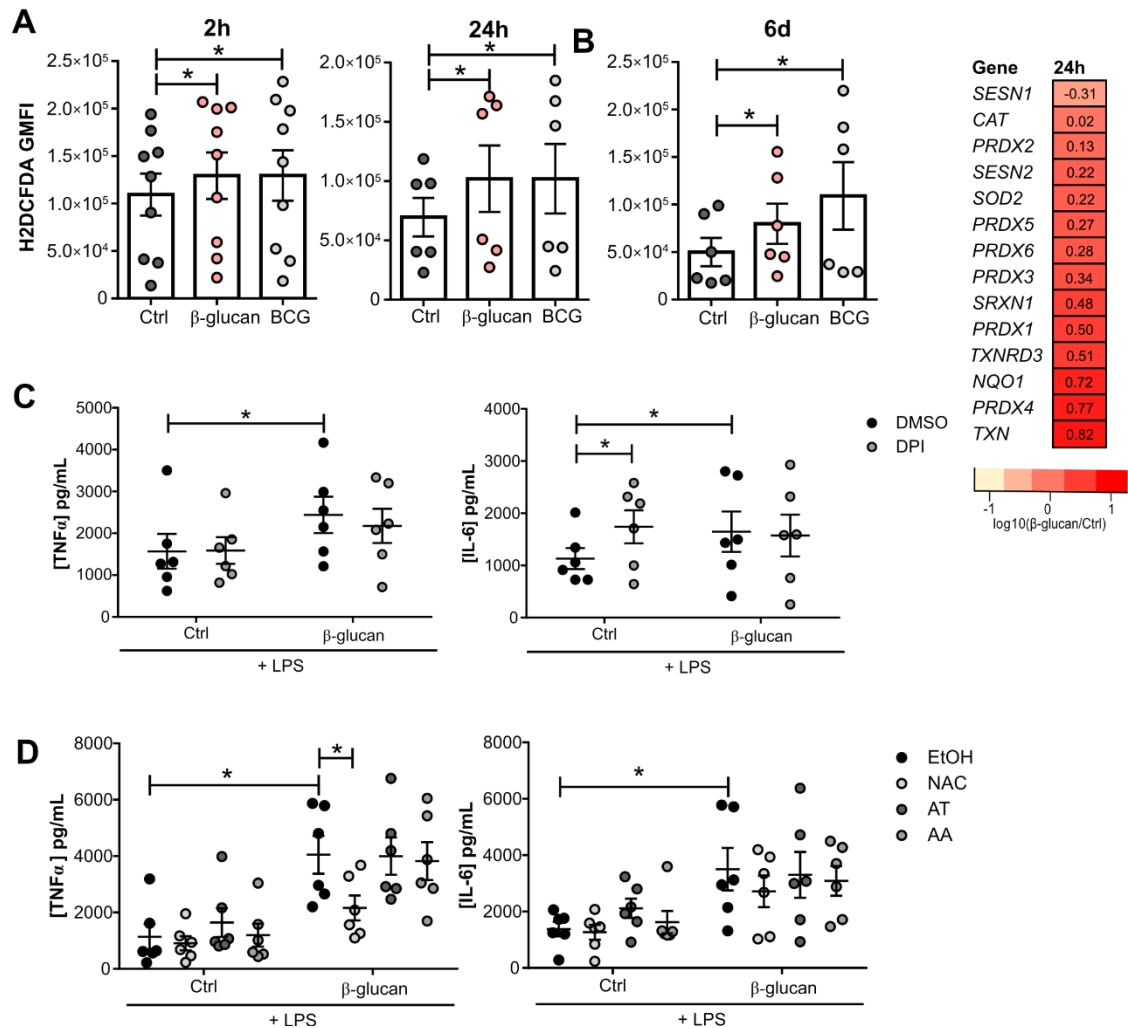


Figure 1 Increased ROS levels of trained monocytes do not contribute to their enhanced pro-inflammatory cytokine production. (A) ROS levels at 2h, 24h and 6 days after exposure of monocytes to β-glucan and BCG (n=6/9 donors, pooled from 2/3 independent experiments, Friedman test Dunn's multiple comparisons test). (B) Expression levels of genes involved in the antioxidant defense in monocytes exposed to β-glucan for 24h in comparison to unstimulated cells. Expression presented as log10(ratio). TNFα and IL-6 produced by β-glucan-trained macrophages after a 1h pretreatment with (C) 0.5μM DPI (n=6 donors, pooled from 2 independent experiments), (D) 1mM NAC, 50μM AT or 0.5μM AA (n=6 donors, pooled from 2 independent experiments, two-way ANOVA, Sidak's multiple comparisons test). (mean±SEM, *p < 0.05).

Glutathione metabolism influences trained immunity responses

Monocytes exposed to β-glucan for 24h show higher levels of the oxidized form of glutathione (GSSG), which is in accordance with the higher ROS levels found at this time point (**Figure. 2A**). After the resting period, the reduced form of glutathione (GSH) tends to be increased in trained macrophages, with no alteration of the concentrations of the

oxidized form. The genes that encode for the subunits of the rate limiting enzyme glutamate cysteine ligase (*GCLC* and *GCLM*) show increased expression at 4h after β -glucan stimulation than in control monocytes.

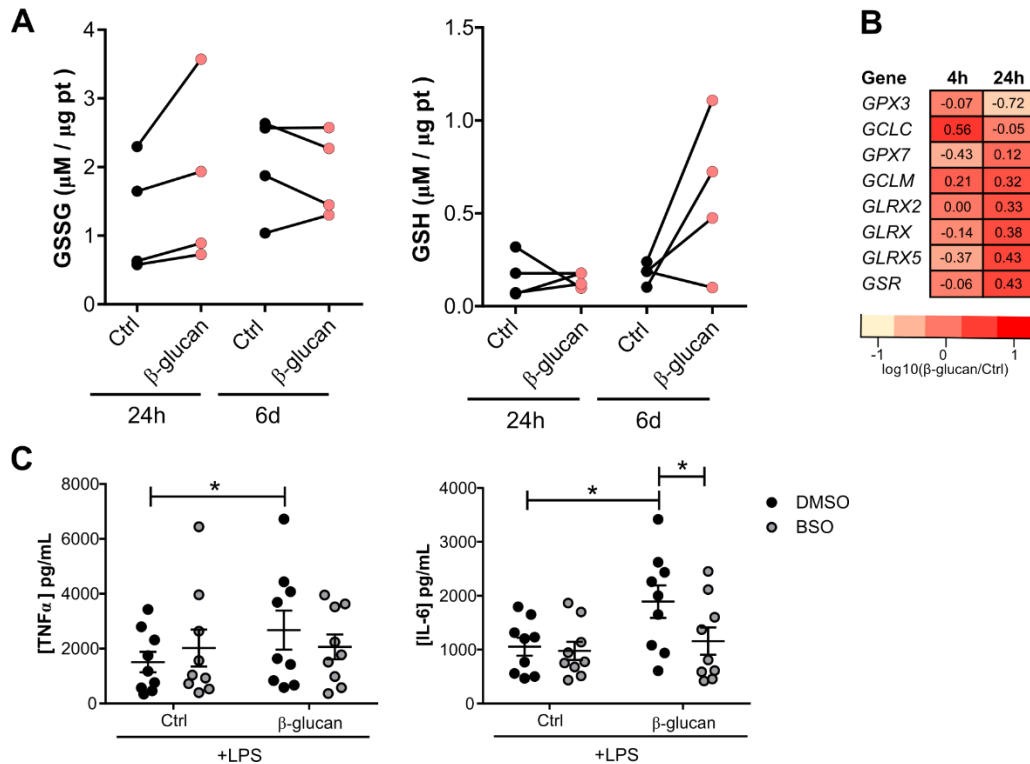


Figure 2 Glutathione levels are modulated upon β -glucan exposure. (A) Reduced and oxidized glutathione intracellular levels in monocytes after 24h exposure with $1\mu\text{g/mL}$ β -glucan and 5 days after the resting period ($n=4$ donors, pooled from 2 independent experiments). (B) Expression of genes involved in glutathione metabolism in monocytes exposed to β -glucan for 4h and 24h. Expression presented as $\log_{10}(\text{ratio})$. (C) TNF α and IL-6 produced by β -glucan-trained macrophages after a 1h pretreatment with $100\mu\text{M}$ BSO ($n=9$ donors, pooled from 3 independent experiments, two-way ANOVA, Sidak's multiple comparisons test).

Glutathione reductase (*GSR*), glutathione peroxidase 7 (*GPX7*) and enzymes of the glutaredoxin family (*GLRX*, 2, 5) are also upregulated at the 24h timepoint (**Figure. 2B**), suggesting not only an increase in synthesis, but also a higher rate of glutathione recycling. Pharmacologically inhibiting the activity of GCL with buthionine sulfoximine (BSO) prior to β -glucan exposure leads to a decrease of IL-6 production after LPS restimulation (**Figure. 2C**). Thus, exogenous modulation of glutathione levels, either supplementation by the addition of the precursor NAC, or its decrease by blocking the enzyme GCL with BSO, dampened the β -glucan increased responsiveness to secondary stimulation.

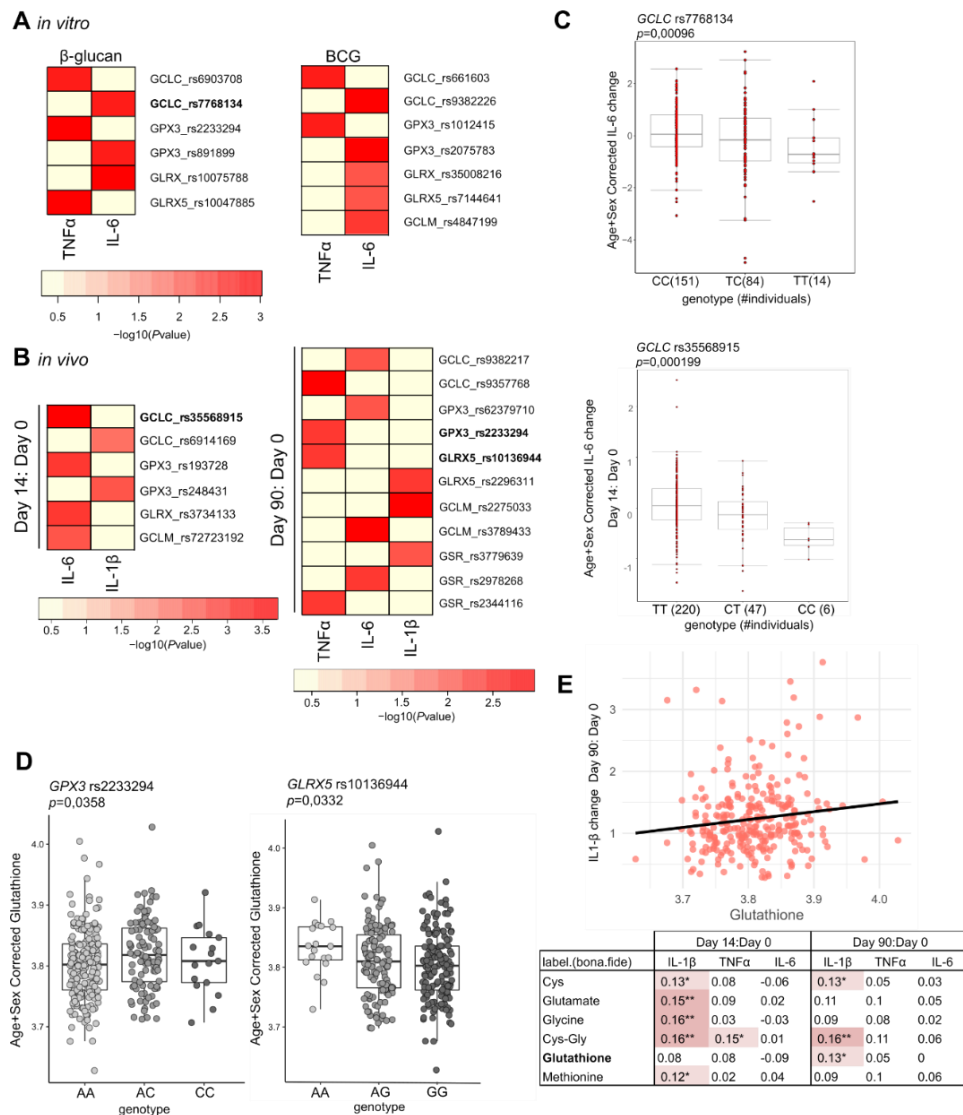


Figure 3 Glutathione is associated with trained immunity features. Heatmap of the p -values of association ($p < 9.99 \times 10^{-3}$) between SNPs mapped to genes involved in glutathione metabolism and the magnitude of cytokine production capacity by monocytes trained *in vitro* with (A) β-glucan and BCG ($n = 251$ healthy volunteers for IL-6 and $n = 238$ healthy volunteers for TNFα) and (B) *in vivo* BCG training responses ($n = 278$ healthy volunteers). (C) Boxplot of the lowest p -value SNP per analysis (rs7768134, rs35568915) (D) Boxplots showing glutathione plasma levels (corrected for age and sex) stratified by genotype for rs2233294 (GPX3) and rs10136944 (GLRX5, $n = 302$). The p -value for each SNP is derived from linear regression model with the SNP of interest as independent variable and glutathione (corrected for age and sex) as the dependent variable. (E) Spearman correlations between circulating metabolites at baseline involved in glutathione metabolism versus fold changes of PBMC-derived *S. aureus*-induced IL-6, IL-1β, and TNFα responses measured at 14 or 90 days after BCG vaccination compared to baseline ($n = 297$ healthy volunteers). The table presents the Spearman's rho for each correlation, and the correlations in red highlight significant positive correlations (* $P < 0.05$, ** $P < 0.01$). The correlation between glutathione concentration and the fold change of IL-1β production at 90 days compared to day 0 is given as an example in a scatter plot.

To further investigate whether genetic variation in genes involved in glutathione metabolism influence trained immunity, QTL mapping was performed using a cohort of 325 healthy individuals. We tested if common SNPs with a minor allele frequency (MAF) > 0.05 in genes relevant for glutathione metabolism were associated to changes in pro-inflammatory cytokine production capacity of monocytes upon β -glucan and BCG *in vitro* training (**Figure. 3A, C**). Similarly, we also assessed the impact of these genetic variants on the *in vivo* trained immunity induced by BCG vaccination of 278 healthy individuals (**Figure. 3B, C**). Several SNPs within a window of 250kb of genes involved in glutathione metabolism were suggestively associated (p value < 9.99×10^{-3}) with up-regulation of IL-1 β , TNF α and IL-6 secretion after training (**Figure. 3A,B**). In the same cohort, we measured the plasma metabolites involved in glutathione metabolism prior to BCG vaccination. SNPs identified in the *in vitro* and *in vivo* analyses were significantly associated to circulating glutathione concentrations (p value < 0.05) (**Figure 3D**). In addition, we found a positive association between plasma glutathione concentration and *ex vivo* IL-1 β production 90 days after BCG vaccination upon *in vitro* exposure to the heterologous stimulus *Staphylococcus aureus* (**Figure. 3E**). The up-regulation of IL-1 β production by BCG vaccination is also positively associated with the circulating concentration of other metabolites involved in glutathione metabolism, such as methionine, cysteine, glutamate, and glycine (**Figure. 3E**). Overall, genes involved in glutathione metabolism containing QTLs for innate heterologous responses, to LPS (**Figure 3A**) or to *S. aureus* (**Figure 3B**) and correlation between glutathione serum levels and *ex vivo* IL-1 β production, both the point to a role of this pathway in the establishment of trained immunity.

Discussion

Macrophages are plastic cells that adapt their phenotype to different environmental stimuli. Trained macrophages rely on a high energy metabolism, with increased glycolysis, TCA cycle and oxidative phosphorylation, to mount a response with enhanced pro-inflammatory cytokine production [6, 21]. In addition to cytokine production, oxidative burst with rapid ROS production is increased in restimulated BCG-trained macrophages [8]. Splenic neutrophils isolated from BCG-treated mice also show increased ROS production [22]. In line with the augmented metabolic rate and the increased capacity to mount an oxidative

burst, we observed that monocytes and macrophages exposed to β -glucan or BCG exhibit an increase in basal ROS production. A similar rise in ROS release was previously reported in oxLDL-trained monocytes [9]. ROS have effector functions in the clearance of pathogens but can also mediate signal transduction [23]. Pharmacological inhibition of the ROS producing enzyme NOX or treatment with antioxidant molecules α -tocopherol and ascorbic acid did not ablate the β -glucan increased cytokine production. However, the ROS scavenger NAC inhibited the production of TNF α enhanced by β -glucan, indicating a possible role of ROS in trained immunity.

Interestingly, NAC is not only an antioxidant molecule, but it also acts as a precursor of glutathione, the main endogenous antioxidant molecule. There are two major cellular redox systems, pyridine nucleotide, such as NAD⁺/NADH, and thiol systems, that include glutathione and thioredoxins. The NAD⁺/NADH ratio has been shown to be increased in β -glucan exposed monocytes in a sustained manner after the differentiation period in trained macrophages [21], similarly to what we report for ROS levels. Here we show that GSH concentrations are also modulated in trained immunity. Most strikingly, the concentration of the GSH reduced form is only enhanced in differentiated trained macrophages, while the levels of the oxidized GSSG are stable. This suggests that trained macrophages have increased GSH stores, which in contrast to NAD⁺/NADH do not partner with the sustained ROS production. The contribution of glutathione metabolism to trained immunity is further strengthened by genetic and metabolic analysis of a cohort of healthy individuals (300BCG). Common genetic polymorphisms within GSH related genes found to influence the magnitude of cytokine production upon *in vitro* and *in vivo* trained immunity ($p < 9.99 \times 10^{-3}$). In addition, two of these QTL loci, *GPX3* at chr5 and *GLRX5* at chr14, were found to be associated with the GSH plasma concentrations ($p < 0.05$).

GSH is an antioxidant molecule, but it is also known to regulate gene expression through different epigenetic mechanisms. GSH participates in post-translational modifications, as observed in the s-glutathionylation of the nucleosome protein histone H3, and is powered by the methionine pathway that ultimately leads to s-adenosyl methionine (SAM) production. In turn, SAM is the major methyl donor used for DNA and histone methylation [7]. The TCA cycle metabolite fumarate accumulates in trained macrophages, and its derivative monomethyl fumarate induces a trained immunity phenotype [5]. In astrocytes, acute exposure of the derivative dimethyl fumarate depletes intracellular GSH, but

increases total GSH at later timepoints [24]. Trained immunity is anchored by epigenic modifications that confer long-term memory [12, 25]. The herein observed increased GSH content of trained macrophages might facilitate the epigenetic rewiring necessary for the establishment of innate immune memory.

Here, we show that pharmacological modulation of glutathione and the redox status of the cell decreases macrophage's heterologous response, as seen by the effect of BSO or NAC on β -glucan enhanced cytokine production. In addition, in a cohort of healthy individuals, GSH metabolism is associated with trained immunity traits. The trained immunity mechanisms that are shaped by GSH metabolism remain to be further explored, but the potential involvement of both metabolic and epigenic landscapes makes this metabolic hub an exciting avenue to investigate in future studies. These insights contribute to the unraveling of the metabolic and epigenetic wiring of trained immunity, allowing for a better understanding of the innate immune impact on health and disease. Ultimately, a better understanding will help identify novel targets of therapy in diseases characterized by dysregulation of innate immune processes.

Conflicts of Interest Statement. MGN is a scientific founder of TTxD. The remaining authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Author Contributions. Conceptualization: AVF and JD-A; methodology: AVF, VACMK, VM, JCA-B, MG; formal analysis: AVF, VACMK, VM, JCA-B; investigation AVF, JAB, SK,

LCJB, SJCFMM, VPM, BN; data curation: VACMK, VM, BN; writing—original draft preparation, AVF; writing—review and editing: all co-authors; supervision: MG, MGN, JD-A; funding acquisition: MG, MGN. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement. The 300BCG study was approved by the local ethics committee CMO region Arnhem- Nijmegen, NL58553.091.16.

Informed Consent Statement. Informed consent was obtained from all subjects involved in the study, according to the approval of the ethical committee Arnhem-Nijmegen (nr. 2010-104).

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Data Availability Statement. RNA-sequencing data presented in this study are available in the NCBI Gene Expression Omnibus under the accession number GSE85243 at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE85243> (accessed on 6 April 2021)

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CHAPTER 3

[RESEARCH PAPER]

FATTY ACID DESATURATION AND LIPOXYGENASE PATHWAYS SUPPORT TRAINED IMMUNITY

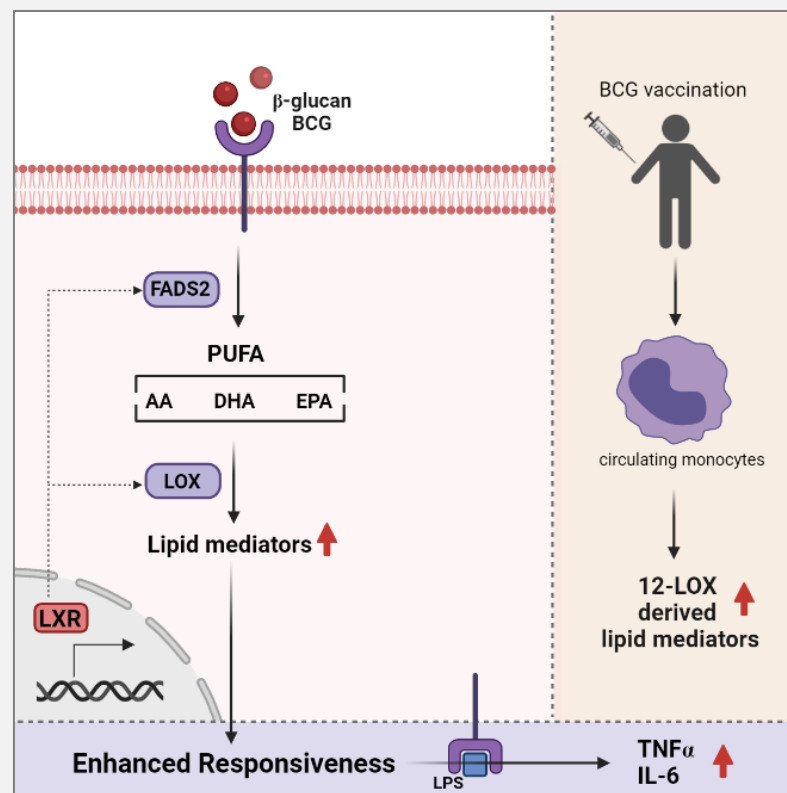
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Submitted

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Abstract

Infections and vaccinations can induce long-term enhanced responses of innate immune cells to heterologous stimuli, establishing a *de facto* innate immunological memory termed *trained immunity*. Monocytes exposed to the *Bacillus Calmette-Guérin* (BCG) vaccine, and to a lesser extent the fungal cell wall component β -glucan, have a trained immunity phenotype, characterized by an increased biosynthesis of different lipid mediators (LMs) derived from long-chain polyunsaturated fatty acids (PUFAs). Pharmacological and genetic approaches showed that long-chain PUFA synthesis and lipoxygenase (LOX)-derived LMs are crucial for the trained immunity responses of human monocytes. Furthermore, monocytes of healthy individuals vaccinated with BCG are enriched in 12-LOX products. The elucidation of the lipid metabolic pathways that promote innate immune memory contributes to our understanding of trained immunity and may help identify therapeutic tools and targets for the modulation of innate immune responses.



Introduction

Adaptive immunity is characterized by the capacity to retain memory to a specific antigen, however, a growing body of evidence attributes memory features to the innate immune system as well – a property termed *trained immunity*. Epidemiological studies have shown that the tuberculosis vaccine BCG increases innate immune cell function and confers non-specific protection to heterologous infections, thus decreasing all-cause mortality [1]. Innate immune cells, such as monocytes, after brief exposure to BCG or the fungal cell wall component β -glucan, exhibit a long-term augmented production of pro-inflammatory cytokines in response to a secondary non-related stimulation [2,3].

Trained immunity is supported by epigenetic modifications that alter gene transcription upon restimulation and metabolic rewiring of diverse metabolic pathways. The metabolic changes of trained immune cells provide energy, signaling molecules, and cellular components that impact innate immune effector functions. Various studies have determined the contribution of glycolysis, the TCA cycle, and oxidative respiration to the establishment of the trained immunity phenotype [4–6]. However, the contribution of other metabolic pathways in the context of trained immunity remains to be fully explored, as is the case of pathways related to lipid metabolism. Several clues from literature point towards the possible relevance of lipid metabolism pathways in trained immunity. For example, cholesterol biosynthesis is increased in hematopoietic stem and progenitor cells of β -glucan trained mice [7], and the metabolite mevalonate, involved in the cholesterol synthesis pathway, amplifies trained immunity in human monocytes [8].

Lipids and lipid mediators (LMs) could act as signaling molecules to establish trained immunity. On this regard, LMs are bioactive signaling molecules produced through the oxidation of polyunsaturated fatty acids (PUFA) by the actions of cyclooxygenases (COX), lipoxygenases (LOX), and epoxygenase (CP450) enzymes. The selective engagement of these pathways allows for a tailored LM response to different inflammatory states [9]. For example, eicosanoids, prostaglandins (PGs) and leukotrienes (LTs) are classically related to a proinflammatory activity, enhancing innate and adaptive immune functions. However, it has become accepted that resolution of acute inflammation is also an active process, regulated, in part, by specialized anti-inflammatory and pro-resolving mediators such as

resolvins, maresin, protectins, and lipoxins [10]. Moreover, recent studies have also started highlighting the tissue regenerative and anti-inflammatory properties of prostaglandin E₂ (PGE₂) [11].

In this study, we hypothesized that LMs influence the induction of trained immunity. We combined lipidomic, genomic, and pharmacological approaches to investigate whether induction of trained immunity in monocytes by BCG or fungal β -glucans relies on the modulation of LM biosynthesis and composition. We show that human monocytes exposed *in vitro* to BCG or β -glucan were enriched for different LM. We identified genetic variations in fatty acid desaturases (*FADS*) and *LOX* genes that are associated with trained immunity phenotype, as mirrored by enhanced cytokine production capacity upon BCG vaccination. Furthermore, we observed that 12-LOX-derived LMs content of circulating monocytes was increased in individuals vaccinated with BCG.

Materials and Methods

Isolation of human peripheral blood mononuclear cells (PBMC) and monocytes. Buffy coats from healthy donors were obtained after written informed consent (Sanquin Blood Bank, Nijmegen, the Netherlands). Isolation was performed by differential density centrifugation using Ficoll-Paque (GE Healthcare). Subsequently, isolation of monocytes was performed with a hyper-osmotic Percoll (Sigma) density gradient centrifugation and washed once with pyrogen-free cold phosphate-buffered saline (PBS). Cells were resuspended and later cultured in RPMI 1640 Dutch modified medium (Invitrogen) supplemented with 5 µg/mL gentamicin (Centraform), 2 mM Glutamax (Gibco), and 1 mM pyruvate (Gibco). To ensure maximal purity, Percoll-isolated monocytes were left to adhere to polystyrene flat bottom plates (Corning) for 1 h at 37 °C 5%, CO₂ and then washed once with warm PBS. For lipid analysis, monocytes were negatively selected from PBMCs (Pan Monocyte Isolation Kit, Miltenyi Biotec) and cultured as mentioned above in supplemented RPMI 1640 Medium with no phenol red (Invitrogen).

***In vitro* trained immunity model.** Monocytes were cultured as previously described [12]. Briefly, monocytes were incubated with 1 µg/mL β-glucan, 5 µg/mL BCG vaccine (BCG-Bulgaria, InterVax, Canada) or 1 ng/mL *Escherichia coli* lipopolysaccharide [13] (LPS; serotype 055:B5, Sigma-Aldrich) in RPMI with 10% human pooled serum. β-1,3-(D)-glucan (β-glucan) from *Candida albicans* was kindly provided by Professor David Williams (College of Medicine, Johnson City, USA). When indicated, cells were exposed to 5 or 15 µM SC-26196 (Abcam), 5 µM GSK2033 (Tocris) 10 or 100 µM Zileuton (Sigma), 2 or 20 µM ML355 (Cayman Chemical), 10 µM PD146176 (Tocris Bioscience), 0.5 mM Aspirin (Sigma), 1 or 50 µM Celecoxib (Sigma), 5 or 10 µM Itraconazole (Janssen) or respective vehicle control in combination with BCG for 24 h. In supplementation experiments, monocytes were incubated with 10 and 100 nM 12-HEPE (Santa Cruz Biotech), 0.1, 1 and 5 µM 17-HDHA (Cayman Chemical), 3, 30 and 300 nM 5-HETE (Cayman Chemical), 0.03, 0.3 and 3 µM 15- HETE (Cayman Chemical), 0.1, 1 and 5 µM 7-HDHA (Cayman Chemical), 1 and 10 nM Leukotriene B₄ (Cayman Chemical) or 10 and 100 pM PGE₂ (Sigma). After 24 h, cells were washed once with warm PBS and left to differentiate in RPMI supplemented with 10% pooled human serum for 5 days. Medium was refreshed at day 3 of culture. At day 6, the monocytes derived macrophages were restimulated with 10 ng/mL LPS for an additional 24

h. All conditions used were tested for cytotoxicity by measuring lactate dehydrogenase in the conditioned media after 24 h incubation according to the manufacturer's instructions (CytoTox96, Promega) (**Figure S1**).

Lipid analysis. Targeted LMs and PUFA analysis were carried out using Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Briefly, 1×10^6 monocytes per sample were washed with PBS and lipid extraction and purification was carried out by solid-phase extraction (SPE) [14][15]. LC-MS/MS analysis was carried out on a QTrap 6500 mass spectrometer (Sciex), connected to a Shimadzu Nexera LC30-system including an autosampler and a column oven (Shimadzu). The employed column was a Kinetex C18 50×2.1 mm, $1.7 \mu\text{m}$, protected with a C8 precolumn (Phenomenex). LC-MS/MS peaks were integrated under manual supervision and area corrected to corresponding internal standard, an in house established mix of deuterated PUFAs (PGE₂ d4, Leukotriene B4 d4, 15-HETE d8, DHAd5), using MultiQuant 2.1 (Sciex). All values are a relative quantitation after IS correction; they are displayed as area ratio and were normalized to protein content measured using Pierce TM BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Cytokine quantification. Cytokine production was determined using commercial ELISA kits for IL-1 β , IL-6 and TNF α (R&D Systems), following the instructions of the manufacturer.

RNA isolation and RT-PCR. Total RNA was extracted by TRIzol (Life Technologies), according to the manufacturer's instructions, and quantified using a Nanodrop 2000 UV-visible spectrophotometer. cDNA was synthesized from 450 ng-1 μg RNA by iScript Reverse Transcriptase (Invitrogen). Relative expression was determined using SYBR Green (Invitrogen) on an Applied Biosciences StepOne PLUS qPCR machine. Fold changes in expression were calculated by the $\Delta\Delta\text{Ct}$ method using *HPRT1* as an endogenous control. The following primers were used:XX *HPRT1*: 5'-GGATTTGAAATTCAGACAAGTTT-3', 5'-GCGATGTCAATAGGACTCCAG-3'; *FADS1*: 5'-CCAACTGCTTCGCAAAGAC-3', 5'-GCTGGTGGTTGTACGGCATA-3'; *FADS2*: 5'-GGATGGCTGCAACATGATTATGG-3', 5'-GCAGAGGCACCCTTTAAGTGG-3'; *SCD*: 5'-TTCCTACCTGCAAGTTCTACACC-3', 5'-CCGAGCTTTGTAAGAGCGGT-3'; *COX1*: 5'-CGCCAGTGAATCCCTGTTGTT-3', 5'-AAGGTGGCATTGACAACTCC-3'; *COX2*: 5'-CTGGCGCTCAGCCATACAG-3', 5'-CGCACTTATACTGGTCAAATCCC-3'.

Animal experimental model. Animal experiments were conducted in the Unit of Animals for Medical and Scientific purposes of the University General Hospital “Attikon” (Athens, Greece) according to EU Directive 2010/63/EU for animal experiments and to the Greek law 2015/2001, which incorporates the Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes of the Council of Europe (code of the facility EL25BIO014, approval number 1853/2015). All experiments were licensed from the Greek veterinary directorate under the protocol number 338087/27-05-2020. 30 C57BL/6 mice that were 8-10 weeks old were used. Animals were housed in groups of 5 animals per enriched type-II cage on a 12 h light-dark diurnal cycle with room temperature between 21 and 23 °C. Water and food were provided *ad libitum*. DSS was added to the drinking water in a concentration of 3 %. Mice were split into groups through a randomization table. Daily intraperitoneal injections of 1 mg of SC-26196 (Abcam) dissolved in dimethyl sulfoxide (DMSO, Sigma) in a volume of 50 µL or vehicle were performed starting on day 1. Analgesia was achieved with paracetamol suppositories. Animals were monitored for weight changes, stool consistency and rectal bleeding (DAI score) for 6 days, after which they were sacrificed using an intramuscular injection of 100 mg/kg ketamine and 10 mg/kg xylazine, followed by cervical dislocation. The experiment was performed in duplicate.

***In vitro* and *in vivo* trained immunity models for genetic analysis.** In the *in vitro* model, adherent monocytes were incubated with 2 µg/mL of β-1,3-(D)-glucan or 5 µg/mL BCG (BCG-Bulgaria, Intervax, Canada), for 24 h at 37 °C in the presence of 10 % pooled human serum. On day 6, cells were re-stimulated for 24 h with LPS 10 ng/mL (Sigma). In the *in vivo* model, healthy adults were vaccinated with a standard dose of 0.1 mL BCG (BCG-Bulgaria, Intervax, Canada) intradermally, and blood was collected before, 14 and 90 days after vaccination. At each visit, 5×10⁵ PBMCs were stimulated with heat-killed *Staphylococcus aureus* (10⁶ CFU/mL) [16].

Genetic analysis. Genetic analysis was performed using a cohort of healthy individuals of Western European descent from the Human Functional Genomics Project. The 300BCG cohort consists of 325 adults from the Netherlands (44% males and 56% females, age range 18 – 71 years). The study approved by the local ethics committee CMO region Arnhem-Nijmegen, NL58553.091.16. DNA samples of individuals (n = 325) were genotyped using the commercially available SNP chip, Infinium Global Screening Array MD v1.0 from Illumina. Genotype information on approximately 4 million single-nucleotide polymorphisms

was obtained upon imputation (MAF > 5% and $R^2 > 0.3$ for imputation quality). Both genotype and cytokine data on *in vitro* trained immunity responses was obtained for a total of 267 individuals from the 300BCG cohort. Three samples were excluded due to medication use (of which one was identified as a genetic outlier), and one sample due to onset of type 1 diabetes during the study. The fold change of cytokine production between trained and non-trained cells was used as a parameter of the trained immunity response, followed by a quality check for cytokine distribution and exclusion of genetic outliers. In the *in vivo* trained immunity model, genotype and cytokine data were obtained for a total of 296 individuals. In addition to the outliers described for the *in vitro* QTL mapping, 18 individuals who were vaccinated in the evening hours were excluded (due to the known circadian effect on BCG vaccination [17]), resulting in 278 samples. Raw cytokine concentrations were log-transformed and ratios of cytokine production between the visits were taken as the fold change of cytokine production. For both *in vitro* and *in vivo* trained immunity models, the fold change of cytokine production was mapped to genotype data using a linear regression model with age and sex as covariates. We used a cutoff of $p < 9.99 \times 10^{-3}$ to identify suggestive QTL associations affecting trained immunity responses. R-package Matrix-eQTL was used for cytokine QTL mapping [18].

Human BCG vaccination for *in vivo* lipid analysis. Healthy adults were vaccinated with a standard dose of 0.1 mL BCG (BCG-Denmark, SSI) intradermally, and blood was collected before and 28 days after vaccination. The study was approved by the local ethics committee CMO region Arnhem- Nijmegen, NL74082.091.20. At each visit, monocytes were negatively selected from PBMCs (Pan Monocyte Isolation Kit, Miltenyi Biotec) and collected in methanol and stored at -80°C . Lipid extraction was performed by collecting the supernatant following protein precipitation with MeOH. Samples were dried under a gentle stream of nitrogen and analyzed using LC-MS/MS as previously described. For PUFA quantification, external calibration lines were constructed. All values were normalized to cell numbers.

Statistics. Data are presented as mean $\pm$ SD or median and interquartile region when indicated. Analysis and visualization was performed using GraphPad Prism 9.0.1 (San Diego, CA, USA). When indicated, data was analyzed using a two-way ANOVA followed by Tukey's multiple comparison test or Sidak's multiple comparison, a one-way ANOVA followed by Dunn's multiple comparisons test or a Wilcoxon signed-rank test. A p -value

below 0.05 was considered statistically significant ($p < 0.05$). Specifically, data derived from the lipid analysis was subjected to Friedman's test followed by Dunn's multiple comparisons test or a two-way ANOVA followed by Sidak's multiple comparisons test, as indicated. In every case a cutoff of 5% for Q for a comparison to be considered significant.

Results

Monocytes trained *in vitro* with BCG display increased concentrations of lipoxygenase-derived metabolites

Trained immunity inducers, such as β -glucan and BCG, trigger long-term metabolic reprogramming of monocytes and macrophages. However, the role of LMs in the induction of trained immunity has not yet been comprehensively investigated. We used an established *in vitro* model of trained immunity [12], in which human peripheral blood monocytes were exposed to β -glucan or BCG for 24h and left to rest for the following 5 days in the absence of any stimulus. A trained immunity cytokine response was subsequently induced by secondary stimulation, by selective TLR-4 stimulation with LPS. In contrast, to induce immune tolerance, monocytes were exposed to low-dose LPS on day 0 of culture.

We investigated changes in LM content at three different time points: upon monocyte collection to determine basal levels, after 24h of treatment, and at day 6 of culture, in the absence of restimulation (**Figure 1, S2, S3**). The concentration of free PUFAs arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosaheptaenoic acid (DHA) was not altered by the different stimulations at the evaluated timepoints. However, despite donor variation, we observed clear trends in the concentrations of different LMs at 24h of exposure (**Figure 1**). BCG triggered the increased production of eicosanoids and docosanoids, primarily associated with 12- and 15-LOX activity, namely 15-HETE and 12-HETE (**Figure 1A**), 15-HEPE, 12-HEPE (**Figure 1B**), 17-HDHA, and 14-HDHA (**Figure 1C**), many of them not reaching significance but presenting clear trends. 5-HETE, a product of 5-LOX activity was also enriched in BCG-exposed monocytes (**Figure 1A**). PGE₂, a product of COX activity, was likewise significantly increased in BCG-exposed monocytes (**Figure 1A**). Interestingly, recent publications on 15-hydroxyprostaglandin dehydrogenase (15-PGDH) inhibitors highlight the role of PGE₂ on cell and tissue regeneration [19,20]. Overall, BCG, and to a lesser degree β -glucan, stimulated an increase in biosynthesis of LMs, particularly 12- and 15-LOX products. In contrast, LPS promoted the depletion of most species, particularly 12-LOX derived products as observed by the significantly decreased concentrations of 12-HETE, 12-HEPE and 14-HDHA, in comparison with BCG stimulated monocytes [20]. These changes are induced early on during monocyte stimulation and were not persistent after differentiation of trained cells at day 6 of culture (**Figure S2, S3**).

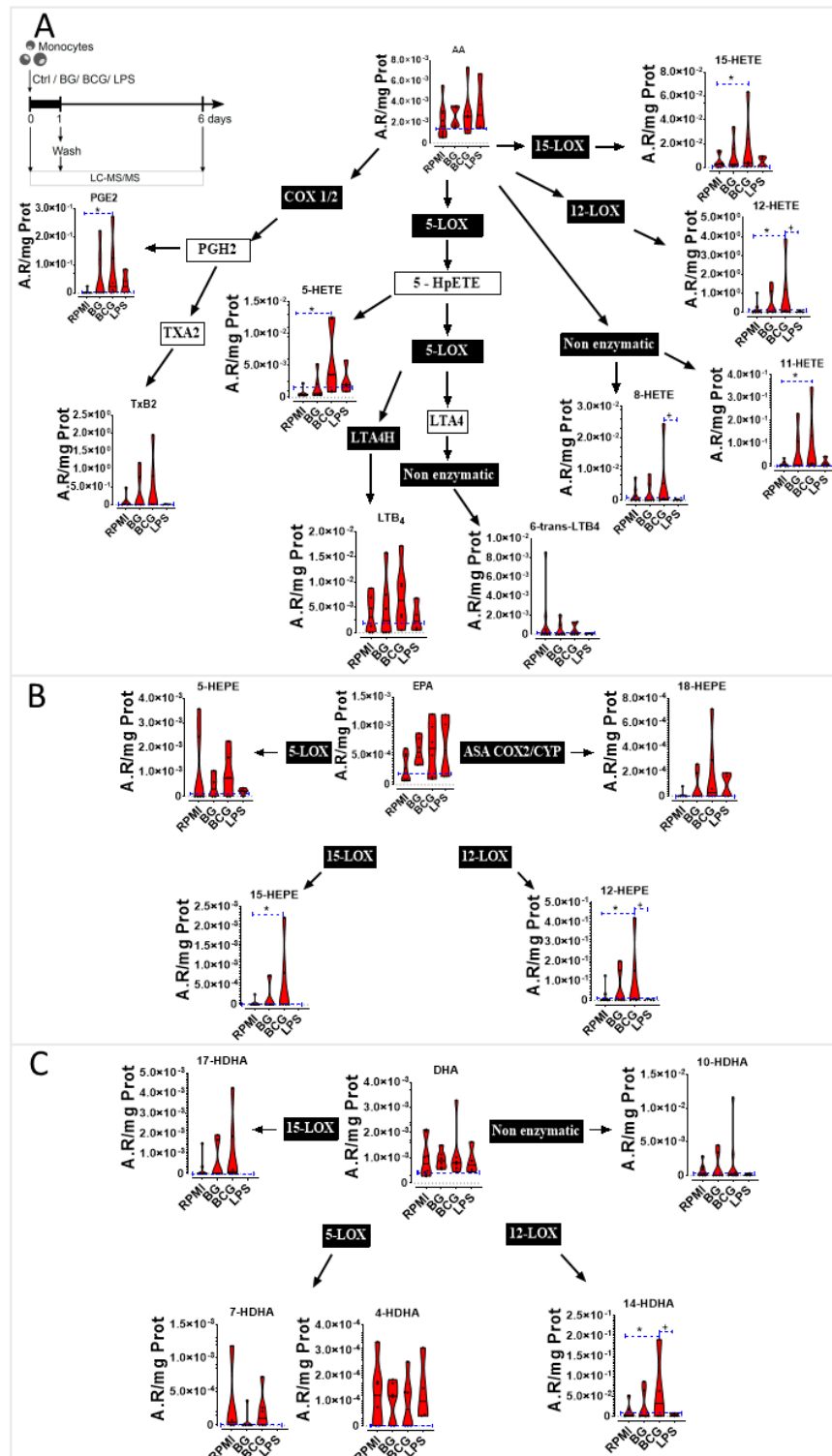


Figure 1 Increased PUFA biosynthesis in BCG-trained monocytes is associated with eicosanoid and docosanoid biosynthesis. (Legend continued next page)

In accordance, a previous study compared human monocytes and differentiated macrophages and observed a decrease in LM synthesis capacity and release in differentiated cells, possibly pinpointing to an exhaustion of PUFA precursor pools (**Figure S2**) [21].

Pharmacological inhibition of polyunsaturated fatty acid desaturase 2 (FADS2) decreases BCG-induced trained immunity *in vitro*

Monocytes exposed to BCG for 24 h exhibit a general increase in concentrations of PUFAs-derived LMs. We then hypothesized that these pathways and the activity of fatty acid desaturases and COX and LOX enzymes play a role in the establishment of BCG-induced trained immunity. We applied an *in vitro* pharmacological approach, in which monocytes were exposed to different enzymatic inhibitors concomitantly with the BCG stimulation for 24 h.

Firstly, we evaluated the importance of fatty acid desaturation. We assessed the expression of the main desaturases stearoyl-CoA desaturase (SCD) and fatty acid desaturases 1 and 2 (FADS1/2). The expression of *SCD* did not appear to be modulated at 4 h or 24 h after BCG exposure, while *FADS1* tended to be decreased and *FADS2* tended to be increased at 4 h (**Figure 2A**). *FADS2* is responsible for desaturation of short-chain PUFAs, such as the essential linoleic acid and alpha-linolenic acid precursors, into long-chain PUFAs, like AA, DHA and EPA. Pharmacological inhibition of *FADS2* by SC-26196 decreased LPS-induced TNF α and IL-6 production in BCG-trained macrophages (**Figure 2B,C**). Curiously, inhibition of *FADS2* increased TNF α production in unstimulated macrophages, contrary to the downregulation seen in BCG-trained cells.

Figure 1 cont. Levels of PUFAs and oxylipids in stimulated monocytes at 24h with BG, BCG, and LPS (n=4-8, pooled from 4 experiments), (A) Arachidonic acid pathway (AA), (B) Eicosapentaenoic acid (EPA), and (C) Docosahexaenoic acid (DHA) pathway. Violin plots showing median and the interquartile region, *, + $p \leq 0.05$ between RPMI and BCG, and BCG and LPS respectively, Friedman's test followed by Dunn's multiple comparisons correction. Area ratios (A.R), corrected for internal standards and protein content are shown.

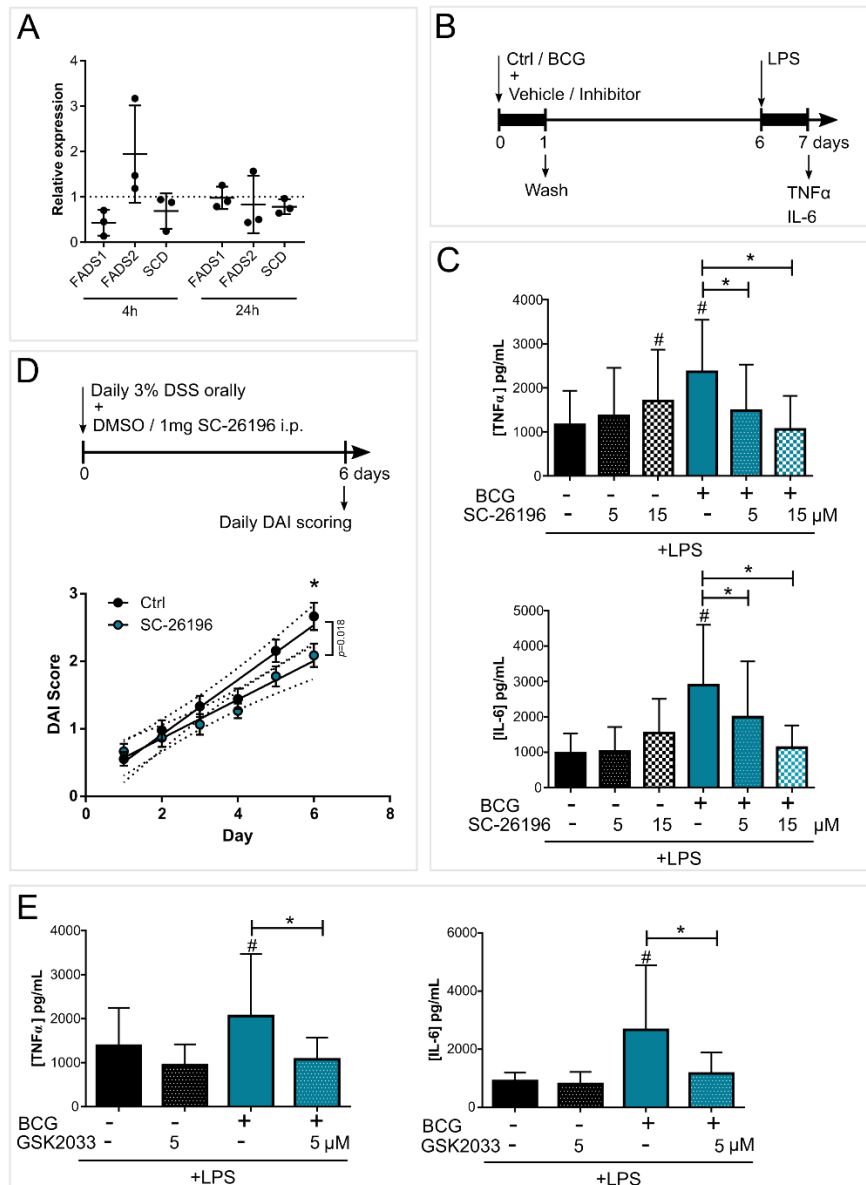


Figure 2 FADS2 activity contributes to the induction of trained immunity. (A) Monocyte *FADS1*, *FADS2*, *SCD* gene expression 4h and 24h after BCG exposure (n=3). (B) Schematic representation of the *in vitro* model used for the pharmacological inhibition of the induction of BCG-trained immunity. (C) Production of TNFα and IL-6 by BCG-trained macrophages incubated with (5 μM, 15 μM) SC-26196 for the first 24h of culture and restimulated at day 6 with LPS for 24 h. (n=6 pooled from 2 independent experiments). (D) Schematic representation of the *in vivo* colitis model used and DAI score (stool consistency, rectal bleeding, and weight loss) of mice with DSS-induced colitis and daily administration of 1 mg SC-26196 or vehicle control (n=15 pooled from 2 independent experiments). (E) Production of TNFα and IL-6 by BCG-trained macrophages incubated with (5 μM) GSK2033 for the first 24 h and restimulated with LPS at day 6 of culture for further 24h. (n=6 pooled from 2 independent experiments). Mean±SD, # vs control, *, * p ≤ 0.05 (C) two-way ANOVA, Tukey's multiple comparisons test, (D) Mann-Whitney U test and linear regression analysis. The slopes are significantly different (p=0.018) (E) two-way ANOVA, Sidak's multiple comparisons test

Epidemiological evidence has pointed to the role of PUFA and macrophages in the development of inflammatory conditions such as ulcerative colitis and Crohn's disease [22,23]. At the same time, a large number of studies suggest the involvement of mycobacteria in the pathophysiology of Crohn's disease [24], and one may speculate that inappropriate induction of trained immunity by mycobacteria in the gut mediates this relationship. Thus, we set out to explore this possibility by means of FADS2 inhibition. DSS treatment induced inflammation, as is reflected in the increase of DAI score over time, while concomitant inhibition of FADS2 led to a significant decrease in DAI score at day 6 (**Figure 2D**). Subsequently, this also led us to interrogate the Liver X receptors (LXRs), which are nuclear receptors that provide transcriptional control of lipid metabolism. The LXR transcription factors are known to control the expression of genes involved in cholesterol, fatty acid, and phospholipid metabolism. Macrophage LXR activation was shown to enhance PUFA synthesis and FADS1/2 gene expression [25]. Interestingly, pharmacological inhibition of LXR also decreased BCG-enhanced TNF α and IL-6 production after LPS stimulation (**Figure 2E**). Jointly, these results underpin the importance of long-chain PUFA for the development of trained immunity.

LOX-derived LM induce trained immunity *in vitro*

We next explored the role of COX- and LOX- activity for the establishment of trained immunity. The inhibition of the COX pathway did not ablate BCG-induced enhanced cytokine production (**Figure 3A**). Although expression of COX2 was increased in monocytes 4 h following BCG treatment (**Figure 3B**), pharmacological inhibition of COX-1, COX-2, or CP450 did not decrease TNF α or IL-6 secreted upon LPS restimulation. Instead, aspirin increased the production of TNF α in both control and BCG-trained monocytes (**Figure 2B, 3A**). Interestingly, exogenous supplementation of monocytes with the COX product PGE₂, which was increased upon BCG 24h stimulation, decreased TNF α and IL-6 production after LPS restimulation (**Figure 3C,D**).

In contrast, inhibition of specific LOX enzymes decreased BCG-enhanced cytokine production (**Figure 2B, 4A**). 5-LOX inhibition decreased TNF α and IL-6 production, while 12-LOX inhibition decreased IL-6 secretion of BCG-trained macrophages. On the other

hand, 15-LOX inhibition potentiated IL-6 production of BCG-trained macrophages and increased TNF α secretion of control cells.

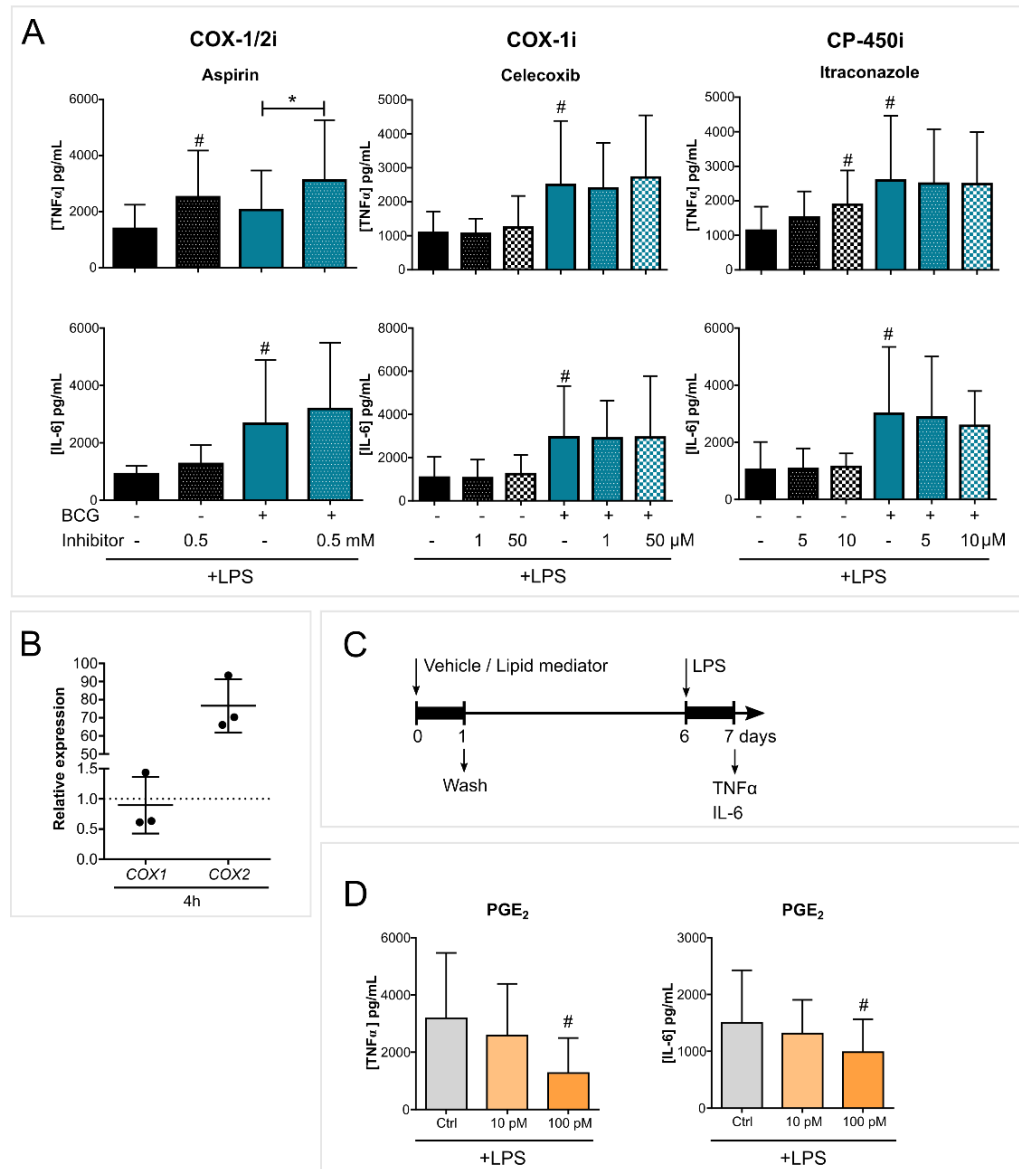


Figure 3 Inhibition of the COX pathway does not decrease BCG-induced cytokine production (A) TNF α and IL-6 secreted by macrophages exposed to BCG and 0.5 mM Aspirin, 1 μ M, 50 μ M Celecoxib or 5 μ M, 10 μ M Itraconazole for the first 24 h of culture and 5 days later stimulated with LPS for 24 h (n=6/9, pooled from 2/3 independent experiments). (B) Relative COX1 and COX2 expressions in monocytes incubated with BCG for 4 h (n=3). (C) Schematic representation of *in vitro* LM supplementation experiments. (D) TNF α and IL-6 secreted by macrophages exposed to 10 or 100 pM PGE₂ for the first 24 h of culture and 5 days later stimulated with LPS for 24 h (n=6, pooled from 2 independent experiments). Mean $\pm$ SD, # vs control, #, * p $\leq$ 0.05. (A) two-way ANOVA, Tukey's or Sidak's multiple comparisons test, (D) one-way ANOVA, Dunn's multiple comparisons correction.

Following these observations, we assessed whether exogenous supplementation of different LOX-derived LMs is sufficient to induce trained immunity (**Figure 4B**). Monocytes were exposed to 5-HETE, 7-HDHA, and leukotriene B₄ (LTB₄), products of 5-LOX activity; 12-HEPE, a product of 12-LOX; or 15-HETE and 17-HDHA, products of 15-LOX for 24h and left to differentiate for 5 days (**Figure 3C, 4B**). 7-HDHA, 15-HETE and 17-HDHA supplementation triggered increased TNF α and IL-6 production upon LPS restimulation of differentiated macrophages for 24h. 12-HEPE exposure significantly increased IL-6 secretion. Although LTB₄ and 5-HETE were enriched in BCG exposed monocytes, exogenous LTB₄ or 5-HETE supplementation did not modulate cytokine production upon restimulation (**Figure 4B**).

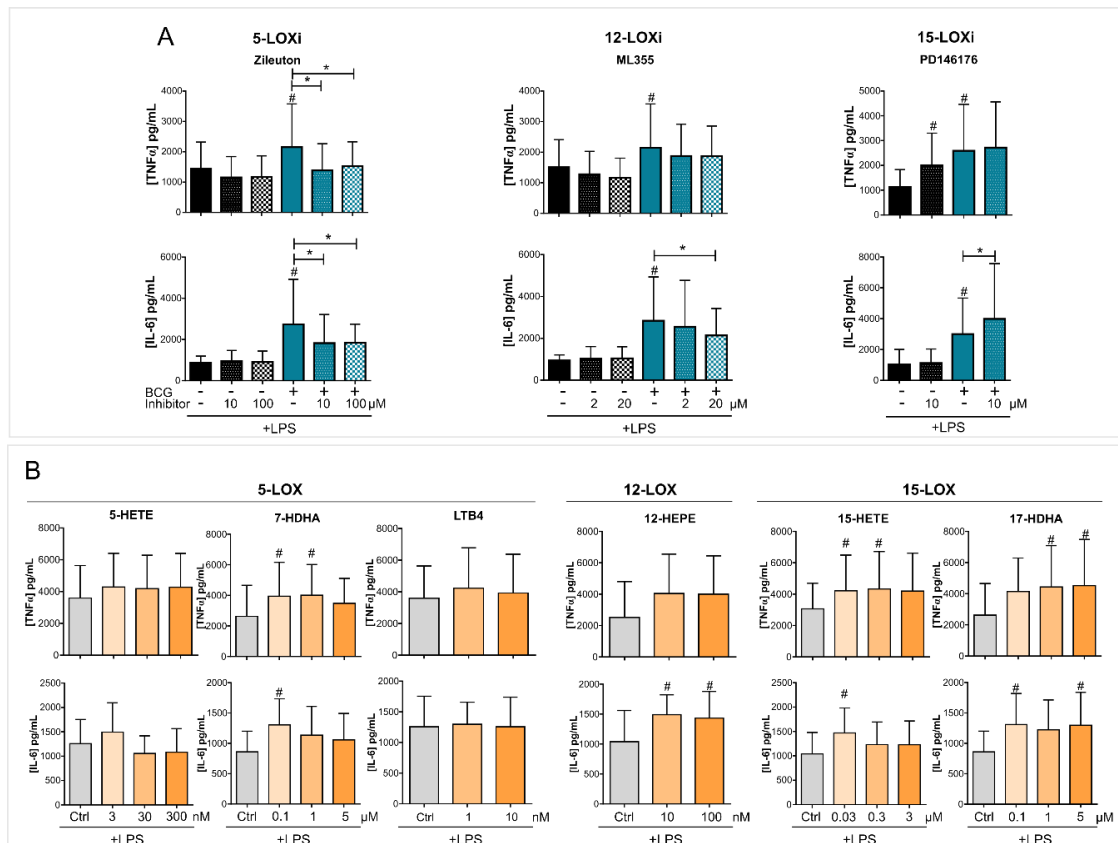


Figure 4. LOX pathways play a role in BCG-induced trained immunity. (A) Production of TNF α and IL-6 by BCG-trained macrophages incubated with (10 μ M, 100 μ M) Zileuton, (2 μ M, 20 μ M) ML355 or 10 μ M PD146176 for the first 24 h and restimulated with LPS for another 24 h at day 6 of culture. (n=6/9 pooled from 2/3 independent experiments). (B) Production of TNF α and IL-6 by macrophages incubated with (3, 30, 300 nM) 5-HETE, (0.1, 1, 5 μ M) 7-HDHA, (1, 10 nM) LTB₄, (10, 100 nM) 12-HEPE (0.03, 0.3, 3 μ M) 15-HETE or (0.1, 1, 5 μ M) 17-HDHA for 24h and restimulated at day 6 of culture with LPS for 24 h. (n=6/9 pooled from 2/3 independent experiments. Mean $\pm$ SD, # vs control, #, * p $\leq$ 0.05 (A) two-way ANOVA, Tukey's or Sidak's multiple comparisons test, (B) one-way ANOVA, Dunn's multiple comparisons correction.

Taken together, the COX pathway does not appear to play a role in the increased responsiveness of BCG-trained macrophages, while LOX-derived LMs generally increase cell responsiveness to restimulation. In line with these results, the inhibition of 5-LOX and 12-LOX activity ablates BCG-enhanced cytokine production, thus pointing to the relevant role of the LOX pathways for the establishment of trained immunity.

SNPs in desaturases and LOX pathway genes influence the induction of trained immunity in healthy human volunteers

To further explore the role of desaturases and LOX pathways in trained immunity, we performed a genetic study in a cohort of 325 healthy volunteers. We assessed whether common SNPs (MAF>0.05) in a window of 250kb around the genes *FADS1*, *FADS2*, *SCD*, *LOX5*, *LOX15* and *LOX12* were associated with changes in pro-inflammatory cytokine production. We performed this analysis not only in an *in vitro* β -glucan or BCG trained immunity model, but also in an *in vivo* BCG vaccination model of trained immunity (**Figure 5A, B**). Taking advantage of models, we identified various SNPs within 250kb of the genes of interest that were suggestively associated ($p < 9.99 \times 10^{-3}$) with changes in TNF α , IL-6, and IL-1 β production. Specifically, variation in *FADS2* (rs9326418, $p=0.00016$) was associated with the increase of TNF α production after β -glucan-induced trained immunity, and *LOX12* (rs12232535, $p=0.00021$) was associated with the potentiation of IL-6 in the *in vitro* BCG trained immunity model (**Figure 5A**). Similarly, variations in *FADS2* (rs11600019, $p=0.00078$) and *LOX12* (rs955461, $p=0.00032$) were associated with the increased fold change of TNF α and IL-6, respectively, after BCG vaccination (**Figure 5B**).

12-LOX derived LM are increased in monocytes of healthy individuals after BCG vaccination

Next, we sought to translate our *in vitro* findings to an *in vivo* setting. We investigated the concentrations of AA-, EPA- and DHA- derived LMs in monocytes from healthy individuals 30 days after BCG vaccination (**Figure 5C, S4**).

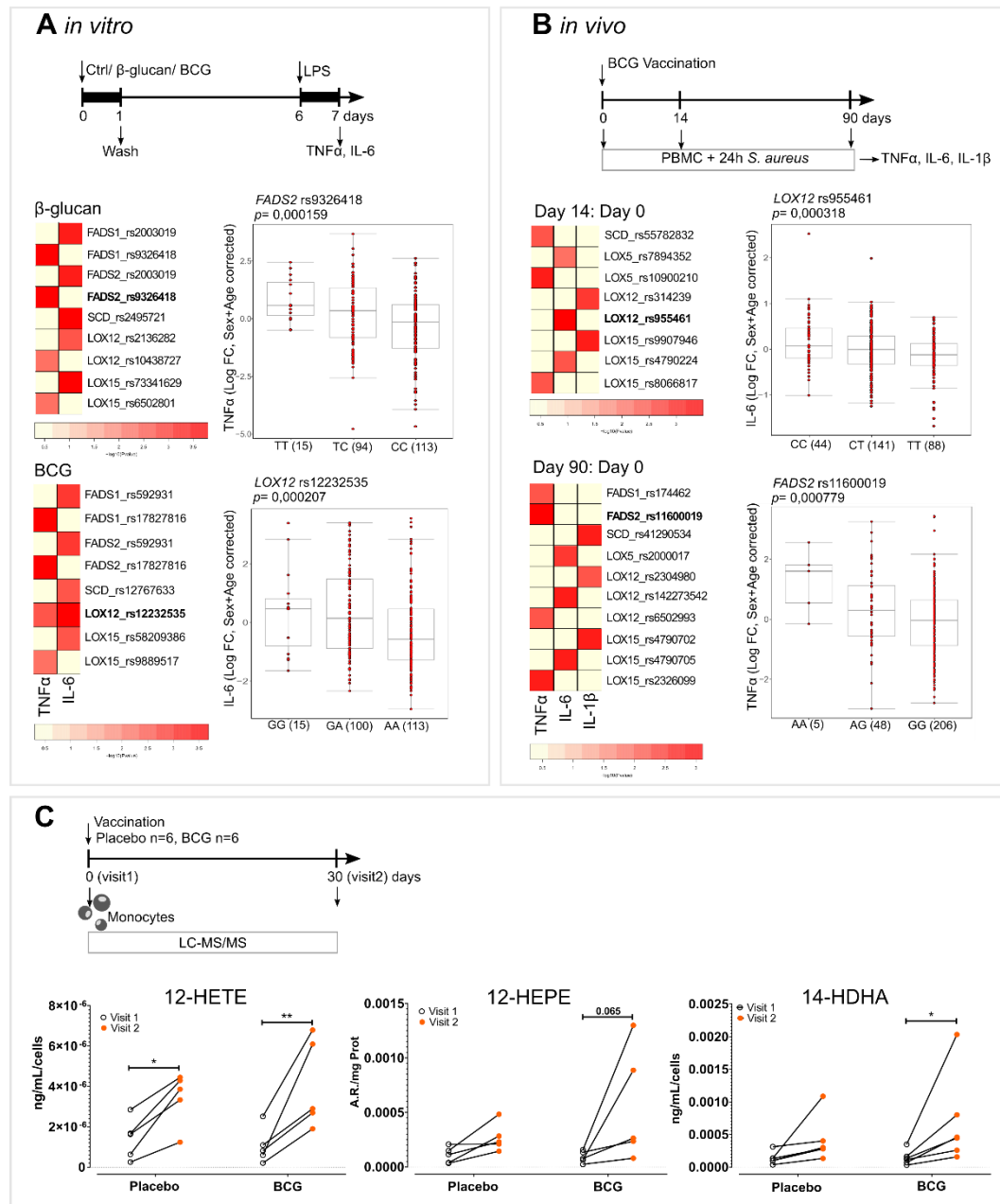


Figure 5 Lipid desaturation and the LOX pathway are involved in the *in vivo* induction of trained immunity. (A,B) Heatmaps of the p values of the association between SNPs around *FADS1*, *FADS2*, *SCD*, *LOX5*, *LOX12* and *LOX15* genes ($\pm$ 250kb) and the fold change of the production of (A) TNF α and IL-6 after *in vitro* exposure to β -glucan or BCG and LPS restimulation, ($n = 238$ healthy volunteers for TNF α and $n = 251$ healthy volunteers for IL-6) and (B) TNF α , IL-6 and IL-1 β upon BCG vaccination and *S. aureus ex vivo* restimulation ($n = 278$ healthy volunteers). Boxplots show selected SNPs with the lowest p -values stratified according to genotype. (C) Symbols and lines plot for concentrations (ng/mL) corrected to cell number or area ratio (A.R.) corrected to protein content of 12-LOX products of AA, EPA, and DHA one month after BCG vaccination ($n=6$), Median, * $p \leq 0.05$, ** $p \leq 0.01$, two-way ANOVA, Sidak's multiple comparisons test.

Remarkably, 12-HETE, 12-HEPE, and 14-HDHA, which are 12-LOX products of AA, EPA, and DHA, respectively, were increased after BCG vaccination (**Figure 5C**). This result is also in agreement with our *in vitro* observations and highlights a potentially important role of 12-LOX products in the *in vivo* BCG-induced trained immunity.

Discussion

Innate immune memory is dependent on the rewiring of different metabolic pathways, such as glycolysis, glutaminolysis, and oxidative phosphorylation [26]; however, the role of lipid metabolism in trained immunity has yet to be comprehensively explored. In this study, we investigated the modulation of monocyte lipid content upon induction of trained immunity and, more specifically, the role of LMs in the induction of trained immunity.

We show that the long-chain PUFA synthesis pathway is relevant for BCG-induced trained immunity. This is in accordance with *in vivo* studies in which peritoneal macrophages of mice infected with BCG have higher PUFA content and arachidonic acid levels than non-infected animals [27]. Curiously, bone marrow progenitor cells of β -glucan trained mice had lower levels of PUFA and AA than the control group [7], pointing to a differential regulation of lipid metabolism by progenitor cells when compared to peripheral monocytes. We observed that long-chain PUFA synthesis is necessary for establishing BCG-dependent trained immunity, as inhibition of fatty acid desaturation by FADS2 decreased BCG's effect on pro-inflammatory cytokine production.

FADS2 inhibition also showed beneficial effects likely related to trained immunity in a colitis mouse model. Inflammatory bowel disease (IBD) has a common genetic burden as mycobacterial diseases [28], and non-tuberculous mycobacteria have been suggested to play a role in its etiology [29–31]. As mycobacteria are strong inducers of trained immunity [32–34] one may hypothesize that inappropriate induction of trained immunity is involved in the pathophysiology of IBD. In addition, in patients with ulcerative colitis, the intestinal mucosa presents increased concentrations of AA and AA-derived LMs, such as PGE₂ and 12-HETE, and decreased DHA and EPA concentrations. The increased AA-derived metabolites were positively correlated with pro-inflammatory cytokine production [35]. Indeed, we show that FADS2 inhibition decreased both trained immunity and the severity of colitis in a murine model. Also, in both *in vitro* and *in vivo* trained immunity models, we have identified associations between genetic variations in *FADS2* gene and pro-inflammatory cytokine production upon heterologous stimulation.

On the other hand, BCG may also induce the modulation of long-chain PUFA-derived LMs via the regulation of LXRs. Macrophage LXR α expression was shown to be induced by a

NOD2 ligand [36], which is a relevant intracellular receptor in the host response elicited by mycobacteria [37] and for BCG-induced trained immunity [3]. Indeed, LXR activity supports BCG-induced trained immunity since LXR antagonism blocks BCG-induced inflammatory responses to TLR2 restimulation [38]. In this line, we also observed a decrease in the response to TLR4 restimulation upon LXR inhibition of BCG-trained macrophages. LXRs are regulators of both lipid metabolism and inflammation. LXR decreases inflammatory responses, since its activation can promote membrane lipid remodelling and increase membrane rigidity, thus blunting the TLR signalling cascade [39]. In mice exposed to LPS, LXR agonist administration led to a decrease in inflammatory gene expression [39], and LXR-deficient macrophages show a defect in efferocytosis and a more pronounced pro-inflammatory phenotype [40]. The impact of LXR signalling on macrophage phenotype appears to be context-dependent but it is an attractive possible regulator of LM synthesis, necessary for the induction of BCG trained immunity.

Downstream metabolism of long-chain PUFAs mainly involves transformation into bioactive signaling lipids through oxidation by the actions of COX, LOX, CP450, and non-enzymatic pathways. Thromboxane and PGs, derived from AA through COX activity, are released by myeloid cells and modulate their migration, differentiation, and effector function [41]. *C. albicans* and BCG increase COX-2 expression and PGE₂ production [42–45]. Indeed, acute stimulation of human monocytes with β -glucan or BCG increased PGE₂ intracellular concentrations, but after the differentiation period, PGE₂ concentrations were unchanged between trained and naive macrophages. However, despite this increase in PGE₂ concentrations upon acute stimulation with trained immunity inducers, inhibition of COX activity did not modulate inflammatory responses of restimulated macrophages. In contrast, PGE₂ exogenous supplementation decreased pro-inflammatory cytokine production induced by LPS five days after the stimulation with the LM, which is in line with the well-known anti-inflammatory effects of PGE₂ [46,47] and aligns well with the concept that “the beginning programs the end” in inflammation resolution [48]. Trained immunity programs rely on the potentiation of glycolysis and oxidative respiration [6], and PGE₂ has been shown to suppress both pathways [49]. Taken together, the COX pathway plays an important immunological role but not in the induction of BCG trained immunity.

In addition to the COX pathway, the LOX enzymes produce AA, DHA and EPA-derived LMs. We show that monocytes exposed to BCG, and to a lower extent β -glucan, are

generally enriched in LOX products. The observed *in vitro* increase in LOX-derived mediators is corroborated by the increase in 12-LOX products in circulating monocytes of BCG vaccinated individuals. Of note, the *in vitro* assays and the genetic study were performed with the BCG-Bulgaria strain while individuals that participated in the *in vivo* lipid analysis were vaccinated with BCG-Denmark strain. Importantly, both strains point to the upregulation of 12-LOX products in BCG-induced trained immunity. Curiously, the 12-LOX product 12-HETE has been suggested as a plasma biomarker for the diagnosis of TB [50], and concentrations of 5-LOX products were also increased in plasma of multidrug-resistant TB patients [51]. In this study, eicosanoids were shown to positively correlate with the plasma concentrations of succinate and fumarate, metabolites also enriched during induction of trained immunity [5,51].

Different LOX-derived metabolites appear to be necessary and sufficient for the responsiveness of macrophages. *In vitro* inhibition of 5- and 12-LOX enzymes downregulated BCG-induced cytokine production, while specific LOX-derived LMs were sufficient to increase responsiveness to TLR-4 stimulation, characteristic of trained immunity. However, we cannot exclude that the lipids we supplemented are metabolized further and thus have an indirect effect on cytokine production. LOX-derived LMs are attributed anti-inflammatory and pro-resolving roles [52], but this activity is context-dependent. In a mouse model of fungal infection with *Aspergillus fumigatus*, 5-LOX-deficient mice showed impaired neutrophil recruitment to the lung and decreased survival. [53]. In contrast, *M. tuberculosis* infection of 5-LOX-deficient mice led to increased resistance [54]. It is important to bear in mind that macrophages are highly dynamic cells, and different inflammatory and infectious conditions may trigger distinct lipid responses. For example, TLR-2 or TLR-4 stimulation promotes COX-2 and 5-LOX pathways in the pro-inflammatory M1 macrophages, while the anti-inflammatory M2 macrophages show increased release of 12-LOX and 15-LOX products [21]. Also, LXR activation in M2 macrophages promotes the expression of 15-LOX and leads to an increase of 12/15-LOX products [55]. In contrast, macrophages supplemented with the AA 12/15-LOX product 12-HETE show increased IL-6 and TNF α expression [56]. Accordingly, IL-6 and TNF α expression is enhanced in peritoneal macrophages isolated from mice overexpressing the 12/15-LOX gene [56].

It is important to note that in humans there are three described 12/15 lipxygenases, *ALOX12*, *ALOX15* and *ALOX15B* [57]. In monocyte/macrophage populations, the

expression of ALOX12 is low or even null [58]. However, the decrease of BCG-enhanced cytokine production induced by 12-LOX inhibition points to a role of this enzyme. Also, it is possible that the increase in 12-lipoxygenated products we observed *in vitro* and *in vivo* BCG exposed monocytes is reflective of increased 15-LOX activity. 15B-LOX is constitutively expressed and produces almost exclusively oxygenated products at carbon 15; on the other hand in addition to 15-lipoxygenated products, 15-LOX is also able to catalyze the synthesis of oxygenated products at carbon 12 [59]. ALOX15 expression is mainly enhanced in M2 macrophages [58] and in macrophages that contribute to the resolution of inflammation [60]. Yet, pharmacological inhibition of 15-LOX did not decrease BCG-enhanced cytokine production, which questions the possible role of this enzyme in the production of 12-lipoxygenated products in BCG exposed monocytes. In summary, further studies are necessary to pinpoint the exact enzymes involved in LM production in BCG-trained immunity.

In conclusion, LMs, in particular LOX products, are essential for the induction of trained immunity. LOX-derived products increased macrophage responsiveness, similar to other key metabolites in trained immunity, such as fumarate and mevalonate, while inhibition of the long-chain PUFA synthesis pathway prevented the trained immunity phenotype. Furthermore, circulating monocytes of BCG-vaccinated individuals, which were previously shown to produce increased levels of pro-inflammatory cytokines upon heterologous stimulation [34], were enriched in 12-LOX products. In summary, the macrophage lipidome appears to directly influence the trained immunity phenotype, and its composition likely reflects immune memory. Further studies are warranted to decipher and correlate higher order lipid species, such as esterified LOX products, with short- and mid-term immune memory as well as the exact enzymes and possible macrophage sub-population responsible for our findings. Nevertheless, this study highlights the relevance of the LOX pathway in trained immunity, and future studies should explore the LOX pathway as a therapeutic tool for the potentiation of the innate immune system or a target to decrease maladaptive inflammatory programs.

Conflict of Interest Disclosure. MGN is a scientific founder of TTxD. The remaining authors declare no conflict of interest. The funders had no role in the design of the study; in

the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results

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Authorship. Conceptualization: AVF, JCA-B, MGN, MG; Methodology: AVF, JCA-B, JD-A, MGN, MG; Formal Analysis: AVF, JCA-B, VM; Investigation: AVF, JCA-B, OB, GK, PAD, RJR, HNO, AZ, SK, VM, JD-A; Writing - Original Draft: AVF, JCA-B; Writing - Review and Editing: all. All authors have read and agreed to the published version of the manuscript.

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Supplemental Figures

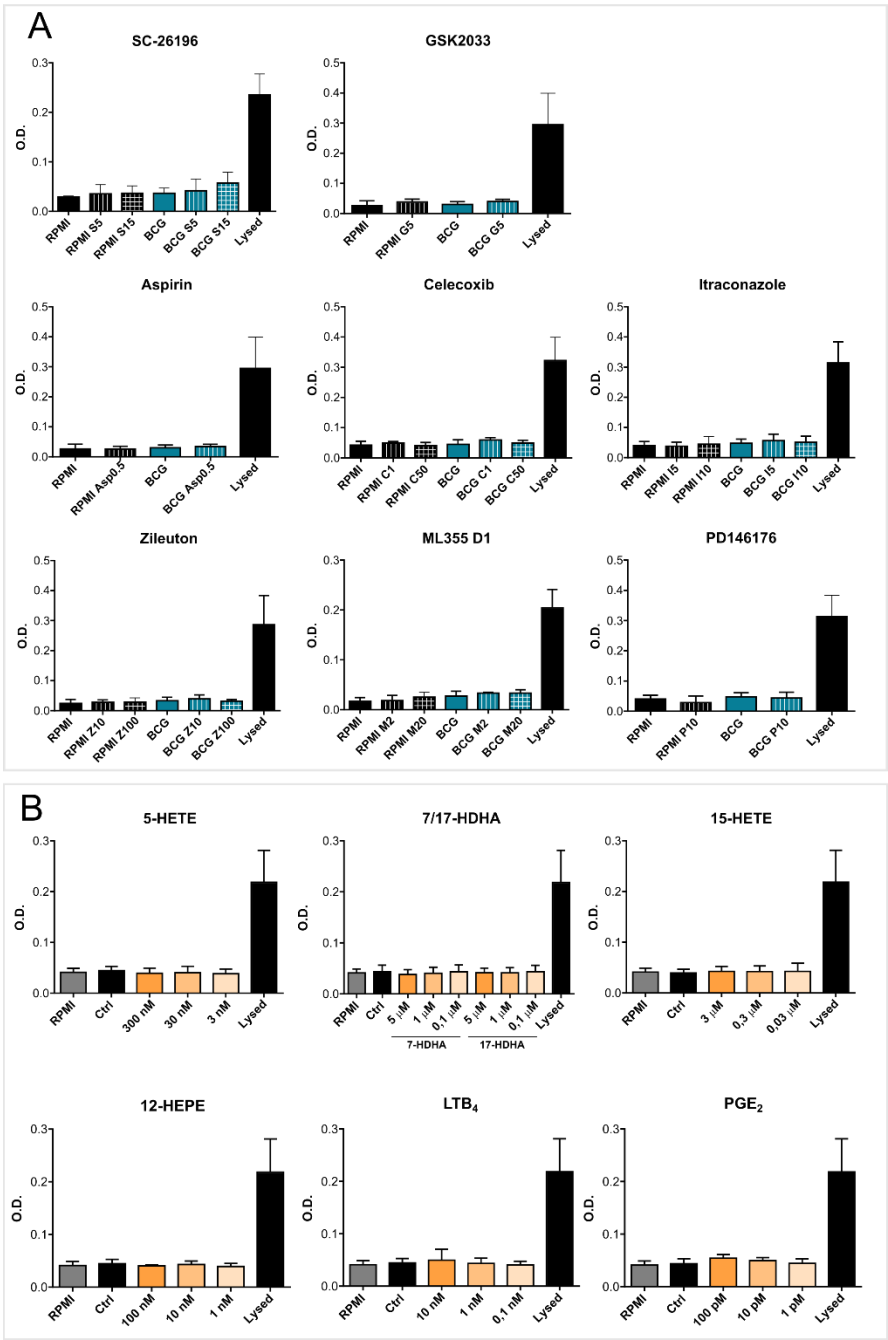


Figure S1 LDH in the conditioned media of monocytes incubated for 24h with (A) Aspirin, Celecoxib, Itraconazole, GSK2033, SC-26196, Zileuton, ML355 or PD146176 in combination with BCG (n=3/6, Mean±SD) and (B) 5-HETE, 7-HDHA, 17-HDHA, 15-HETE, 12-HEPE, LTB₄ or PGE₂ (n=6, Mean±SD).

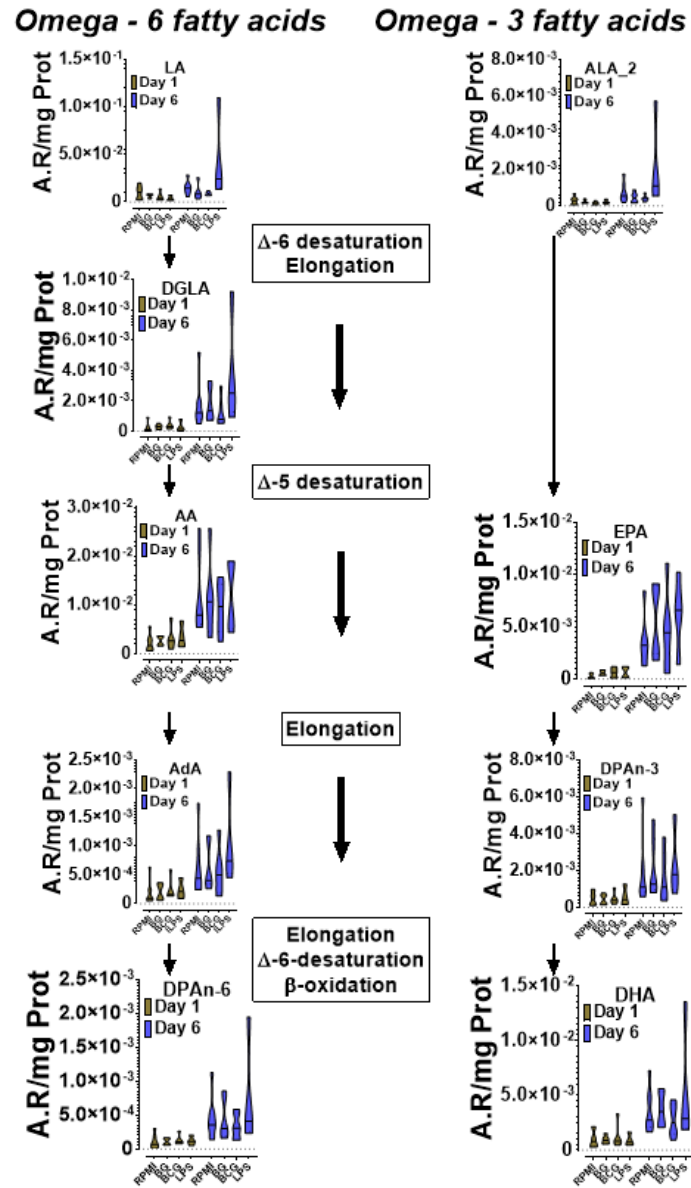


Figure S2 Area ratios (A.R) levels of PUFAs corrected for internal standards and protein in the omega-6/3 pathways in stimulated monocytes at 24h with BG, BCG, and LPS and after 6 days of culture (n=4-8), Median and the interquartile region.

Complete pathway analysis of LMs pathway (*in vitro* assay)

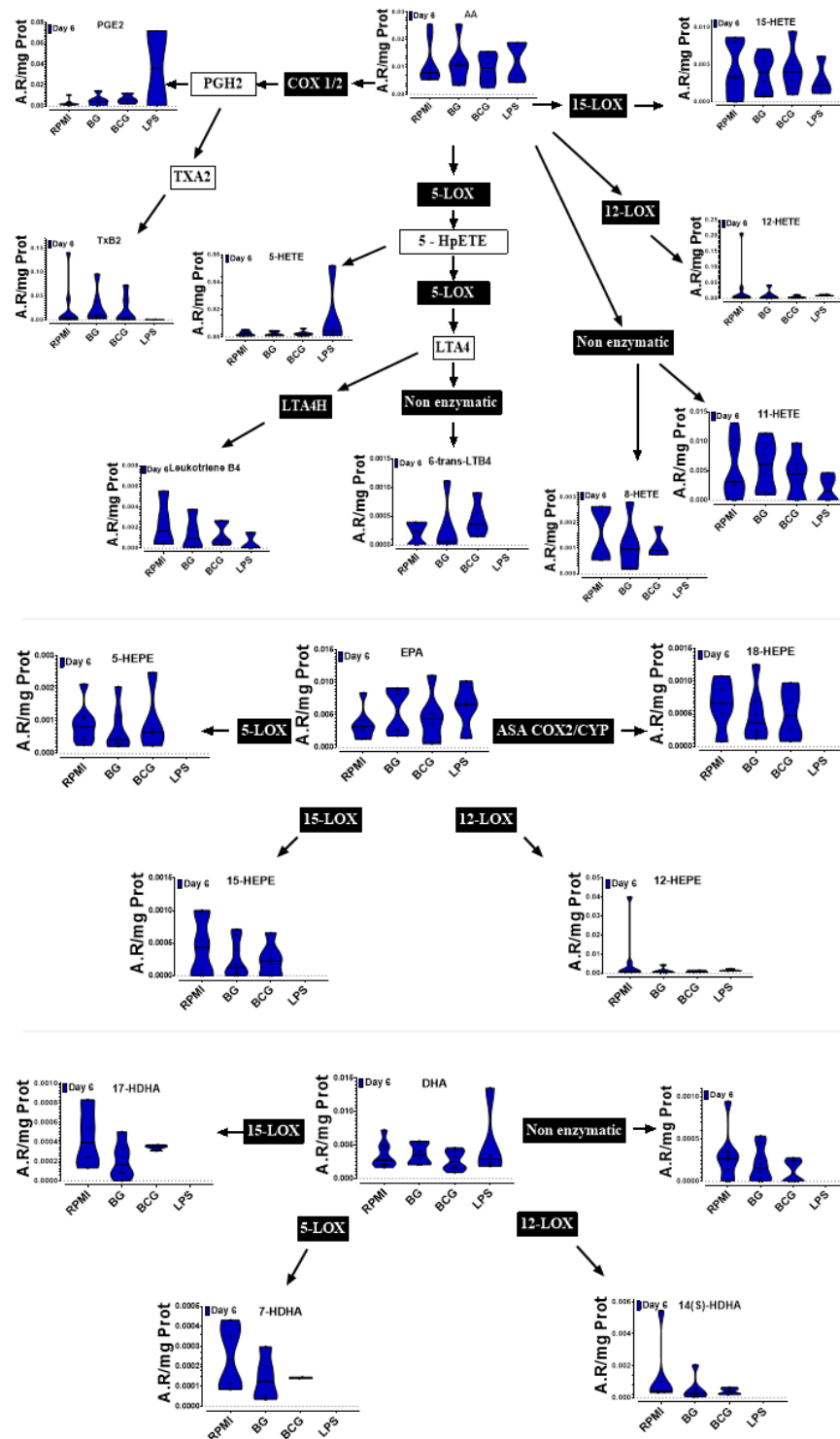


Figure S3 Area ratio (A.R) levels corrected to protein levels of PUFAs and oxylipids of macrophages at day 6 of culture after in vitro exposure to BG, BCG or LPS (n=4-8), Median and the interquartile region.

Complete pathway analysis of LMs and omega fatty acids (*in vivo* assay)

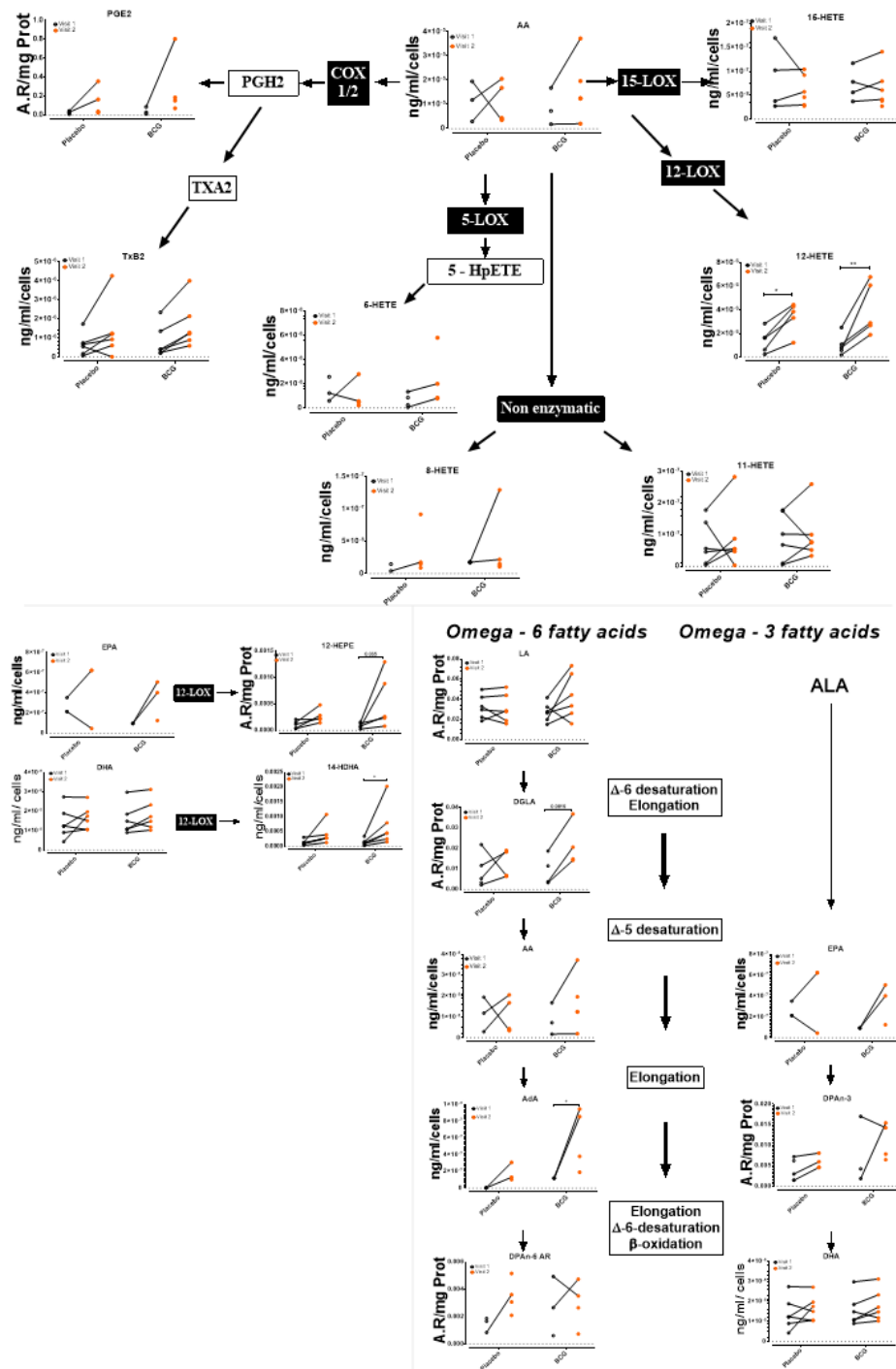


Figure S4 Concentrations (ng/mL) corrected to cell number or Area ratio corrected to protein levels of PUFAs and oxylipids of *in vivo* BCG-trained response in monocytes (n=6), * $p \leq 0.05$, ** $p \leq 0.01$, two-way ANOVA, Sidak's multiple comparisons test.

CHAPTER 4

[RESEARCH PAPER]

LIMITED ROLE OF THE SPLEEN IN A MOUSE MODEL OF TRAINED IMMUNITY: IMPACT ON NEUTROPHILIA

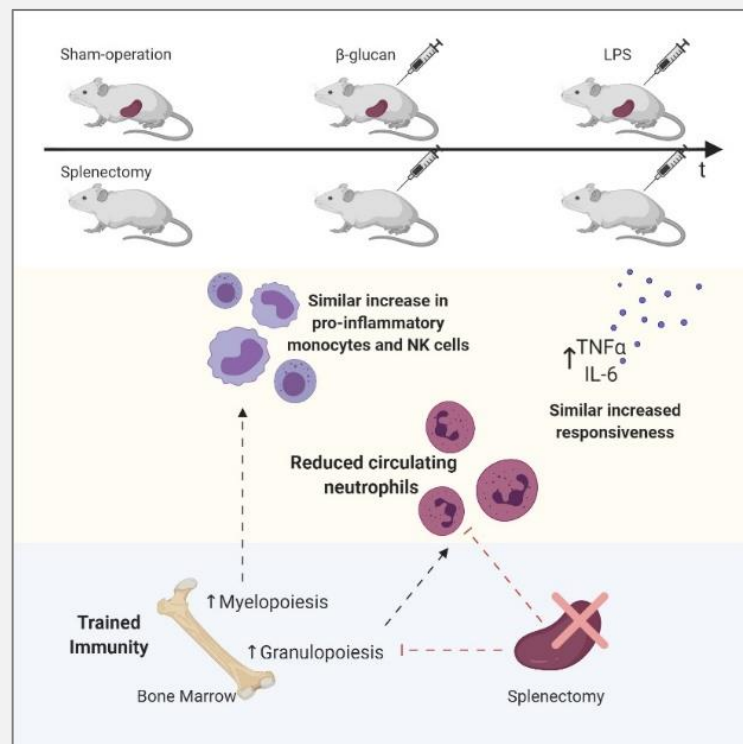
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Abstract

Trained immunity is a de facto memory of innate immune cells, resulting in a long-term increase in innate host defense mechanisms after infection. The long-term heterologous protection conferred by trained immunity is mediated through epigenetic and functional reprogramming of hematopoietic stem and progenitor cells. Since the spleen is a reservoir of undifferentiated monocytes and is considered the prime organ for extramedullary hematopoiesis, we investigated the role of the spleen in the establishment of trained immunity. A β -glucan-induced trained immunity mouse model was performed in previously sham-operated or splenectomized animals. Removal of the spleen did not modulate the pro-inflammatory cytokine production of *in vivo* trained peritoneal cells, nor did it ablate the increased percentage of pro-inflammatory circulatory monocytes and natural killer cells seen in trained animals. However, spleen removal prevented neutrophilia, an important characteristic of trained immunity. These data point to a limited role of the spleen in trained immunity. The pathophysiological relevance of the spleen in the induction of neutrophilia during trained immunity remains to be fully explored.



Introduction

Immunological memory is classically attributed to the adaptive immune system. However, a growing body of evidence in human and murine models indicates that the innate immune system has long-term memory characteristics, also termed trained immunity [1]. During induction of trained immunity after certain challenges, innate immune cells respond stronger to a secondary event, conferring protection against heterologous pathogens. Trained immunity has been described primarily in monocytes, macrophages and natural killer (NK) cells after exposure to different stimuli, such as the *Bacillus Calmette–Guérin* (BCG) vaccine and the *Candida albicans* cell wall component β -glucan [2, 3]. Due to the short half-life of monocytes in the circulation, the long-lasting protection secured by innate immune memory is thought to be assured centrally with changes in the hematopoietic immune cell progenitors. Mice exposed to BCG or β -glucan show long-term change in the transcriptional and functional program of bone marrow immune progenitors with a bias towards myelopoiesis, which ultimately generates trained myeloid cells [4, 5]. Similar observations were recently made also in humans after BCG vaccination [6].

The spleen is a secondary lymphoid organ, which stores macrophages and recycles erythrocytes. The spleen serves also as a reservoir of bone marrow-derived undifferentiated monocytes that can be expanded and rapidly deployed to the circulation [7], and is also a hematopoietic organ. Hematopoiesis is an orchestrated process in which a pool of hematopoietic stem progenitor cells (HSPCs) gives rise to multi-lineage blood cells. HSPCs are mostly located in the bone marrow, however, extramedullary hematopoiesis might occur under emergency conditions. HSPCs mobilized from the bone marrow seed extramedullary sites, proliferate, and mature. Specifically, pro-inflammatory monocytes and neutrophils have been shown to be produced by the spleen in mice models of atherosclerosis and endotoxin challenge [8].

Trained immunity relies on an HSPCs expansion and skewing, leading to trained peripheral myeloid cells. We therefore hypothesized that the spleen, as a site of extramedullary hematopoiesis and reservoir of monocytes, is an organ that might contribute to the increased systemic responsiveness conferred by stimuli inducing trained immunity. Taking advantage of the well-established β -glucan trained immunity mouse model, we aimed to explore the role of the spleen in the induction of innate immune memory.

Materials and Methods

Animals and ethical statement. Eight weeks old female C57BL/6JRj from Janvier Labs (Le Genest Saint Isle, France) were used for all experiments. Animals were allowed one week to acclimatise to the facilities before the experiment. Mice were housed in groups of 5 animals per enriched type-II cage on a 12 h light-dark diurnal cycle with room temperature between 21 and 23 °C. Water and food were provided *ad libitum*. Procedures were approved by the Central Committee on Animal Experiments, Radboud University (2016-0100).

Splenectomy and *in vivo* training model. Animals were randomized, and half had their spleens removed by electrocauterization (Bvi medical, Waltham, MA, USA). In sham-operated mice, the abdominal wall was opened and then closed after identifying the spleen. All surgical procedures were done under inhalation anaesthesia (1% isoflurane) on a heat pad. Body temperature was monitored. Pain was mitigated pre- and post-operatively. 10mg/kg Carprofen was provided in drinking water two days before surgery and continued until two days after surgery. In addition, 1h prior to surgery 5mg/kg Carprofen was injected subcutaneously. To allow for the inflammation derived from the surgery to resolve, mice were housed for further 70 days without intervention. β -glucan from barley (Sigma-Aldrich, St. Louis, MO, USA) was administered intraperitoneally to 40 mice at day 0 and at day 3. PBS was used as a vehicle control. Animals were euthanised at day 7 or at day 7 plus 3 hours after intraperitoneal injection of 10 μ g *Escherichia coli* lipopolysaccharide (*E. coli* LPS; serotype 055:B5, Sigma-Aldrich). Sacrifice involved deep inhalation anaesthesia (5% isoflurane) followed by cervical dislocation. Two independent experiments were performed.

Peritoneal cell isolation. Peritoneal cells were collected by peritoneal lavage. Briefly, the inner skin lining of the peritoneal cavity was exposed and 5mL of ice-cold PBS was injected into the cavity using a 27g needle. The peritoneum was gently massaged to dislodge attached cells. The PBS solution was then collected avoiding organs and fat tissue. Cells were kept on ice in PBS 3% FBS (Hyclone, Logan, UT, USA).

Splenocyte isolation. Single-cell suspensions from spleen were prepared using previously described methods [9]. Briefly, spleens were cut and incubated in RPMI with 300 U/mL of grade II DNase I (Roche, Basal, Switzerland) and 0,2 mg/mL Liberase TM (Roche) for 20

min at 37 °C with intermittent shaking. Digested tissue was gently squeezed in a sterile 100µm cell strainer (Fisher Scientific, Waltham, MA, USA). Red blood cells were lysed with a solution of 155mM NH₄Cl and 10mM KHCO₃.

Flow cytometry analysis. Lysed EDTA-anticoagulated peripheral blood and 10⁶ splenocytes were incubated with Fc block anti-CD16/32 (Biolegend, San Diego, CA, USA) before the addition of the antibody mix. Fluorophore-labelled anti-mouse antibodies against CD19-PE (Becton Dickinson, Franklin Lakes, NJ, USA), Ly6G-PE (Becton Dickinson), CD49b-PE (Biolegend), CD90.2-PE (Becton Dickinson), CD11b-PE-Cy7 (Becton Dickinson), CD11c-APC, Cy7 (Becton Dickinson), Ly6C-APC (Becton Dickinson), MHCII-BB515 (Becton Dickinson), CD64-BV421 (Biolegend) were diluted to appropriate working concentrations as provided by the manufacturer. Live/Dead fixable aqua stain (Invitrogen, Waltham, MA, USA) was added. Samples were measured in a BD LSR II Flow cytometer. Analysis was performed in FlowJo V10. The gating strategy [9] can be found in **Figure S1**.

Ex-vivo immune cell stimulation. 0.5x10⁶ splenocytes or 0.5x10⁶ peritoneal cells were seeded per well in round or flat bottom 96well plates, respectively, in RPMI 1640 Dutch modified (Invitrogen, Waltham, MA, USA) 10% FCS (Hyclone), 5 µg/mL gentamicin (Centraform, Etten-Leur, The Netherlands), 2mM L-glutamine (Gibco, Waltham, MA, USA) and 1mM pyruvate (Gibco). Peritoneal cells were left to adhere for 2h and then washed. Cells were stimulated with 10ng/mL LPS (*E. coli* LPS; serotype 055:B5 Sigma-Aldrich) or 10µg/mL Pam3Cys-SKKKK (EMC microcollections, Tübingen, Germany).

Measurement of cytokines. Cytokine production was determined in conditioned media and serum using IL-10, KC, TNFα and IL-6 ELISA kits (DuoSet R&D Systems, Minneapolis, MN, USA). In mouse plasma samples, M-CSF and GM-CSF were measured by Milliplex Luminex assay (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed in Graph Prism 6 (USA). Data are presented as mean ± SD and analysed using a two tailed Mann Whitney test, or a two-way ANOVA test followed by Sidak's multiple comparisons test as indicated. A p-value below 0.05 was considered statistically significant (*p < 0.05).

Results and Discussion

To determine if the spleen plays a role in the establishment of trained immunity, mice were sham-operated or splenectomized, and subsequently given two doses of β -glucan that has been previously shown to induce trained immunity [10]. Seven days after the first β -glucan injection, an *in vivo* restimulation with a non-related stimulus, lipopolysaccharide (LPS), was administered intraperitoneally (**Figure 1A**). In addition, splenocytes and peritoneal cells were isolated from control and β -glucan trained mice and restimulated *ex vivo*. The differences in the production of the pro-inflammatory cytokines TNF α and IL-6 by splenocytes collected from trained mice and *ex vivo* stimulated with the TLR2 agonist Pam3Cys did not reach statistical significance when compared to splenocytes isolated from PBS treated mice (**Figure 1B**). *Ex vivo* stimulation with Pam3Cys of *in vivo* trained peritoneal cells derived from sham-operated mice did not produce a significantly increase in TNF α concentrations, whereas the trained splenectomized group secreted more TNF α than the splenectomized control group. Reversely, IL-6 production in β -glucan exposed sham-operated mice was increased, while IL-6 levels were not significantly different between the splenectomized groups (**Figure 1C**). TLR4 stimulation with LPS revealed an increased production of TNF α by peritoneal cells isolated from β -glucan treated mice, independently of splenectomy, while IL-6 levels were not modulated (**Figure 1C**). Thus, peritoneal cells of β -glucan treated mice generally demonstrate an increased responsiveness to stimulation, characteristic for trained cells. However, removal of the spleen did not consistently alter this enhanced responsiveness. Likewise, splenectomized and sham-operated mice show similar plasma IL-10, IL-6 and TNF α concentrations 3h after LPS intraperitoneal injection (**Figure 1D**). However, contrary to what was previously reported [2, 11], β -glucan exposed mice do not exhibit increased cytokine levels upon LPS injection. Differences in mice sub-strains and housing conditions play a role in immune responses, but possibly more impactful is the surgical intervention we performed. Unlike previous studies, mice underwent sham or splenectomy operations, also including anaesthesia and analgesia, which might have influenced systemic immune responses and account for the disparity in this parameter. Taken together, these data indicate that the spleen or spleen-derived myeloid cells do not modulate the capacity to mount a cytokine response against heterologous restimulation, particularly at a cellular level.

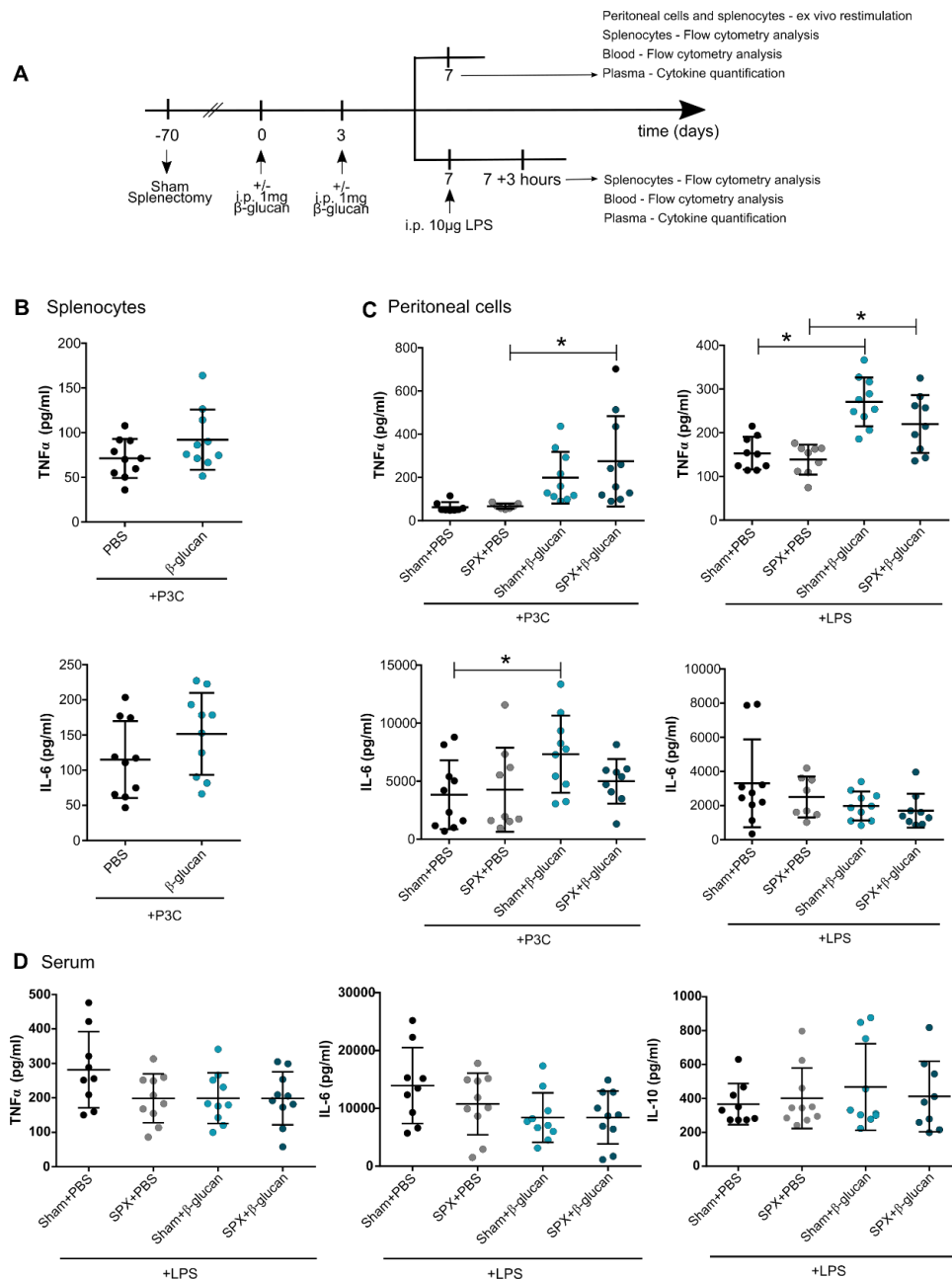


Figure 1 The effect of the spleen on the long-term cytokine production (A) Schematic representation of the *in vivo* training model. (B) splenocytes and (C) peritoneal cells were isolated 7 days after β -glucan administration. TNF- α and IL-6 secretion was determined after *in vitro* restimulation with LPS or/and P3C. TNF- α and IL-6 production by splenocytes restimulated by LPS were below the detection limit (* $p<0.05$, two-way ANOVA, Sidak's multiple comparisons test) (D) IL-6, TNF α and IL-10 measured in mice plasma 3h after LPS intraperitoneal injection. $n=10$ pooled from two independent experiments, mean $\pm$ SD. Grubbs' test was applied for outlier exclusion. (i.p. intraperitoneal, LPS lipopolysaccharide, P3C Pam3Cys-SK4KKK, SPX splenectomy).

Another important component of the trained immunity response is represented by the skewing of the hematopoietic stem cells towards myelopoiesis and granulopoiesis, giving rise to peripheral trained myeloid cells [4, 5]. This bias is associated with a granulocyte-macrophage colony-stimulating factor (GM-CSF) signalling in the bone marrow [4]. Granulocyte colony-stimulating factor (G-CSF) levels were not altered. In contrast, if we account for the mice that showed GM-CSF concentrations below the detection limit, there is a trend for an increase of GM-CSF concentrations in the circulation of β -glucan trained mice. However, GM-CSF concentration does not appear to be significantly influenced by splenectomy (**Figure 2**).

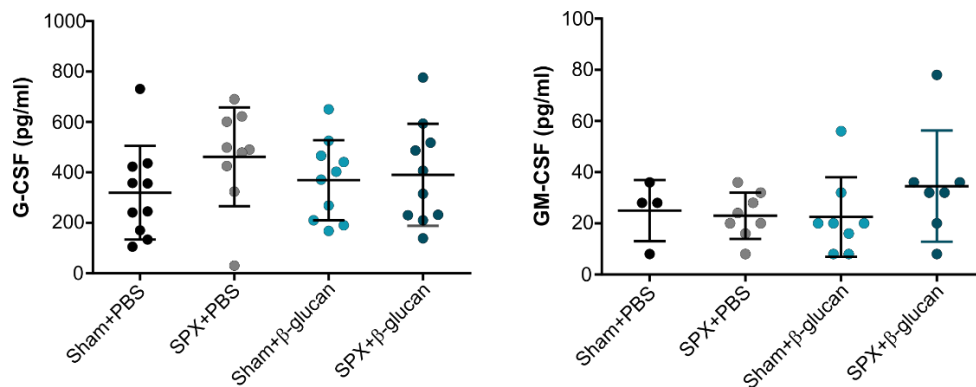


Figure 2 The impact of splenectomy on G-CSF and GM-CSF plasma concentrations. Levels of G-CSF and GM-CSF were determined in the serum of mice 7 days after β -glucan administration (n=10 pooled from two independent experiments). GM-CSF quantification values below the limit of detection: Sham+PBS n= 6, SPX+PBS n=1, Sham+ β -glucan n=2, SPX+ β -glucan n=3. (SPX splenectomy)

The hematopoietic bias that contributes to systemic trained immunity is reflected in the populations represented in the peripheral blood. Thus, we assessed by flow cytometry (**Figure S1**) whether β -glucan exposure modulates splenic and peripheral blood immune cell populations, and whether the absence of the spleen influences these changes. Our analysis was focused on cells of the innate immune system, but cells of the adaptive immune system, such as B and T cells were not influenced by splenectomy (**Figure 3 and 4**). Splenic cell populations in the β -glucan exposed mice show a trend for an increased percentage of the pro-inflammatory Ly6C^{hi} monocytes, especially after *in vivo* LPS administration (**Figure 3A and 5Ai**). The percentage of peripheral blood Ly6C^{hi} monocytes was also increased in the β -glucan treated group, but only significantly in the splenectomized groups (**Figure 5Bi**). In contrast, the more homeostatic Ly6C^{low} monocytic population was not modulated by β -glucan either in the spleen or in peripheral blood (**Figure**

5Aii and 5Bii). In peripheral blood, LPS *in vivo* administration abrogates the distinct Ly6C^{hi} monocytes percentages induced by β -glucan training (**Figure 4B and 5Bi**).

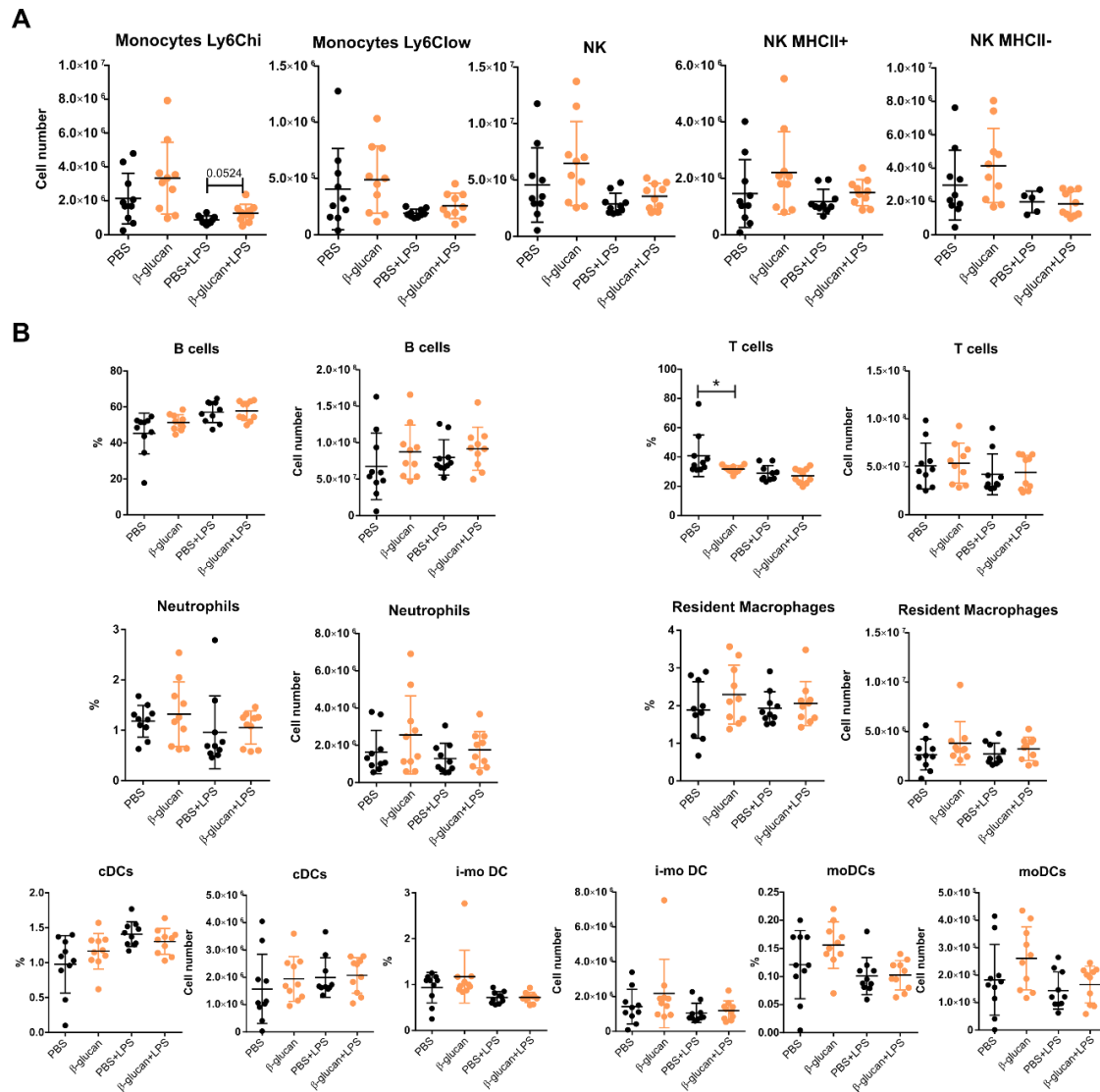


Figure 3 Splenocyte populations 7 days after β -glucan administration before and 3h after LPS injection. (A) Normalised for cell number and (B) percentage and cell number (n=10 pooled from two independent experiments, mean $\pm$ SD, *p<0.05 Mann Whitney test). (LPS lipopolysaccharide).

The spleen is a reservoir for undifferentiated monocytes [7], but it does not seem to replenish the pool of circulating monocytes during trained immunity. However, monocytes have the potential to differentiate into dendritic cells during inflammation [12]. The

monocyte-derived dendritic cells (moDCs) and immature moDCs (i-moDCs), characterized by decreased ability in antigen presentation, are not increased in β -glucan exposed mice (**Figure 5Biii and 5Biv**). However, trained splenectomized mice show an enhanced percentage of professional antigen-presenting cells conventional DCs (cDCs), while β -glucan exposure alone produces no modulation (**Figure 5Bv**).

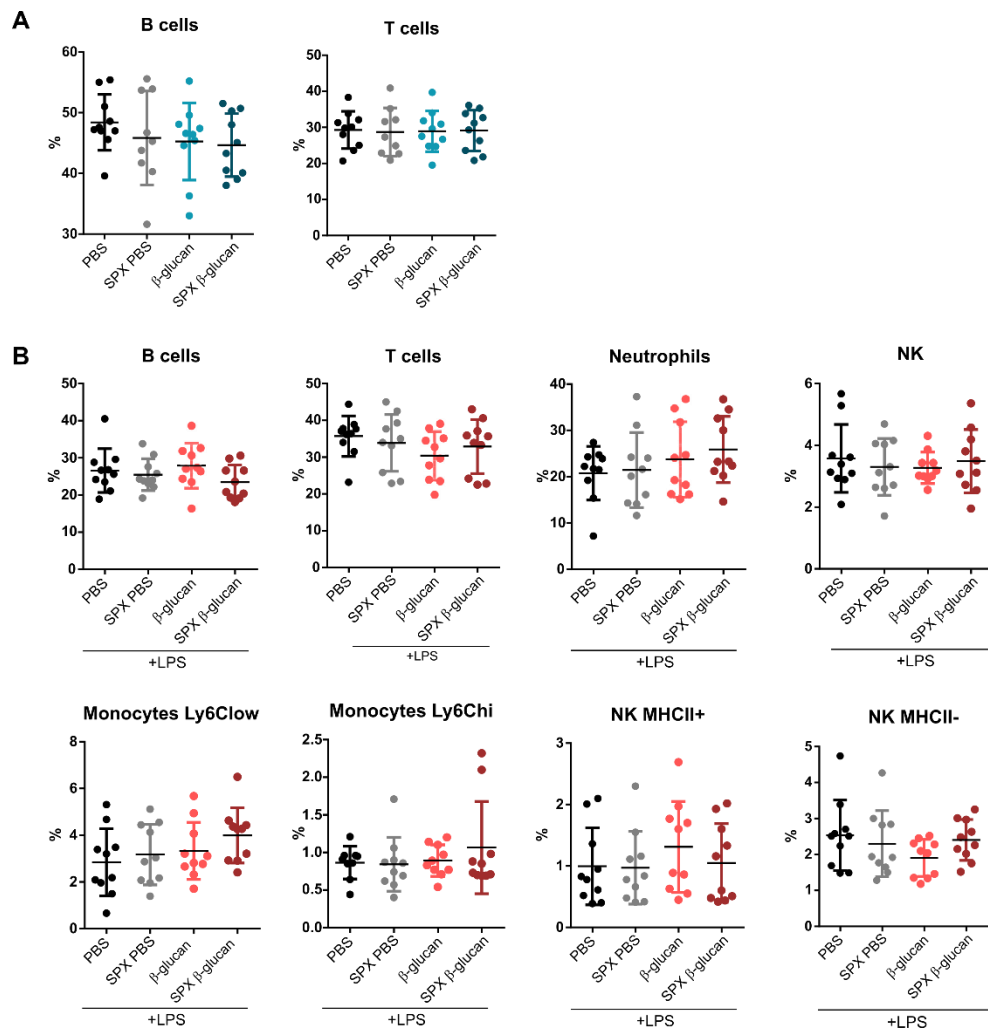


Figure 4 Peripheral blood populations. (A) Percentages found 7 days after β -glucan and (B) 3h after LPS challenge (n=10 pooled from two independent experiments, mean $\pm$ SD). (LPS lipopolysaccharide, SPX splenectomy)

However, the DCs populations assessed were not modulated in the spleen of trained mice (**Figure 3B**). The *in vivo* origins and differentiation of DCs are controversial but *in vitro*, DCs can be generated from monocytic precursors exposed to exogenous GM-CSF and type I interferons, both highly secreted by activated natural killer (NK) cells. DCs have been shown

to participate in a crosstalk with NK cells, where NK cells promote DC maturation while DCs in turn potentiate NK cell activation [13][14]. In addition, innate immune memory and adaptive immune features are also attributed to NK cells [15, 16].

As previously observed in BCG vaccinated individuals [15], peripheral NK cell percentage was not modulated by β -glucan administration (**Figure 5Bvi**). However, the more pro-inflammatory NK MHCII⁺ subpopulation was significantly increased in trained animals, but this increase was not dependent on the spleen (**Figure 5Bvii**). In accordance, splenic NK populations tend to be increased in trained mice and are significantly enhanced after β -glucan and LPS stimulation (**Figure 5Aiii**). This augmented splenic NK percentage is likewise reflected in the increase of the NK MHCII⁺ subpopulation (**Figure 5Aiv**), while the NK MHC⁻ population is not modulated after LPS (**Figure 5Av**). In peripheral blood, β -glucan exposure and spleen absence do not modulate the percentage of NK cells MHC⁻ population (**Figure 5Bviii**), and these cells are also not changed in the peripheral blood of LPS restimulated mice (**Figure 5C**). Overall, β -glucan training increases the systemic percentages of pro-inflammatory cells such as Ly6C^{hi} monocytes and NK MHC⁺ in a spleen independent manner. In contrast, cDCs appear to be significantly overrepresented in trained animals only in the absence of the spleen, highlighting a possible role of this organ on DC differentiation during trained immunity. The increase in DC population in the blood of trained splenectomized mice could also point to the spleen as a site for inhibition of DC differentiation. For example, in a mouse model of autoimmune encephalomyelitis, spleen removal has been shown to prevent the induction of tolerance. This was attributed to the decrease of polymorphonuclear myeloid-derived suppressor cells, shown to be derived from the spleen [17]. Another innate immune cell population that has been implicated in trained immunity are the neutrophils. A cohort of BCG vaccinated new-born infants exhibited an increase in neutrophil counts in comparison to non-vaccinated children [6], and in adults the function of neutrophils is increased after induction of trained immunity by BCG vaccination [18]. In accordance with the previously described trained immunity enhanced granulopoiesis [4], the percentage of neutrophils is increased in the peripheral blood of β -glucan-treated mice (**Figure 5Bix**). Interestingly, β -glucan-induced neutrophilia was reduced in splenectomized trained mice, suggesting a role for the spleen in this process.

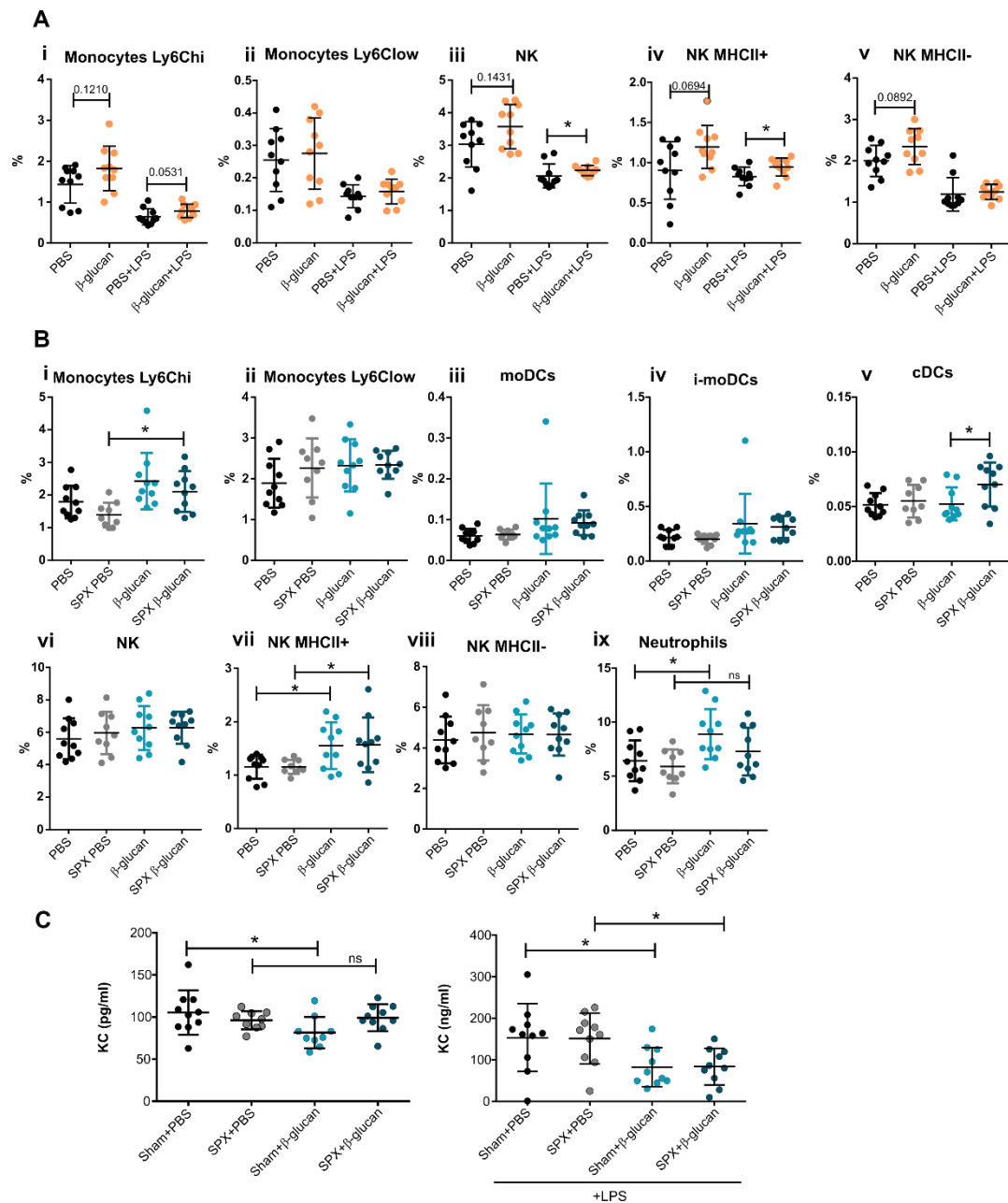


Figure 5 Training effect of β -glucan percentage on splenic and peripheral blood immune cell populations. (A) splenocyte populations were assessed 7 days after β -glucan administration before and 3h after LPS injection. Peripheral blood populations were evaluated (B) 7 days after β -glucan administration (two-way ANOVA, Sidak's multiple comparisons test). $n=10$ pooled from two independent experiments. (C) KC concentration in mouse serum 7 days after β -glucan administration with and without 3h LPS restimulation. Outliers were excluded with Grubbs' test ($n=10$ pooled from two independent experiments). (mean $\pm$ SD. * $p<0.05$, (A) Mann Whitney test, (B,C) Two-way ANOVA, Sidak's multiple comparisons test.) (LPS lipopolysaccharide, SPX splenectomy, ns non-significant).

However, the percentage of neutrophils in the spleen of β -glucan treated mice is not changed (**Figure 3B**), arguing against a direct role of the spleen as a reservoir of neutrophils from which they are mobilized after stimulation. Alternatively, the spleen may influence neutrophilia by modulating bone marrow granulopoiesis. Future studies are warranted to investigate these possibilities and the impact of the spleen on the function of neutrophils during induction of trained immunity. Keratinocyte cell–derived chemokine (KC), also known as CXC chemokine ligand 1 (CXCL1), is a potent neutrophil chemoattractant. Contrary to the increase in circulating neutrophils, serum of β -glucan exposed mice has significant decreased KC concentrations (**Figure 5C**). Circulating neutrophils and KC levels of splenectomized trained mice are similar to splenectomized control mice. Upon LPS restimulation, KC concentration of trained animals is decreased, independently of the absence of spleen. These results reveal that the increase in neutrophil percentages is not correlated by an increase in recruitment to the circulation by KC, and that the decrease in circulating KC concentration depends on the presence of the spleen.

In conclusion, *in vivo* induction of trained immunity by β -glucan enhances the pro-inflammatory response of splenocytes and peritoneal cells. β -glucan exposure tends to augment circulatory GM-CSF concentrations, that might contribute to an increase in myelopoiesis and granulopoieses. However, the induction of GM-CSF is exerted in a spleen-independent manner, and splenectomized mice build a largely intact trained immunity response. Ultimately, trained animals present an increase in pro-inflammatory circulatory monocytes and NK cells, which does not require splenic involvement.

Our study has potential limitations. The use of a mouse model does not fully mirror human immune responses, spleen microanatomy and its systemic role [19]. Also, our model with spleen removal prior to β -glucan exposure allows for the evaluation of the role of the spleen on the establishment and maintenance of trained immunity, but it does not permit to evaluate its contribution solely to the long-term maintenance of trained immunity. In this regard, HSPC populations were not explored in this study. However, the spleen does not appear to play a crucial role in β -glucan-induced central trained immunity, since circulating immune populations do not strongly differ in the absence of the spleen. These findings might be translated to patients who undergo splenectomy, suggesting that the establishment of innate immune memory responses will not be generally compromised.

However, here we show that a feature linked to trained immunity that was influenced by splenectomy was neutrophilia, pointing to a possible regulatory role of spleen in this process. This possible novel role of the spleen in the regulation of circulating immune cell populations gives new insights into the systemic induction of memory. Of note, trained immunity induced activation of neutrophils has been recently shown to have an important role in the anti-tumoral effects of β -glucan [20]. This unveils a possible clinical implication of the spleen in the establishment of trained immunity derived neutrophilia, which advocates further exploration.

Disclosures. M.G.N. is a scientific founder of TTxD. All other authors declare no conflicts of interest.

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Supplemental Figures

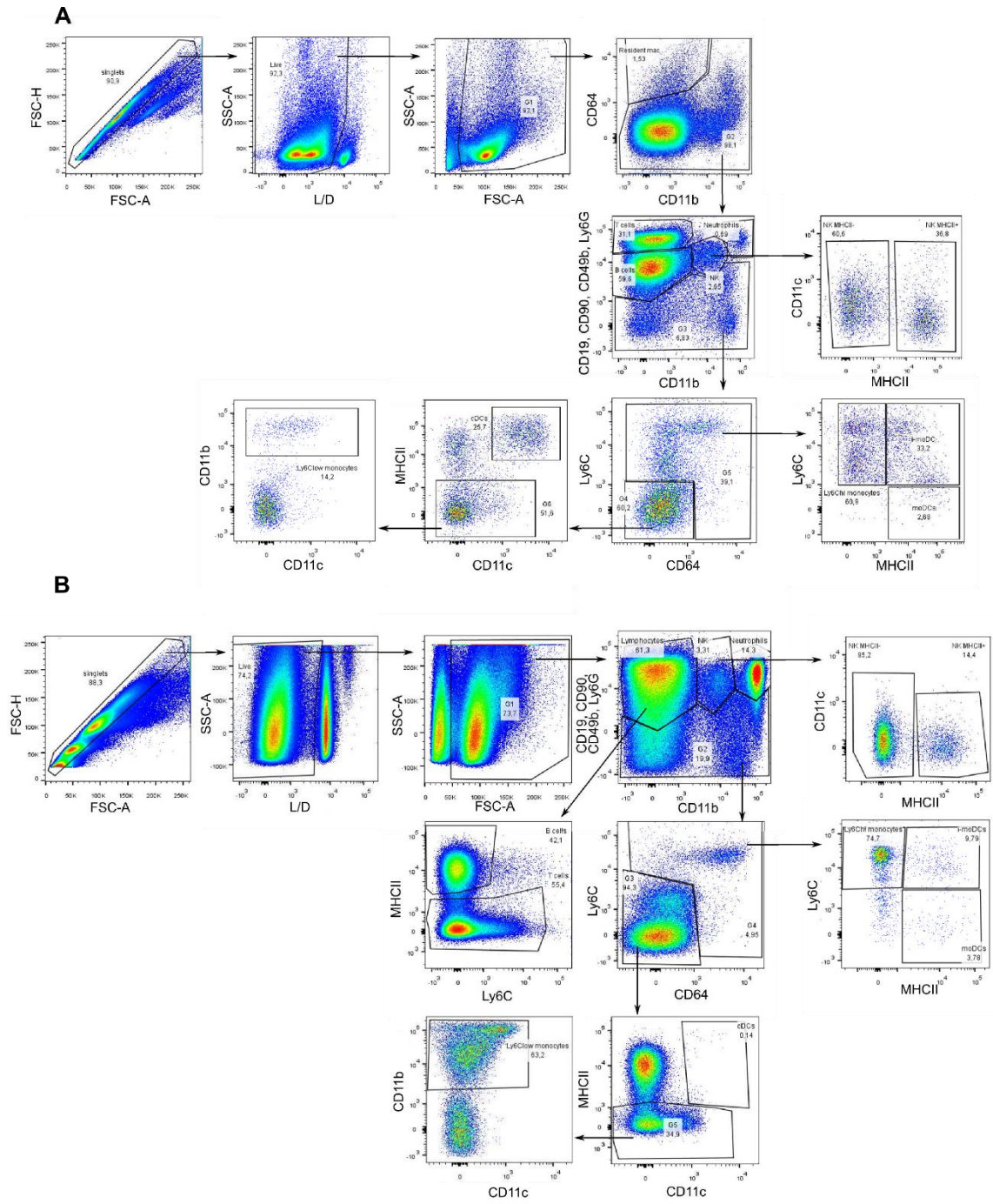


Figure S1 Gating strategies. Applied for analysis of (A) splenic and (B) peripheral blood populations

CHAPTER 5.1

[EDITORIAL]

ITACONATE AS AN IMMUNE MODULATOR

Anaísa V Ferreira, Mihai G. Netea, Jorge Domínguez-Andrés

Aging (Albany NY). 2019; 11(12): 3898–3899.

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The immune system has been implicated in a myriad of different contexts, such as cancer, neurological disorders and aging. Tissue homeostasis is maintained through multiple regulatory mechanisms, among which the immune system. Deregulation of immune responses can in turn lead to tissue pathology and disease. Thus, understanding the mechanisms that regulate immune responses has been a focus of different fields within the scientific community. In recent years, an intriguing metabolite has been explored for its broad immunomodulatory properties, itaconate.

Itaconate has long been manufactured from the fungi *Aspergillus* species and used as a multi-functional industrial biomaterial. However, the last years have uncovered novel facets of itaconate in mammalian systems. Activated immune cells, chiefly of the myeloid lineage, have an increased expression of the immune-responsive gene 1 (IRG1) mitochondrial enzyme, which catalyses the decarboxylation of the Krebs cycle intermediate cis-aconitate to itaconate. On one hand, itaconate has direct antibacterial properties, since it can inhibit bacterial growth in glucose-poor conditions. Notably, some bacteria can degrade itaconate to pyruvate and acetyl-CoA and thus survive in the eukaryotic host. These detoxifying pathways could also confer advantage against itaconate-producing fungi. On the other hand, in inflammatory conditions, the rising levels of itaconate have an extremely relevant role in keeping in check the inflammatory response. Lipopolysaccharides and type I interferons induce increased IRG1 expression and the production of the latter is limited by itaconate, indicating a regulatory negative feedback loop [1].

The molecular mechanisms under control by itaconate vary, but the net function is considered to be anti-inflammatory. Itaconate acts as a competitive succinate dehydrogenase (SDH) inhibitor, impairing the electron transport chain (ETC) with a concomitant succinate accumulation [2]. SDH is both an enzyme of the Krebs cycle, oxidizing succinate to fumarate, and the component of complex II of the ETC, feeding electrons derived from succinate oxidation to the ubiquinone pool. Itaconate has also been shown to inhibit fructose-6-phosphate 2-kinase and thereby possibly blocks the increased glycolysis of pro-inflammatory macrophages. Itaconate reacts with thiols, forming adducts with glutathione, the major cellular antioxidant, and producing higher levels of reactive oxygen species (ROS) [3]. The antioxidant Nrf2 pathway is then activated in the presence of itaconate, possibly indirectly due to the increased ROS production, but also due to direct alkylation of cysteine residues of the Nrf2 inhibitor Keap1 [1]. Ultimately, there is enhanced

expression of antioxidant and a decreased expression of pro-inflammatory genes, due to the induction of Nrf2 and the transcription factor ATF3 [3], respectively. Interestingly, it has been suggested that itaconate increases the level of MHC class I in macrophages. However, its influence on antigen presentation remains to be explored. In addition, itaconate could conceptually regulate gene expression through epigenetic alterations. It sequesters glutathione, thereby possibly influencing histone glutathionylation and the levels of the methylation substrate S-adenosyl methionine (SAM). Moreover, an intermediate of itaconate catabolism has been negatively implicated in vitamin B12 metabolism [4]. The reduced levels of B12 affects methionine and folate cycles, relevant for not only glutathione synthesis and redox homeostasis but also for SAM levels and histone and DNA methylation.

The immunosuppressive effects of itaconate have been explored in various disease models, such as cardiac ischemia reperfusion injury, psoriasis, cancer and infection. In *in vivo* mouse models, intravenous administration of itaconate reduces myocardial infarct size [2], while the induction of psoriasis with itaconate treatment prevented the typical scaling and edema of the skin [3]. In a peritoneal tumor mouse model, high itaconate levels in tumor associated macrophages promote tumor progression. Itaconate plays important roles during infection, decreasing tissue injury in a *Mycobacterium tuberculosis* infection mouse model [5] and increasing macrophage bactericidal activity in a zebrafish model. Interestingly, itaconate altered neuronal metabolism to suppress viral replication in a mouse model of Zika virus infection [6], indicating that the modulatory effects of itaconate are not curtailed to cells of the myeloid lineage.

Itaconate could also be a possible modulator of maladaptive immune programs. In sepsis, the exacerbated inflammatory response to infection is counteracted by the induction of tolerance, which can lead to immune paralysis and an increased susceptibility to secondary infections. Monocyte immune tolerance is promoted by itaconate, which decreases the production of pro-inflammatory mediators. Heme oxygenase 1, a cytoprotective enzyme that limits inflammation, is expressed during tolerance and has also been shown to regulate IRG1 expression. In contrast, the immune system can be hyperactivated. Particularly, the innate immune system can be primed to exhibit an increased responsiveness upon a second non-related insult, mirroring an immunologic memory which was coined trained immunity [7]. One stimulus that can exert trained immunity in monocytes is the *C. albicans* cell wall component β -glucan. It has been recently described by our group that β -

glucan-induced training can prevent endotoxin tolerance [8]. Exposure to β -glucan inhibits the increased IRG1 expression and thus preserves a functional SDH pathway and the integrity of the Krebs cycle. Interestingly, exposure to itaconate can also inhibit the induction of β -glucan trained immunity. In this model, itaconate is also considered to have anti-inflammatory effects, regulating tolerance positively while decreasing trained immunity.

In conclusion, itaconate metabolism could be both a possible therapeutic target and a tool in inflammatory and autoimmune diseases with altered immune responses. Further exploration of the molecular, metabolic and epigenetic changes induced by itaconate in different cell types and disease models will be of great importance, in order to better establish the potential of the modulation of this promising metabolite and open new therapeutic avenues

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CHAPTER 5.2

[RESEARCH PAPER]

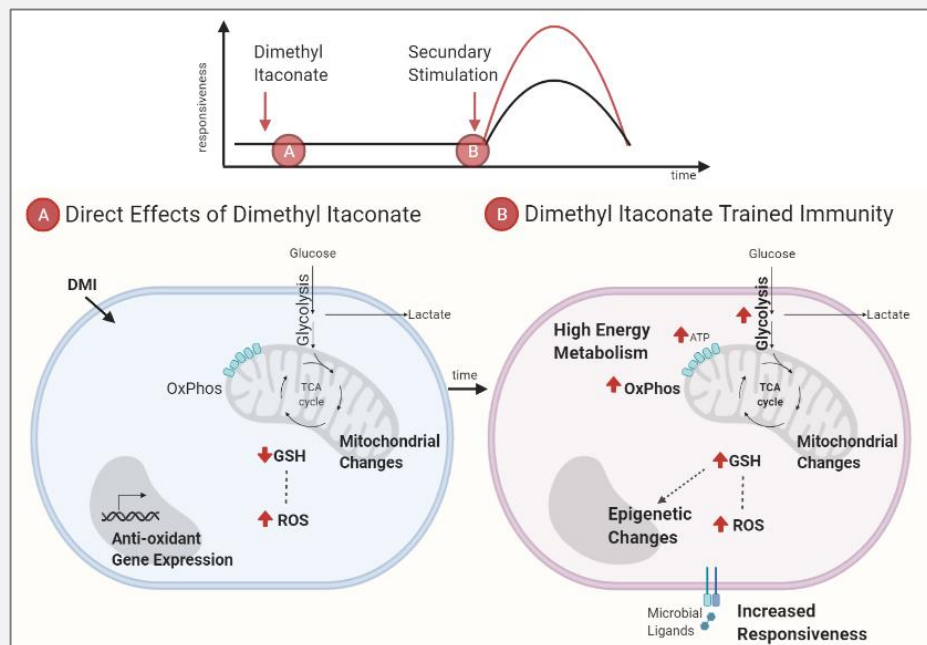
ITACONATE AND ITS DERIVATIVES INDUCE LONG-TERM INNATE IMMUNE RESPONSES AND CONFER PROTECTION AGAINST INFECTIONS THROUGH TRAINED IMMUNITY

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In preparation

Abstract

Itaconate is an immunomodulatory metabolite produced by immune cells under microbial stimulation and pro-inflammatory conditions. Itaconate and its derivatives has antioxidant and anti-inflammatory effects in distinct disease models. Here, we show that dimethyl itaconate (DMI), a derivative of itaconate previously linked to suppression of inflammation and widely employed as an alternative to the endogenous metabolite, can induce long-term epigenetic and metabolic changes in human monocytes, characteristic of trained immunity. Treatment of monocytes with DMI altered their mitochondrial activity and cellular redox state, while increasing responsiveness to stimulation with microbial ligands. Mice trained with DMI present a trend for increased survival to infection with *Staphylococcus aureus*. Additionally, itaconate levels in human plasma correlate with enhanced *ex vivo* pro-inflammatory cytokine production. Collectively, these findings demonstrate that itaconate and its derivatives display both short-term anti-inflammatory characteristics and the capacity to induce long-term trained immunity. This pro-and anti-inflammatory dichotomy of DMI is likely to induce complex immune responses and should be contemplated when considering itaconate and its derivatives in a therapeutic context.



Introduction

Itaconate is an intriguing metabolite that has been recently explored for its broad immunomodulatory properties in mammalian systems. Under pro-inflammatory conditions, activated immune cells increase the expression of the immune-responsive gene 1 (IRG1) enzyme, which decarboxylates the Krebs cycle intermediate cis-aconitate to itaconate [1]. Upregulating itaconate concentrations is an important metabolic feedback on inflammation [2, 3]. The transcription factor NRF2 is activated by itaconate, leading to the induction of the antioxidant response and a decrease in the expression of pro-inflammatory genes [4]. Itaconate also acts as a competitive succinate dehydrogenase (SDH) inhibitor, blocking the progression of the Krebs cycle from succinate to fumarate. Impairment of SDH, or complex II of the electron transport chain (ETC), compromises succinate oxidation and thus the transfer of electrons to the ubiquinone pool [2]. Itaconate also inhibits the glycolytic enzyme fructose-6-phosphate 2-kinase [5], while the derivative 4-octyl itaconate (OI) inhibits glyceraldehyde-3-phosphate dehydrogenase, and thereby blocks the increased glycolysis in pro-inflammatory macrophages [6].

The immunosuppressive effect of itaconate and its derivatives dimethyl itaconate (DMI) and OI have been described in various disease models, such as cardiac ischemia-reperfusion injury, psoriasis, and infection. In *in vivo* mouse models, DMI reduces myocardial infarct size [2], prevents the scaling and skin edema typically seen in psoriasis [4], and mice that lack *IRG1* show increased susceptibility to *Mycobacterium tuberculosis* infection due to enhanced pathological inflammatory responses [7]. Besides these anti-inflammatory properties, itaconate also presents direct antimicrobial activity by inhibiting isocitrate lyase, an enzyme found in different pathogenic microorganisms such as *M. tuberculosis*, *C. albicans* or *S. aureus* [1, 8, 9]. In contrast, *S. aureus* and *P. aeruginosa* have also adapted to itaconate-rich environments by using it as a carbon source for the production of biofilms, which are associated with chronic infections of these microorganisms [10, 11]. These properties make itaconate and its derivatives one of the most promising groups of molecules with therapeutic potential in pathologies characterized by exacerbated inflammatory responses [12]. The anti-inflammatory properties of itaconate have also been reported in trained immunity [13]. The innate immune system, most notably monocytes, can exhibit an increased responsiveness to a second unrelated stimulus following an earlier immune

insult, a *de facto* innate immune memory [14]. In this regard, treatment with DMI is able to dampen the induction of trained immunity in human monocytes stimulated with β -glucan, a cell-wall component of *C. albicans* [13].

These previous studies investigated the effects of itaconate and its derivatives in an established inflammatory context. In this work however we demonstrate that the anti-inflammatory effects of itaconate and its derivatives are context-dependent and that DMI, in the absence of other stimuli, is also able to trigger trained immunity, highlighting the dual role of DMI as a molecule with anti- and pro-inflammatory characteristics.

Materials and Methods

Isolation of human peripheral blood mononuclear cells (PBMC) and monocytes. Buffy coats from healthy donors were obtained after written informed consent (Sanquin Blood Bank, Nijmegen, the Netherlands). Samples were anonymized to safeguard donor privacy. PBMC isolation was performed by differential density centrifugation over Ficoll-Paque (GE Healthcare). Subsequently, isolation of monocytes was performed with a hyper-osmotic Percoll (Sigma) density gradient centrifugation and washed once with pyrogen-free cold phosphate buffered saline (PBS). Cells were resuspended in RPMI medium Dutch modified (Invitrogen) supplemented with 5µg/mL gentamicin (Centraform), 2mM Glutamax (Gibco), and 1mM pyruvate (Gibco). In order to ensure maximal purity, percoll-isolated monocytes were left to adhere to polystyrene flat bottom plates (Corning) for 1h at 37°C 5% CO₂ and then washed once with warm PBS.

In vitro trained immunity model. 10⁵ monocytes were seeded on flat-bottom 96-well plates (Greiner) and incubated, in RPMI with 10% human pooled serum, with 5mM itaconate (Sigma), 100µM 4-octyl itaconate (kindly provided by Richard C. Hartley, University of Glasgow, UK), 250µM dimethyl itaconate (Sigma), 250µM methyl-fumarate (Sigma), 1µg/mL β-glucan for 24h at 37°C. β-1,3-(D)-glucan (β-glucan) from *Candida albicans* was kindly provided by Professor David Williams (College of Medicine, Johnson City, USA). In some experiments, 0.5mM 5'-deoxy-5'-methylthioadenosine (MTA) (Sigma) 0.5µM diphenyleneiodonium (DPI) (Sigma), 50µM α-tocopherol (AT) (Sigma) or 1mM N-acetyl cysteine (NAC) (Sigma), 500µM MitoTempo (MT) (Sigma), 25µM DL-buthionine sulfoximine (BSO) (Sigma), were added to the cells 1h prior to 250µM DMI. After 24h, conditioned media was collected, cells were washed once with warm PBS and left to differentiate in RPMI supplemented with 10% pooled human serum for 5 days, with one medium refreshment at day 3 of culture. At day 6, conditioned media was collected, cells were restimulated with 10ng/mL *Escherichia coli* lipopolysaccharide (LPS; serotype 055:B5, Sigma) for an additional 24h, after which conditioned media was stored at -20°C. Cytotoxicity was determined by measuring lactate dehydrogenase in the conditioned media after 24h incubation according to the manufacturer's instructions (CytoTox96, Promega).

Cytokine quantification. Cytokine production was determined using commercial ELISA kits IL-1 β , IL-6, TNF α , IL-10 and IL-1Ra (R&D Systems) following the instructions of the manufacturer.

Chromatin immunoprecipitation (ChIP). Monocytes cultured for 6 days were fixed with 1% formaldehyde (Sigma) and sonicated. 1 μ g of fixed and sonicated chromatin was immunoprecipitated using 1mg of H3K4me3 or H3K9me3 Antibodies (Diagenode). Chromatin was purified with Qiaquick MinElute PCR purification Kit (Qiagen). Data was expressed as percentage of input samples. Primer pairs employed for quantitative PCR were:

Gene.#	Forward (5'-3')	Reverse (5'-3')
<i>IL6.1</i>	TCGTGCATGACTTCAGCTTT	GCGCTAAGAAGCAGAACCAC
<i>IL6.2</i>	AGGGAGAGCCAGAACACAGA	GAGTTTCCTCTGACTCCATCG
<i>TNFA.1</i>	CAGGCAGGTTCTCTTCCTCT	GCTTTCAGTGCTCATGGTGT
<i>TNFA.2</i>	GTGCTTGTTCTCAGCCTCT	ATCACTCCAAAGTGCAGCAG

Seahorse XFb metabolic flux analysis. 10⁷ monocytes were cultured in 10 cm Petri dishes (Greiner) and trained with DMI as described above. At day 6 of culture, cells were detached with Versene solution (ThermoFisher Scientific), and 10⁵ cells were seeded in cartridges in DMEM with 0.6mM glutamine, 5 mM glucose, and 1 mM pyruvate, pH 7.4. Cells were left to rest in a non-CO₂-corrected incubator at 37°C for 1h. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a Cell Mito Stress Kit in an XFp Analyzer (Seahorse Bioscience), with final concentrations of 1 μ M oligomycin (Sigma-Aldrich), 1 μ M FCCP (Sigma-Aldrich), and 0.5 μ M rotenone/antimycin A (Sigma-Aldrich). OCR measurements were used to calculate ATP production (basal OCR - OCR after oligomycin), H⁺ leak (OCR after oligomycin – OCR after antimycin A/ Rotenone), and spare respiratory capacity (SRC) (OCR after FCCP – basal OCR).

Chronic granulomatous disease (CGD) patient samples. Blood was collected from age-matched healthy controls who did not suffer from infectious or inflammatory diseases and

from CGD patients harboring homozygous mutations in the NCF1 gene (p47-phox) or X-linked mutation in CYBB gene (Gp91-phox), in which severely impaired ROS production has been demonstrated. All participants gave written informed consent. Inclusion of healthy controls was approved by the local institutional review board (CMO region Arnhem-Nijmegen, #2299 2010/104). According to the Dutch law, approval by an ethics committee was not required since the blood samples were part of regular clinical care. All experiments were performed and conducted in accordance with good clinical practice and the Declaration of Helsinki. PBMCs and monocytes were isolated and cultured as aforementioned.

Lactate, glutathione, and ATP quantification. Lactate was quantified in day 6 conditioned media, that were deproteinized with perchloric acid. The oxidation of lactate produces H₂O₂ which through the action of horseradish peroxidase converts Amplex Red to the fluorescent resorufin (Thermo Fisher Scientific). To determine intracellular glutathione concentration, 10⁶ monocytes were lysed with 0.5% NP-40 in PBS at 5h and 6 days of culture and samples were deproteinized with 100% TCA and neutralized with 0.5M NaHCO₃, following the manufacturer instructions (Abcam). ATP levels were determined after macrophages were detached and incubated with or without 1μM oligomycin (Sigma-Aldrich) for 30min. The manufacturer's instructions were then followed (Sigma).

RNA isolation and RT-PCR. Total RNA was extracted by TRIzol (Life Technologies), according to the manufacturer's instructions, and quantified using a Nanodrop 2000 UV-visible spectrophotometer. cDNA was synthesized from 450ng-1μg RNA by iScript Reverse Transcriptase (Invitrogen). Relative expression was determined using SYBR Green (Invitrogen) on an Applied Biosciences StepOne PLUS qPCR machine. Fold changes in expression were calculated by the $\Delta\Delta C_t$ method using HPRT1 as an endogenous control. The following primers were used:

Gene	Forward (5'-3')	Reverse (5'-3')
<i>HPRT1</i>	GGATTTGAAATTCAGACAAGTTT	GCGATGTCAATAGGACTCCAG
<i>CAT</i>	TGGGATCTCGTTGGAAATAACAC	TCAGGACGTAGGCTCCAGAAG
<i>GCLC</i>	GCAGTGGTGGATGGTTGTGGCAAG	CCTTCCTTCCCATTTGATGATGGTGT

<i>GSR</i>	TTCCAGAATACCAACGTCAAAGG	GTTTTCGGCCAGCAGCTATTG
<i>HMOX</i>	GCAGTCAGGCAGAGGGTGATAGAAG	TGGTCCTTGGTGTCATGGGTCAG
<i>NQO1</i>	AGATGCTGACTGGCACTGGTGGTT	AATTGCAGTGAAGATGAAGGCAACA
<i>SOD</i>	GGTGGGCCAAAGGATGAAGAG	CCACAAGCCAAACGACTTCC
<i>TXN</i>	GTGAAGCAGATCGAGAGCAAG	CGTGGCTGAGAAGTCAACTACTA
<i>TXNDR1</i>	GGCTCTATGCAGGTTCCACTGTCAA	GCCACAAGCACCATATTCCAAAGG

Total ROS production quantification. Superoxide anion levels were evaluated using luminol-enhanced chemiluminescence and determined in a luminometer (Biotek Synergy HT). 10^5 monocytes were incubated with 250 μ M DMI, 100 μ M OI, or RPMI alone for 1h followed by the addition of opsonized zymosan (1mg/ml). Luminol (Sigma) was added to each well in order to start the chemiluminescence reaction. Each measurement was carried out in quadruplicates. Chemiluminescence was determined every 145s at 37°C for 1h. Luminescence was expressed as relative light units (RLU) per second and as area under the curve (AUC). The fluorescent probe H2DCFDA was also used to determine reactive oxygen species levels. Monocytes/macrophages were cultured for 2h, 24h, or 6 days, washed, detached with cold PBS, and incubated with 5 μ M H2DCFDA (Life Technologies) in PBS for 30min at 37°C. Cells were analyzed by flow cytometry (CytoFlex, Beckman Coulter).

Mitochondrial membrane potential and mitochondrial ROS measurements. Monocytes cultured for 24h or 6d were washed and detached with cold PBS. Approximately 500000 cells were stained in 250 μ L of 5 μ M MitoSOX Red (Invitrogen) in HBSS 1mM Ca, 100nM tetramethylrhodamine ethyl ester perchlorate (TMRE, Sigma) in RPMI for 30min at 37 °C and 5% CO₂. Dyes allow for the evaluation of mitochondrial ROS production and mitochondrial membrane polarization, respectively. As a control, 10 μ M carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone (FCCP) was added to TMRE stained cells. Cells were analyzed by flow cytometry (CytoFlex, Beckman Coulter).

Itaconate and its derivatives induce long-term innate immune responses and confer protection against infections through trained immunity

Flow cytometry analysis. Analysis was performed in CytoFlex (Beckman Coulter) and using Kaluza 2.1 software (Beckman Coulter). Cell aggregates were gated out based on the forward scatter (FSC)-height versus FSC-area plot, monocytes/macrophages were gated according to FSC and side scatter (SSC), or as a CD14⁺ population, when mentioned.

In vivo trained immunity model. Animal experiments were conducted in the Unit of Animals for Medical and Scientific purposes of the University General Hospital “Attikon” (Athens, Greece). All experiments were licensed from the Greek veterinary directorate under the protocol number 338087/27-05-2020. 40 C57BL/6 mice 8-10 years old were used. Animals were housed in groups of 5 animals per enriched type-II cage on a 12 h light-dark diurnal cycle with room temperature between 21 and 23 °C. Water and food were provided ad libitum. Mice received a single intra venous injection of 2×10^5 live *C. albicans* blastoconidia (UC820 strain) or/and daily intraperitoneal injections of 20mg DMI for 4 days. Pyrogen-free phosphate-buffered saline (PBS) injection was used as control alone. Seven days after the first injection, mice were infected intravenously with 5×10^6 cfu/mouse *Staphylococcus aureus*. Survival was monitored for 14 days.

BCG Cohort. Healthy adults were included between April 2017 and June 2018 as part of the 300BCG study, which was approved by Arnhem-Nijmegen Medical Ethical Committee (NL58553.091.16) and performed as previously described. Participants were vaccinated with a standard dose of 0.1mL BCG (Bulgaria strain, Intervax, Canada) intradermally in the left upper arm, and EDTA blood was collected before and 90 days after vaccination. 5×10^5 PBMCs were stimulated with 10^6 CFU/mL heat-killed *Staphylococcus aureus* at 37°C with 5% CO₂ for 24h. Metabolite levels of the same individuals were measured in plasma collected prior to BCG vaccination. The metabolic features were measured and annotated by General Metabolics (Zurich, Switzerland) using flow injection time-of-flight mass (flow-injection TOF-M) spectrometry [15]. Non-targeted metabolites were annotated according to the human metabolites database (HMDB). All metabolomic measurements were performed in duplicates, and the average value was calculated for each sample per metabolite. A regression analysis corrected for age and sex was for itaconate and ex vivo cytokine fold changes in relation to before vaccination responses.

Statistical analysis. Data are presented as mean $\pm$ SEM and analyzed using a Wilcoxon signed-rank test, Two-way ANOVA or Log-rank Mantel-Cox test when indicated. A p-value below 0.05 was considered statistically significant, as shown by asterisks (*p < 0.05).

Results and Discussion

Itaconate and its derivatives increase the responsiveness of human monocytes to secondary stimulation

In order to explore the possible role of itaconate and its derivatives as inducers of long-term inflammatory responses, we used an established *in vitro* model of trained immunity [16]. Human peripheral blood monocytes were exposed to itaconate, OI, or DMI for 24 hours and left to differentiate for the following 5 days in the absence of any stimulus, after which macrophages were restimulated with the TLR4 agonist lipopolysaccharide (LPS) for 24h (**Figure 1A**). Contrary to the previously described anti-inflammatory characteristics of itaconate, exposure to itaconate or OI led to increased amounts of IL-6 and TNF α upon restimulation, respectively (**Figure 1B, C**). DMI exposed macrophages (DMI-Mf) showed increased TNF α and IL-6 secretion after LPS restimulation, in a similar magnitude as the well-described inducer of trained immunity β -glucan (**Figure 1D; left panels**). DMI-Mf also showed enhanced TNF α and IL-6 production upon TLR2 activation with the agonist Pam₃Cys (P3C) (**Figure 1D; right panels**). Similarly, the Krebs cycle intermediate fumarate has previously been shown to induce trained immunity in monocytes [17], by increasing the production of IL-6 (**Figure 1E**). This pro-inflammatory feature of DMI was not observed at the level of cytokine production after acute stimulation. After exposing human monocytes to DMI for 24h, production of the pro-inflammatory cytokines IL-1 β , TNF α and IL-6 could not be detected, while the secretion of the anti-inflammatory cytokine IL-10 was reduced and IL-1Ra was increased (**Figure S1A**).

DMI induces epigenetic modulation in human monocytes

The long-term responsive phenotype in trained cells is underlined by epigenetic reprogramming. Trained cells exhibit changes in histone marks at promoter regions of pro-inflammatory genes to favor open chromatin and transcription, which promotes the enhanced responsiveness observed upon a non-related stimulation [18, 19]. DMI-Mf exhibited enriched deposition of the histone 3 trimethylation of lysine 4 (H3K4me3) mark and decreased deposition of the H3K9me3 mark at the promoter regions of *TNFA* and *IL6* (**Figure 1F**).

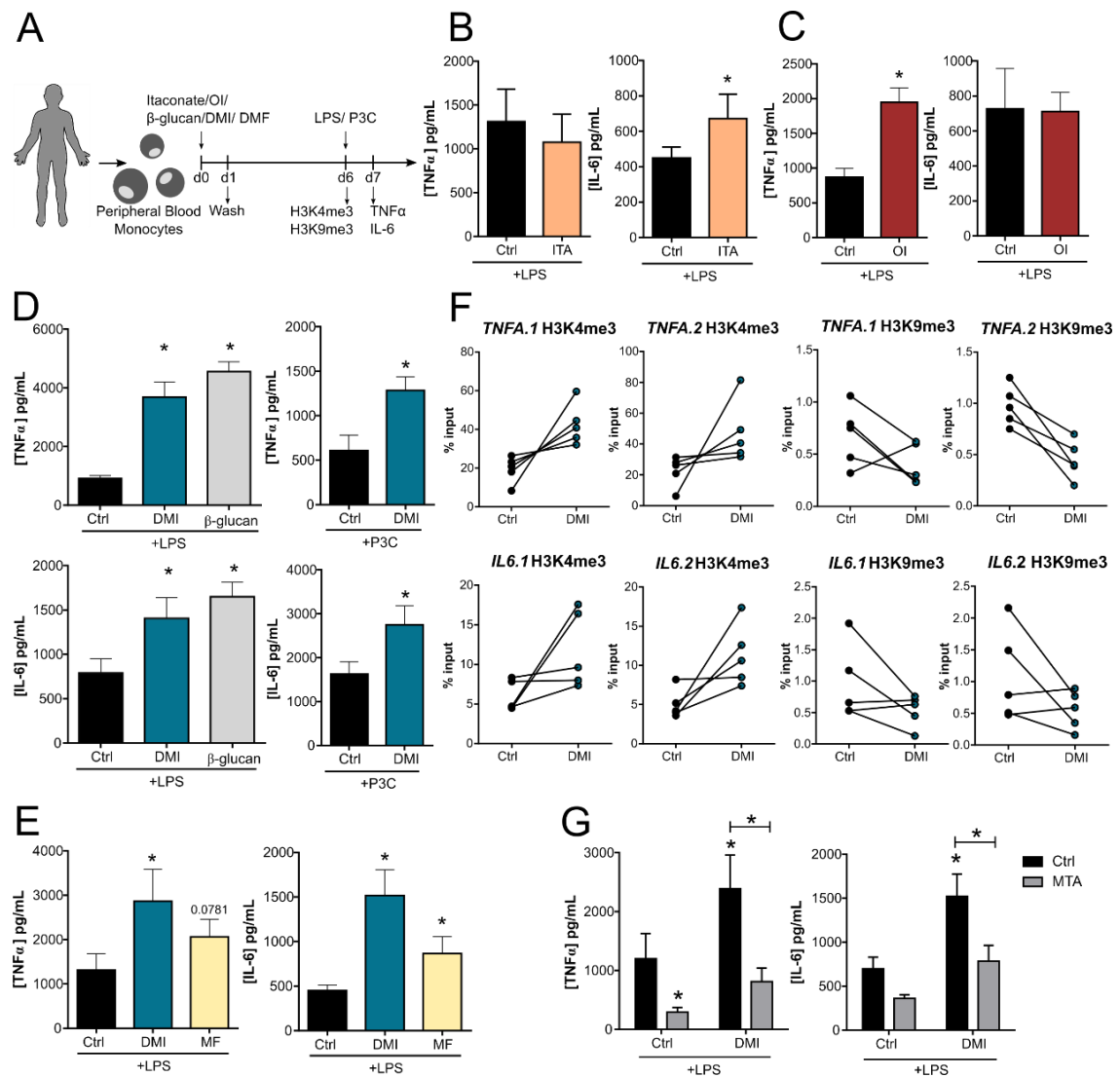


Figure 1 DMI increases TNFα and IL-6 production upon restimulation. (A) Schematic depiction of the *in vitro* training protocol. (B, C) TNFα and IL-6 production by monocytes exposed to 5mM itaconic acid (ITA) or 100μM 4-octyl itaconate (OI) for 24h and restimulated with 10ng/mL LPS 6 days later for another 24h (n=6-7 pooled from 2 independent experiments). (D) TNFα and IL-6 production by 250μM dimethyl itaconate (DMI) treated monocytes, stimulated 6 days later with 10ng/mL LPS or 10μg/mL P3C for another 24h (n=9-12 pooled from 3, 4 independent experiments). (E) TNFα and IL-6 production by monocytes exposed to 250μM DMI or 250μM methyl-fumarate (MF) for 24h and restimulated with 10ng/mL LPS 6 days later for another 24h (n=7 pooled from 2 independent experiments). (F) Deposition of H3K4me3 and H3K9me3 marks at the promoter sites of TNFA and IL6 of DMI exposed monocytes at 6 days of culture (n=5 pooled from 2 independent experiments). (G) TNFα and IL-6 production by monocytes treated with 0.5mM MTA and 250μM DMI for 24h and restimulated with LPS at day 6 of culture for another 24h. (n=7, pooled from 2 independent experiments). (Mean ± SEM, *p<0.05, Wilcoxon signed-rank test; (G) Two-way ANOVA, Sidak's multiple comparisons test).

These epigenetic alterations suggest poised transcription of the *TNFA* and *IL6* genes and a decrease in heterochromatin at these regions. The relevance of epigenetic changes is further supported by decreased responsiveness in cells exposed to the protein methylation inhibitor methylthioadenosine (MTA) (**Figure 1G**). Cellular metabolism shares an intimate link with the epigenetic landscape. Namely, TCA cycle-derived metabolites exhibit signaling functions and are involved in the regulation of the activity of chromatin-modifying enzymes that promote histone and DNA demethylation and histone acetylation, thus modulating transcriptional activity. Itaconate inhibits the enzymatic activity of succinate dehydrogenase (SDH) [20], leading to a break in the TCA cycle and an accumulation of its substrate succinate [21]. Changes in the TCA cycle and further metabolic alterations may account for the epigenetic change elicited by dimethyl itaconate. Another attractive layer of epigenetic regulation induced by itaconate is the possibility of itaconylation of protein lysine residues, similarly to acetylation or succinylation [3, 22]. Thus, we demonstrate that exposure to the immunomodulator DMI leads to epigenetic reprogramming akin to the changes elicited by the prototypical inducers of trained immunity.

DMI stimulates glycolytic and mitochondrial energy metabolic rate

The enzyme SDH is not only involved in the TCA cycle but also known as complex II of the mitochondrial respiratory chain. Acute exposure to itaconate inhibits oxidative respiration by blocking the flow of electrons from the oxidation of succinate to the ubiquinone pool, as shown by a decreased oxygen consumption rate [20]. Glycolysis is also modulated by itaconate, where upon LPS exposure, the produced itaconate inhibits the glycolytic enzyme fructose-bisphosphate aldolase A, which ultimately impairs the glycolytic rate [22]. Similarly, OI decreases the activity of the glycolytic enzyme GAPDH in LPS-activated macrophages downregulating glycolysis [6]. OI also alkylates lactate dehydrogenase A that regulates the glycolytic rate [3]. In contrast, when bone marrow-derived macrophages are not activated and have been exposed to DMI, they exhibit an increase in glycolysis [2]. Using the *in vitro* model of trained immunity, and compatible with the effect of acute DMI exposure, we found that DMI-Mf displayed an increased basal and maximal extracellular acidification rate (ECAR) (**Figure 2A**).

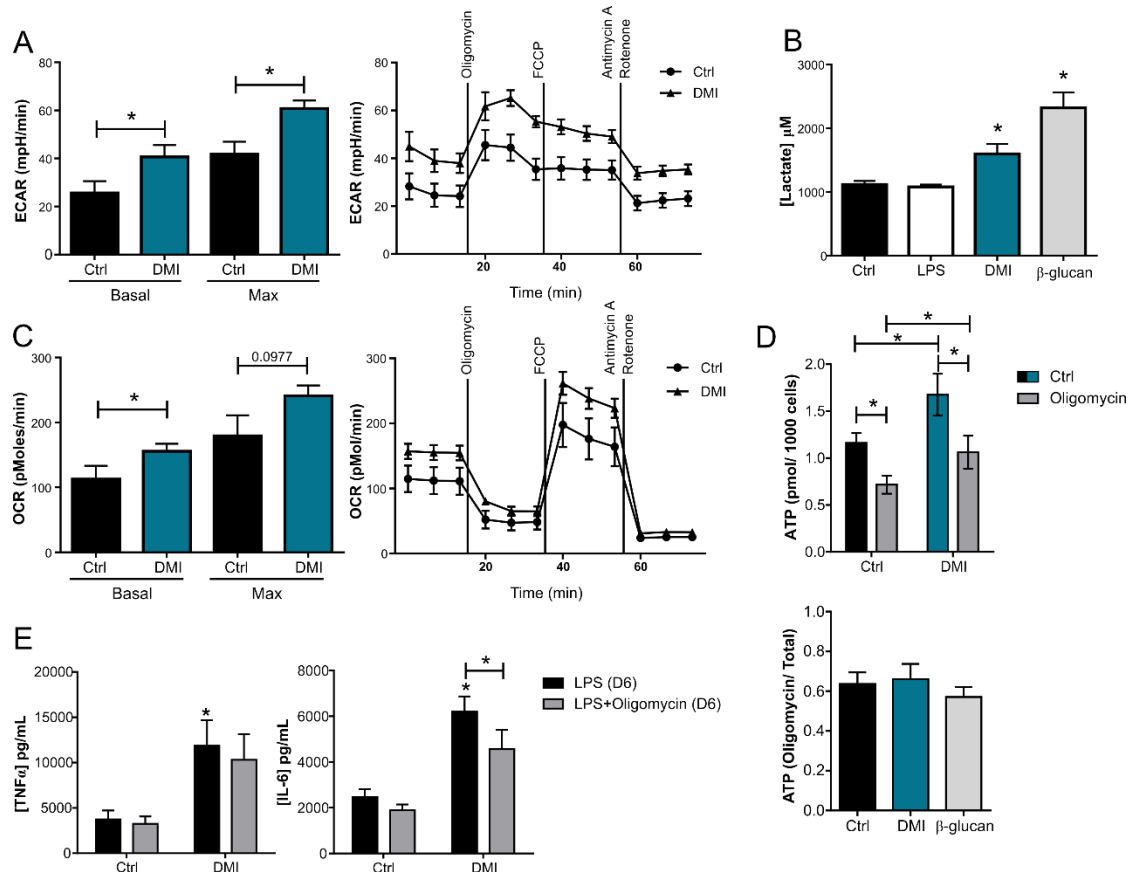


Figure 2 DMI alters glycolysis, oxidative phosphorylation, and ATP concentration in human monocytes. (A) Basal and maximum (Max) extracellular acidification rate (ECAR) was determined 5 days after 24h stimulation with DMI by extracellular flux measurements (n= 9 pooled from 3 independent experiments). (B) Lactate secretion by monocytes 5 days after 24h stimulation with LPS, DMI or β -glucan (n=9, pooled from 3 independent experiments). (C) Oxygen consumption rate (OCR) of monocytes were determined 5 days after 24h stimulation with DMI, also by extracellular flux measurements (n= 9 pooled from 3 independent experiments). (D) ATP was quantified at day 6 of culture in monocytes that were stimulated with DMI or β -glucan for 24h. Prior to the collection at day 6, half the cells were exposed to 1 μ M of oligomycin for 30min (n=3/7 pooled from 2/3 independent experiments) (E) TNF α and IL-6 production by human monocytes treated with DMI for 24h and restimulated with 10ng/mL LPS and 1 μ M oligomycin 5 days later (n= 6 pooled from 2 independent experiments). (Mean $\pm$ SEM, * p <0.05, Wilcoxon signed-rank test, (D, E)* p <0.05 Two-way ANOVA, Sidak's multiple comparisons test).

In accordance, extracellular lactate levels were enhanced in DMI-Mf compared to non-stimulated cells (**Figure 2B**), indicating that DMI treatment enhances glycolysis in human monocytes [23]. In addition, despite the previously reported acute inhibition of itaconate on oxidative respiration, DMI-Mf displayed an increased oxygen consumption rate (OCR)

(**Figure 2C**). The parallel increase in ECAR and OCR suggests that DMI treatment increases both the glycolytic and mitochondrial energy metabolic rate in macrophages. The latter is supported by the fact that DMI-treated cells displayed a higher ATP content (**Figure 2D; top panel**). To determine the relative contribution of mitochondria to the total ATP production, the activity of the mitochondrial ATP-producing synthase (F_0F_1 -ATPase) was prevented using oligomycin. This inhibition reduced ATP content in both control and DMI-Mfs, and the relative contribution of glycolysis and mitochondria to the total ATP level was similar between groups (**Figure 2D; bottom panel**). The contribution of F_0F_1 -ATPase activity to DMI-Mf responsiveness is observed by the decreased production of IL-6 by cells stimulated with LPS and oligomycin (**Figure 2E and S2A**).

We conclude that short DMI exposure induces a metabolic rewiring that is characterized by increased glycolytic and mitochondrial ATP generation. These findings are in accordance with the profile of β -glucan or BCG trained monocytes. Inhibition of glycolysis ablates β -glucan trained immunity [24] and genetic variation in genes of the ETC are associated with changes in BCG trained immunity traits [25].

DMI induces mitochondrial perturbations in human monocytes

Acute DMI treatment inhibits mitochondrial oxygen consumption, whereas long term DMI treatment enhances this parameter. To further explore this dichotomy, and given the fact that mitochondria can represent significant sources of cellular ROS [26] we assessed ROS levels by quantifying the oxidation of a mitochondria-targeted Hydroethidium (HEt) variant (MitoSOX). Monocytes exposed to DMI for 24h displayed increased HEt oxidation, indicative of increased levels of HEt-oxidizing ROS (**Figure 3A; left panel**). However, the DMI-induced increase in HEt oxidation was transient, since DMI-Mf displayed a slight decrease in HEt oxidation (**Figure 3A; right panel**). In a zebra fish model of infection with *Salmonella enterica*, IRG1 depletion in macrophage-lineage cells decreased HEt oxidation and compromised bactericidal activity [27]. Compatible with these results, a study on a zebra fish model of gouty inflammation demonstrated that IRG1 depletion decreased macrophage-specific HEt oxidation [28]. Human macrophages trained with oxidized low-density lipoprotein show increased HEt oxidation, and the mitochondria-targeted antioxidant

MitoTEMPO (MT) decreases the augmented pro-inflammatory cytokines produced by these cells [29]. However, pre-treatment with MT did not decrease DMI enhanced cytokine production (**Figure 3B**). To further assess mitochondrial function, we used TMRE. This fluorescent cation accumulates in the mitochondrial matrix across the mitochondrial inner membrane (MIM) in a manner dependent on the trans-MIM mitochondrial membrane potential ($\Delta\Psi$). To determine the minimal TMRE fluorescence signal, $\Delta\Psi$ was dissipated using the mitochondrial uncoupler FCCP (**Figure S3A**). Following normalization of the TMRE signals on the FCCP-treated condition, no effect of acute DMI treatment was observed (**Figure S3B; left panel**). In contrast, the TMRE signal was increased in macrophages previously exposed to DMI and β -glucan (**Figure S3B; right panel**), suggesting a more negative (hyperpolarized) $\Delta\Psi$. Similarly, timelapse analysis of TMRE fluorescence demonstrated a significantly higher signal in DMI-treated vs. control cells (**Figure S3C**). Acute inhibition of the mitochondrial F_0F_1 -ATPase (i.e. OXPHOS complex V) by oligomycin significantly increased TMRE fluorescence in DMI-Mfs and apparently increased this signal in control cells (**Figure S3C**). These results, combined with the fact that oligomycin inhibited OCR in control and DMI-Mf (**Figure 2B; right panel**), strongly suggest that the F_0F_1 -ATPase operates in its forward (ATP-producing) mode under our experimental conditions. This supports the conclusion that the ETC is more active after the 5 days resting period, allowing the F_0F_1 -ATPase to actively contribute to the increase in cellular ATP content in DMI-Mfs.

DMI increases general ROS levels in human monocytes

Itaconate is also known to promote the activation of antioxidant responses. Itaconate and its derivatives activate the transcription factor Nrf2, which is the master regulator of the antioxidant response [4]. The inhibitor of Nrf2, KEAP1, has cysteine residues alkylated by itaconate, allowing Nrf2 to initiate the transcriptional antioxidant response [3, 22]. As previously reported, the expression of the Nrf2 target genes *NQO1* and *HMOX1* were upregulated in DMI exposed monocytes (**Figure 3C**). Also, monocytes exposed to DMI prior to stimulation with zymosan had decreased chemiluminescent due to luminol oxidation when compared to zymosan-activated cells, indicative of lower general (gen)ROS levels (**Figure 3D**). However, monocytes that were exposed to DMI alone, in the absence of other

stimuli, show increased oxidation of the ROS species indicator H₂DCFDA than control cells, which points to higher basal genROS levels in DMI treated cells. Increased H₂DCFDA oxidation is present not only after acute DMI stimulation of 2h and 24h (**Figure 3E; left and middle panel**), but also after the resting period of 5 days (**Figure 3E; right panel**). In accordance with our results, LPS tolerized RAW264.7 cells that overexpress *IRG1* present higher levels of genROS than the tolerized control cell line, while *IRG1* silencing decreased genROS levels in the same model [30]. *IRG1* overexpression in the human monocytic cell line THP-1 led to an increase in genROS levels [31], and the mouse microglia cell line BV2 exposed to DMI had an enhanced genROS production as well [4].

To determine if ROS contribute to the induction of increased responsiveness in DMI-Mf, we performed genetic and pharmacological approaches. Chronic granulomatous disease (CGD) patients are characterized by a failure to mount a respiratory burst to combat pathogens due to mutations in genes of the NADPH oxidase (NOX) complex [32]. Trained monocytes derived from CGD patients produced lower amounts of TNF α and IL-6 after LPS restimulation compared to healthy controls (**Figure 3F**). However, the decreased responsiveness is not specific for DMI-Mf since the non-stimulated cells from CGD patients also responded less than controls (**Figure 3G**). To clarify the role of NOX in DMI-trained immunity, we pharmacologically inhibited NOX activity with diphenyleneiodonium (DPI) and found no differences in TNF α and IL-6 secretion after restimulation (**Figure 3H**). On the other hand, genROS scavenger molecules N-acetyl cysteine (NAC) and α -tocopherol (AT) significantly modulated the responsiveness to LPS in DMI-Mfs (**Figure 3I**). AT further enhanced TNF α and IL-6 production in DMI-Mfs, but it also increased IL-6 secretion in control cells. In contrast, NAC decreased TNF α production. The opposing effect of NAC and AT on cytokine production may be due to these molecules' distinct scavenging properties. NAC is not only an effective antioxidant molecule but it also acts as a precursor of glutathione, the main endogenous antioxidant molecule, while AT is present in lipid structures and inhibits lipid peroxidation, preventing damage of membranes [33]. Collectively, we show that DMI has antioxidant features when combined with other stimulants, as previously described.

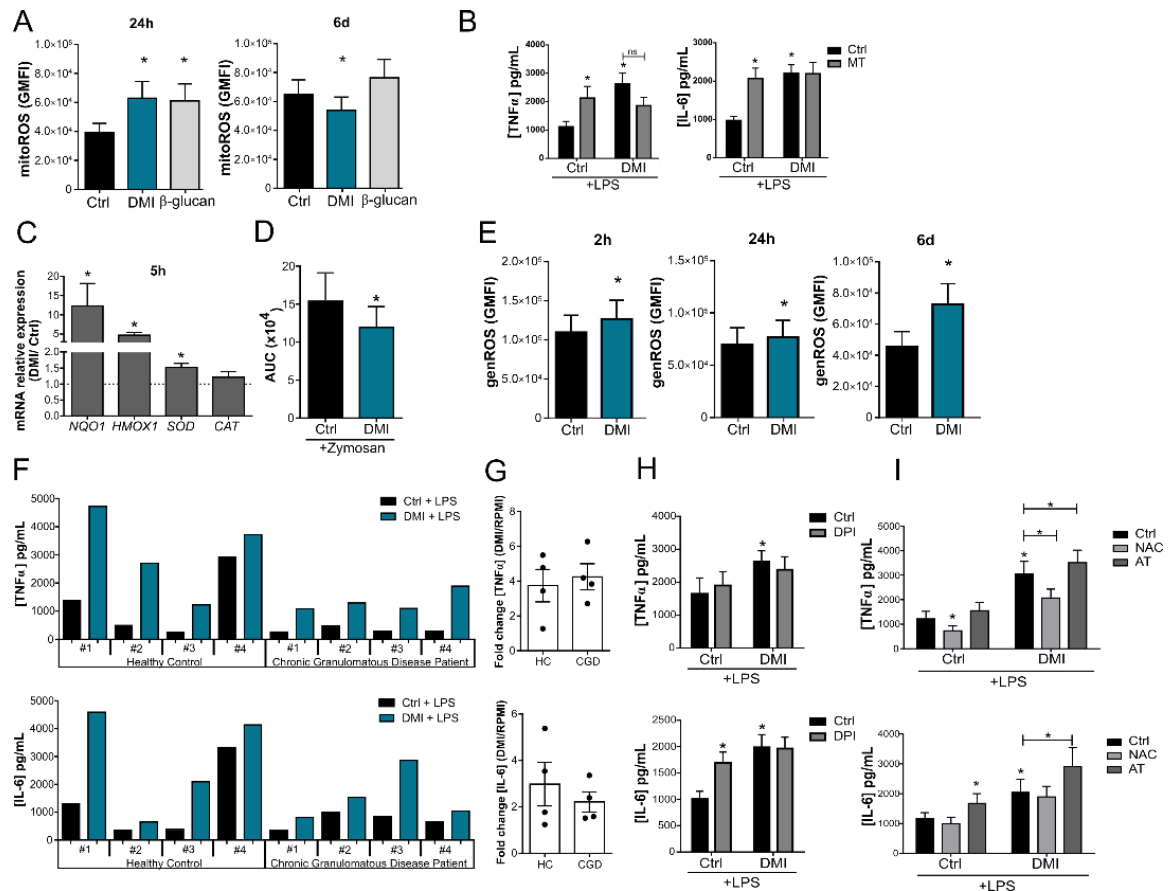


Figure 3 DMI modulates reactive oxygen species (ROS) production. (A) Mitochondrial ROS levels (MitoSox) in monocytes exposed to DMI for 24 and at 6 days of culture (n=7-9 pooled from 4 independent experiments). (B) TNFα and IL-6 production by monocytes pre-treated with 500μM MitoTempo (MT) exposed with DMI for 24h and restimulated with 10ng/mL LPS after 5 days for 24h (n=12 pooled from 4 independent experiments) (C) Expression of genes of the antioxidant response *NQO1* (NAD(P)H:quinone oxidoreductase 1), *HMOX1* (heme oxygenase-1), *SOD* (superoxide dismutase) and *CAT* (Catalase) in monocytes exposed to DMI for 5h (n=6 pooled from 2 independent experiments). (D) ROS levels in monocytes pre-treated with DMI for 30min and then stimulated with zymosan (n=6 pooled from 2 independent experiments). (E) Basal levels of ROS in monocytes exposed to DMI for 2h, 24h or after 6 days of culture (n=6-9 pooled from 2-3 independent experiments). (F, G) TNFα and IL-6 production by monocytes from healthy controls and chronic granulomatous disease patients after treatment with DMI for 24h followed by 10ng/mL LPS restimulation at day 6 of culture for another 24h. (H, I) TNFα and IL-6 production by human monocytes pre-treated for 1h with (H) 0.5μM diphenyleneiodonium (DPI), (I) 50μM α-tocopherol (AT) or 1mM N-acetyl cysteine (NAC), exposed to DMI for 24h and restimulated with 10ng/mL LPS after 5 days for 24h. n=9 pooled from 3 independent experiments. (Mean ± SEM, *p<0.05, Wilcoxon signed-rank test, (B, H, I) *p<0.05, Two-way ANOVA, Sidak's multiple comparisons test).

However, when DMI is used in isolation, genROS levels are sustainably increased, possibly reflecting the highly energetic metabolism of DMI-Mf and thus leading to a higher genROS production. Alternatively, increased ROS levels could indicate a decrease in the capabilities

of the cell to scavenge ROS. In the case of β -glucan and BCG trained macrophages, ROS levels were also increased and the antioxidant response genes were upregulated [34,35].

Glutathione metabolism plays a role in DMI-induced responsiveness

DMI-induced high energy metabolism and ROS increase could be linked to a modified cellular redox status. Glutathione (GSH) is the most abundant thiol that scavenges ROS and protects against oxidative stress. It was recently shown that both itaconate and DMI react with thiols. They form conjugates with GSH, decreasing its intracellular levels while increasing ROS [4]. In addition to the antioxidant role of GSH, this molecule also influences epigenetic mechanisms, possibly bridging the metabolic effects of DMI with the epigenetic changes observed. GSH not only regulates S-adenosyl methionine (SAM) levels, which is used by DNA methyltransferases and histone methyltransferases, but also can directly lead to a post-translational modification of histones, a process termed glutathionylation [36]. Moreover, an intermediate of itaconate catabolism, itaconyl-CoA has been shown to compromise vitamin B12 metabolism [37, 38], affecting the methionine cycle. In turn, methionine is relevant for glutathione synthesis, redox homeostasis and SAM levels. Recently, variations in genes involved in the glutathione pathway have been shown to modulate β -glucan and BCG trained immunity traits [34] and bone marrow derived macrophages isolated from β -glucan trained mice have increased levels of glutathione [35]. In addition, the decrease in TNF α production of DMI-Mfs in the presence of the GSH precursor NAC (**Figure 3I**), possibly points to the importance of this pathway in the context of innate immune memory. We hypothesized that GSH plays a role in DMI-induced trained immunity. The expression of glutamate-cysteine ligase catalytic subunit (*GCLC*) and glutathione reductase (*GSR*) was upregulated after 5h and 24h of DMI exposure, pointing to an increase in synthesis and reduction of GSH, respectively (**Figure 4A; top panels**). The expression of the thiol antioxidant thioredoxin (*TXN*) was also increased together with the expression of the reducing enzyme *TXNDR1* (**Figure 4A; bottom panels**). Indeed, GSH intracellular levels were decreased in monocytes exposed to DMI for 5h (**Figure 4B; left panel**). Inhibition of the GSH synthesizing enzyme γ -glutamyl cysteine ligase (*GCL*) by buthionine sulfoximine (BSO) further decreases GSH levels (**Figure 4B; left panel, 4C**).

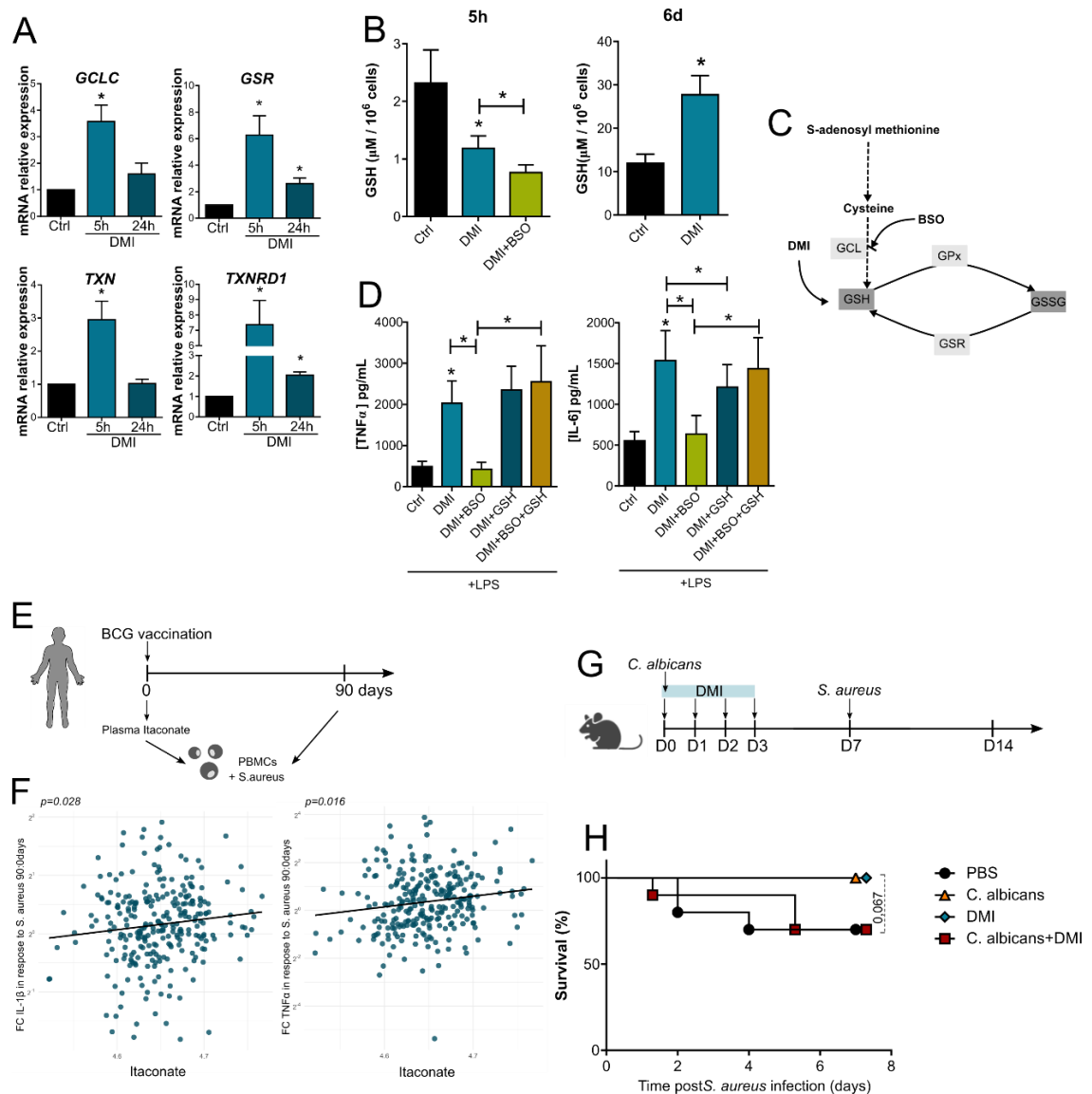


Figure 4 DMI induced trained immunity depends on modulation of glutathione, and itaconate correlates with *in vivo* trained immunity features. (A) Expression of *GCLC* (γ-glutamyl cysteine ligase catalytic subunit), *GSR* (Glutathione-Disulfide Reductase), *TXN* (thioredoxin) and *TXNRD1* (thioredoxin reductase 1) in monocytes exposed to DMI for 5h and 24h (n= 6-9 pooled from 2-3 independent experiments). (B) GSH levels in monocytes exposed to 250μM DMI, 25μM buthionine sulfoximine (BSO) or both for 5h. GSH concentration was also measured at day 6 of culture in DMI exposed cells (n=8 pooled from 3 independent experiments). (C) Schematic representation of glutathione metabolism and the action of DMI and BSO (Glutamate-Cysteine Ligase (GCL), Glutathione peroxidase (GPx), Glutathione Reductase (GSR), reduced glutathione (GSH), oxidized glutathione (GSSG)). (D) TNFα and IL-6 production by human monocytes pre-treated for 1h with 25μM BSO, 1mM EtGSH, or a combination of 25μM BSO and 1mM EtGSH, exposed to DMI for 24h and restimulated with 10ng/mL LPS after 5 days for 24h (n=6 pooled from 3 independent experiments). (Legend continued next page)

In contrast, DMI-Mf show increased GSH concentrations when compared to non-trained cells (**Figure 4B; right panel**). Interestingly, dimethyl fumarate, which has a similar chemical structure to DMI, also has acute anti-inflammatory properties [39]. Fumarate, a Krebs cycle metabolite accumulates in trained cells and the derivative methyl fumarate induces trained immunity in human monocytes (see **Figure 1E**) [17]. Dimethyl fumarate, like DMI, was reported to deplete intracellular glutathione [39, 40]. In line with the increased concentration of GSH in DMI-Mf, long exposure to DMF potentiated the enzymatic activity of γ -glutamyl cysteine ligase and enhances GSH levels in astrocytes [41]. We observed that modulation of glutathione levels in DMI-Mf modulates cellular responsiveness to restimulation (**Figure 4D**). The DMI-increased TNF α and IL-6 production was strongly prevented by BSO pre-treatment, which was reversed by the concomitant addition of ethyl (Et)GSH. The addition of EtGSH to DMI-treated monocytes led to a decrease in IL-6 secretion compared to DMI-Mf. The presence of BSO did not induce cellular toxicity (**Figure S2B**). Thus, DMI-induced responsiveness depends on a tightly balanced regulation of GSH concentration. In DMI-treated monocytes, if GSH is further decreased by BSO or, in contrast, if GSH is increased by exogenous supplementation, the cytokine production triggered by LPS is decreased.

Itaconate and DMI associate with trained immunity *in vivo*

We next investigated the association of itaconate and DMI with trained immunity features *in vivo*. Firstly, we explored circulating plasma itaconate concentrations in a cohort of 274 healthy volunteers vaccinated with BCG (**Figure 4E**).

Figure 4 (cont.) (E) Schematic representation of the BCG vaccination study. Plasma was collected before vaccination and peripheral blood mononuclear cells (PBMCs) were isolated before (0 days) and 3 months (90days) after BCG vaccination. PBMCs were stimulated with *S. aureus* for 24h and secretion of pro-inflammatory cytokines was quantified. (F) Scatter plot depicting the correlation between baseline itaconate concentration in plasma and the fold change of IL-1 β and TNF α produced by PBMCs after *ex vivo* stimulation at 90 days compared to day 0 (n= 274 healthy volunteers). (G) Graphic representation of mouse survival study. Mice were injected with a single dose of 2x10⁵CFU *C. albicans* intra venously and/or with 20mg DMI intraperitoneally daily for four days. At day 7, mice were infected with 5x10⁶CFU *S. aureus* intravenously and survival was monitored for the following 7 days. (H) Survival rate of mice in response to systemic *S. aureus* infection following administration of PBS, *C. albicans* and/or DMI (n= 10) (PBS vs DMI, PBS vs *C. albicans*, p=0.0669 Log-rank Mantel-Cox test). (Mean $\pm$ SEM, *p<0.05, Wilcoxon signed-rank test).

Peripheral blood mononuclear cells were collected before and 90 days after BCG vaccination and stimulated *ex vivo* with *Staphylococcus aureus* for 24h. The fold change of the production of pro-inflammatory cytokines between these two timepoints relays the magnitude of response enhanced by BCG vaccination. After correction for age and sex, itaconate concentration in plasma before vaccination positively associated with the fold change of IL-1 β ($p=0.028$) and TNF α ($p=0.016$) production after BCG vaccination (**Figure 4F**). Itaconate concentrations have previously been correlated with a reduction in rheumatoid arthritis disease progression [42], and idiopathic pulmonary fibrosis patients have reduced concentrations of itaconate in the lungs [43]. Interestingly, it has been recently shown that induction of trained immunity by BCG vaccination is associated with lower systemic inflammation [44], and it is tempting to speculate that in an *in vivo* human BCG trained immunity context, higher itaconate concentrations may be a predictor of individuals who will develop greater trained immunity features.

To further assess the functional relevance of our findings, we explored the potential of DMI to induce protection against infection *in vivo*, by using a mouse model of trained immunity. Mice received daily DMI injections for 4 days and/or were infected with a sublethal dose of *Candida albicans*, which triggers a systemic induction of trained immunity in the animals [45]. After 7 days, mice were subjected to infection with the Gram-positive bacteria *Staphylococcus aureus*. (**Figure 4G**). In line with our findings *in vitro*, DMI administration showed a tendency for increased protection of mice against a challenge with *S. aureus*, to a similar extent as a sublethal infection with *C. albicans*. This result may reflect the ability of DMI to induce trained immunity *in vivo*, offering protection against heterologous infections (**Figure 4H**). Similar to what was demonstrated *in vitro* [13], the protection conferred by *C. albicans* is ablated when mice are concomitantly exposed to DMI. The effect of DMI on mice survival demonstrates that, when in the absence of another stimulation, DMI may exert immunostimulatory properties, while showing anti-inflammatory characteristics when combined with a pro-inflammatory stimulus. The same duality is observed in its antioxidant properties when combined with zymosan stimulation and oxidant properties when added alone (see **Figure 3D, 3E**).

In this study, we show that DMI is an inducer of trained immunity. DMI-Mf show increased pro-inflammatory cytokine production upon TLR2 and TLR4 restimulation. The possible protection of DMI-injected mice against *S. aureus* infection further supports the ability of

DMI to enhance immune functions. We show that DMI-induced innate immune memory is accomplished through epigenetic reprogramming coupled to a high energy metabolism, with increased glycolysis, oxidative phosphorylation, and ATP concentrations, which ultimately may allow for increased effector functions in macrophages. The potentiation of distinct metabolic pathways provides the building blocks and the signaling messengers that shape cellular responses. Trained immunity triggered by β -glucan [24] or BCG [19] is characterized by metabolic changes similar to what was observed here for DMI-trained macrophages. The metabolic changes of DMI treated cells manifest with a modified redox state as ROS levels are increased in a sustained manner. We previously demonstrated that monocytes trained with β -glucan or BCG also show increased ROS levels [34] while β -glucan trained cells have a higher NAD⁺/NADH ratio when compared with controls [24]. Genetic variations in genes of the glutathione pathway, the main endogenous anti-oxidant molecule, were associated with changes in β -glucan and BCG trained immunity traits [34]. Here we show that glutathione concentrations are modulated in DMI exposed cells, either decreased upon acute exposure or enhanced in trained differentiated macrophages.

A quickly growing number of studies are highlighting the potential of itaconate and its derivatives for the suppression of inflammation in different pathological contexts, such as pulmonary fibrosis [43] and psoriasis [4]. But it is important to note that itaconate and its derivatives do not produce similar biological response, and so DMI and OI do not appear to mimic the endogenous molecule. For example, unlike itaconate, the synthetic itaconate derivatives DMI and OI neither decrease SDH activity nor lead to succinate accumulation [21,47]. In addition, distinct *in vitro* models reveal different inflammatory profiles of DMI. Mice bone marrow-derived macrophages pretreated with DMI for 3h followed by 4h LPS stimulation produced more TNF α than LPS-stimulated macrophages, while a 12h DMI pretreatment produced no differences in TNF α production after LPS [21]. Taken together, we demonstrate that the immunomodulatory functions of itaconate and its derivatives are of great complexity and far from being fully understood. The mechanisms of action of DMI comprise and influence fundamental cellular processes, such as metabolism, epigenetics and the redox state of the cell. These pathways are highly dynamic and can be differently modulated depending on the context, triggering both pro- and anti-inflammatory mechanisms. Further exploration of the molecular, metabolic, and epigenetic changes induced by itaconate and derivatives in different cell types and disease models will be of

great importance, to fully assess the potential of the modulation of immune responses by this promising metabolite and open new therapeutic avenues.

Conflict of Interest Statement: WJHK is a scientific advisor of Khondrion B.V. (Nijmegen, The Netherlands). This company was not involved in the data analysis and interpretation, writing of the manuscript, and in the decision to submit the manuscript for publication.

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Supplementary Figures

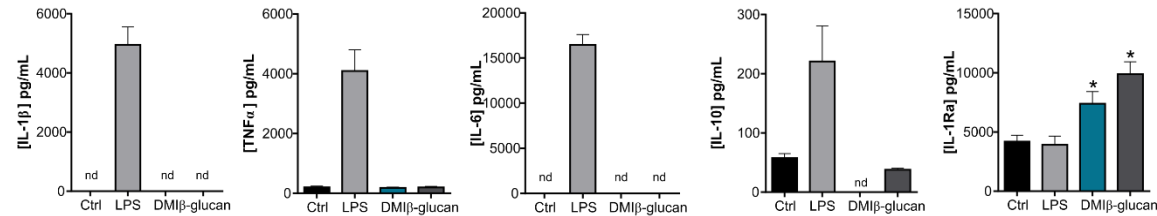


Figure S1 Cytokine secretion by monocytes upon acute stimulation. IL-1 β , TNF α , IL-6, IL-10, and IL-1Ra secretion by human peripheral blood monocytes stimulated for 24h with LPS, DMI, or β -glucan. Mean $\pm$ SEM, n=3-6 pooled from 1-2 independent experiments. *p<0.05, Wilcoxon signed-rank test.

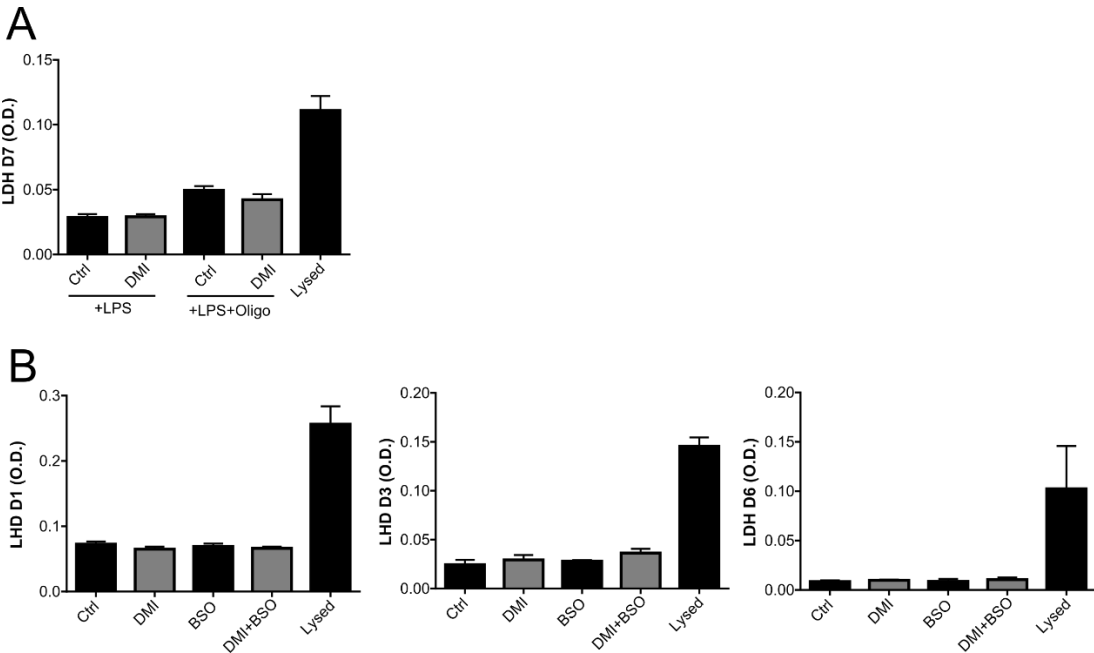


Figure S2 Cytotoxicity of oligomycin and buthionine sulfoximine (BSO). (A) monocytes were exposed to DMI for 24h and were restimulated after 5 days with 10ng/mL LPS and 1 μ g/mL oligomycin for 24h. After the last 24h, the cytotoxicity assay was performed. (B) Cytotoxicity of 100 μ M BSO and DMI on monocytes exposed for 24h, measured after 1, 3 and 6 days of culture. Lysed cells are included as a control. n=3/6 pooled from 1/2 independent experiments. Mean $\pm$ SEM.

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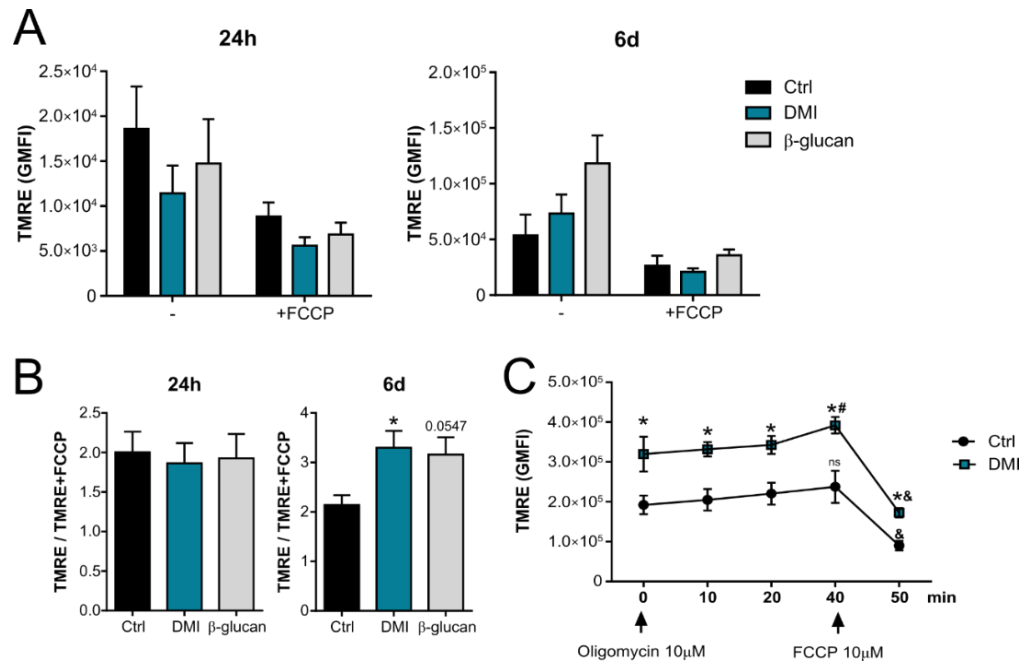


Figure S3 The influence of DMI on mitochondrial membrane potential. Monocytes were cultured (A) 24h in the presence of DMI and left to rest for an additional 5 days. Mitochondrial membrane potential was accessed by TMRE staining in the presence or absence of 10 μM FCCP. (B) TMRE staining was normalised with TMRE staining of FCCP treated cells (TMRE GMFI/ TMRE TMRE (+FCCP) GMFI) (n=5-7, pooled from 3 independent experiments), *p<0.05, Wilcoxon signed-rank test. (C) Cells pre-exposed to DMI were stained with TMRE at day 6 of culture. TMRE fluorescence was measured for 40min after the addition of 10μM oligomycin. 10 μM FCCP was added after 40min and fluorescence measured after 10min. (n=3, pooled from 3 independent experiments). (Mean ± SEM, vs Ctrl *p<0.05, vs 0min #p<0.05, 40min vs 50min &p<0.05. Two-way ANOVA, Sidak's multiple comparisons test).

CHAPTER 6

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The present thesis aimed to explore the mechanisms underlying the regulation of trained immunity. We focused on one innate immune cell - the monocyte. Monocytes are a heterogeneous cell population that originates in the bone marrow, derived from the differentiation of hematopoietic stem cells, and migrates to the circulation. Circulating monocytes can become activated and migrate to tissues, to the site of infection or damage. There, monocytes and macrophages display antimicrobial features, rapidly producing reactive oxygen species, secreting cytokines and chemokines to potentiate the immune response, and ultimately phagocytosing and eliminating pathogens. In a trained immunity context, macrophages produce increased amounts of ROS and cytokines [1] and exhibit an enhanced killing capacity [2,3]. Macrophages are also antigen-presenting cells, contributing to the activation of T cells and the induction of adaptive immune responses. However, the influence of trained immunity on T cell responses might be an yet underexplored promising avenue of research [4]. For example, in a randomized controlled trial, BCG vaccination prior to influenza vaccination enhanced antibody responses against influenza H1N1 strain [5].

Mechanisms involved in cellular and systemic trained immunity

The central role of monocytes in innate immunity and specifically in long-term memory has warranted the focus of recent research. In this thesis, we investigated the contribution of two different metabolic pathways in the establishment of trained immunity. In **Chapter 2**, we report that modulation of the glutathione pathway is crucial for monocyte trained immunity. On the other hand, in **Chapter 3**, we show that the monocyte lipidome can manipulate the trained immunity phenotype. Importantly, we detected an upregulation of LOX- derived mediators in both *in vitro* and *in vivo* models.

Monocytes isolated from individuals vaccinated with BCG showed an increase in 12-LOX products, 30 days after vaccination when compared to before vaccination. Curiously, the 12-LOX product 12-HETE was also altered in the placebo-treated group, revealing that this metabolite might have some effects linked to seasonality. The possible influence of the circadian rhythm was reduced by controlling vaccination and blood collection times [6]. Curiously, a recent study reported that LOX enzymes, particularly 12-LOX, may have non-enzymatic roles. The authors show that 12-LOX plays a direct role in cell signaling, via the

inhibition of AMPK activity in a non-enzymatic manner [7]. AMPK is also involved in the induction and maintenance of trained immunity, since AMPK is a negative regulator of mTOR and ultimately glycolysis [8]. This was confirmed with the use of metformin, a known AMPK activator [8]. A human trial using oral metformin showed that metformin treatment decreased macrophage responsiveness in an *ex vivo* BCG-trained immunity model [9]. Pharmacological inhibition of 12-LOX, not only prevents its catalytic activity but also ablates 12-LOX inhibition of AMPK [7]. We observed that 12-LOX inhibition decreased stimulated IL-6 production, possibly reflecting both the effect of 12-LOX products on trained immunity and the role of 12-LOX in AMPK inhibition. 12-LOX derived mediators appear to be relevant since the exogenous supplementation of 12-HEPE is sufficient to increase macrophage responsiveness. But the possible role of 12-LOX in AMPK regulation in the context of trained immunity remains to be explored.

In contrast to LOX-derived mediators, inhibition of COX activity does not compromise BCG-induced trained immunity. Instead, monocytes exposed to the COX inhibitor aspirin exhibited higher stimulated secretion of TNF α . Aspirin is a non-steroidal anti-inflammatory drug that, contrary to what we observed, has been reported to inhibit the activation of LPS stimulated macrophages through the inhibition of NF- κ B and blunting TNF α mRNA transcription [10,11]. This illustrates that one molecule may play a dual role, depending on timing of exposure and cellular inflammatory context. Due to the availability of aspirin for common human use, in the future it would be interesting to fully evaluate aspirin as an inducer and enhancer of trained immunity.

The insights that trained immunity relies on glutathione and lipid metabolism can be translated to possible therapeutic approaches to potentiate innate immune functions. However, the overactivation of the innate immune system may also become maladaptive. In **Chapter 3**, we explored the role of FADS2 inhibition in inflammatory bowel disorders. Clinical trials showed some favorable effects of PUFA in inflammatory bowel disease [17,18]. Interestingly, in individuals with specific genetic variations in the *FADS2* gene, the intake of a higher dietary ratio of AA to EPA, DHA and DPA was shown to be a risk factor for Crohn's disease [14]. In this study, we have identified suggestive associations between genetic variations in *FADS2* and *in vitro* pro-inflammatory cytokines production upon heterologous stimulation in a trained immunity setting. Trained immunity features were previously suggested in colitis, as pointed by the worsening inflammation of a mouse model

of colitis upon oral β -glucan administration [15]. Thus, we used a chemically induced colitis model, in which we show that the pharmacological inhibition of FADS2 decreased mouse morbidity. However, in this experimental model, FADS2 inhibition is not exclusive to myeloid cells. Namely, intestinal stromal cells, including mesenchymal stem cells and fibroblasts, may also contribute to the decrease in morbidity induced by FADS2 inhibition. These cells possess immune properties and are long-lived, when compared to immune cells, and are thus an interesting candidate for memory retention [16].

In this line, trained immunity features have been recently identified in non-immune cells. Trained immunity is present in peripheral innate immune cells, but its longevity is sustained by hematopoietic stem cells. Upon trained immunity, HSCs are transcriptionally [17] and metabolically distinct [18]. Consistently to what was described in peripheral trained cells, HSC of β -glucan trained animals had increased glycolysis. However, HSCs derived from these animals presented lower PUFA levels [18], which was not observed in our human *in vitro* and *in vivo* BCG trained immunity models. This disparity suggests that different cell types undergo distinct metabolic programs to promote long-term memory. Also, different trained immunity inducers might trigger separate metabolic programs, although this does not seem to be the case for PUFA modulation since our *in vitro* model showed comparable results between BCG and β -glucan exposed monocytes. It is also possible that this disparity is due to differences between species or time of analysis. In this scenario, monocytes were isolated from humans vaccinated with BCG 30 days previously, while HSCs were collected from mice 24h after β -glucan administration. This comparison highlights that the metabolic changes supportive of trained immunity are possibly dependent on cell type, context, and inducer. Other non-immune cells were also newly implicated in trained immunity. A recent study reported that the progeny of male mice exposed to a sublethal dose of *C. albicans* were protected against heterologous infections, possibly due to DNA methylation differences in sperm DNA of the previously infected mice [19]. Since alterations in metabolism are interwoven with epigenetic modifications, it would of great interest to explore the possible metabolic effects of trained immunity on germ cells.

The spleen is crucial for the generation of adaptive responses, with the presentation of antigens and activation of T and B cells. The spleen is also a reservoir for undifferentiated monocytes and is the prime organ for extramedullary hematopoiesis [20,21]. In **Chapter 4**, we investigated the impact of the spleen on the induction of trained immunity. Despite the

relevance of the spleen for the immune response, we showed that spleen removal does not influence monocyte, T or B circulating pools in mice exposed to β -glucan. Instead, the absence of the spleen prevents neutrophilia, typical of trained immunity. Other studies report that trained neutrophils also showed increased glycolytic activity, mitochondrial respiration, ROS production and phagocytic and killing capacity, increasing mice survival to *Listeria monocytogenes* infection and promoting neutrophil anti-tumor activity [22–24]. In the future, it would be relevant to establish if spleen removal prior to induction of trained immunity is confined to decreasing the percentage of circulating neutrophils or if the activity of these cells is also altered. Only then would we be capable of inferring the potential clinical consequences to patients that have undergone a splenectomy.

Trained immunity as a strategy to improve immune impairments

Trained immunity is an attractive tool to increase innate immune responses in immunocompromised individuals. Indeed, modulation of known metabolic or epigenetic pathways could be explored as a therapeutic strategy to induce trained immunity. The clinical relevance of these approaches was stressed in a BCG vaccination human trial in which vaccination decreased new respiratory infections in elderly individuals [25]. Also, different trials are now exploring the impact of BCG vaccination on the incidence of COVID-19 (NCT04537663, NCT04328441, NCT04327206). This highlights the existing need for the identification of inducers and enhancers of trained immunity.

In **Chapter 5.1 and 5.2**, we explored the immunomodulatory effects of itaconate and identified DMI as a trained immunity inducer. Mechanistically, we demonstrate that DMI triggered similar metabolic alterations when compared to the prototypical trained immunity inducers BCG or β -glucan. Namely, potentiation of glycolysis [8], oxidative phosphorylation [26] and ROS levels [1].

In accordance with what we observed for β -glucan-induced trained immunity in **Chapter 2**, the modulation of GSH levels contribute for the establishment of DMI-trained immunity. Of note, a recent study reported that itaconate exogenous supplementation also increased ROS levels and decreased GSH concentration. The enhanced ROS levels was accompanied by an increase in NOX activity [27]. However, in our *in vitro* trained immunity

model, NOX activity does not appear to be relevant for the enhancement of DMI-induced macrophage responsiveness, as demonstrated by the pharmacological and genetic approaches used for NOX inhibition. Indeed, previous studies have shown that itaconate derivatives do not fully mimic endogenous itaconate. For example, contrary to itaconate, DMI does not inhibit SDH activity and does not promote succinate accumulation [28]. Another potential metabolic similarity between DMI and BCG or β -glucan trained immunity is the involvement of lipid metabolism (see **Chapter 3**). Phagocytes deficient in IRG1 infected with *M. tuberculosis* have increased lipid droplet formation [29]. In addition, exogenous itaconate decreases acetyl-CoA concentrations [28]. DMI could have a different effect than what is observed for itaconate, and so further studies are required. In an *in vitro* trained immunity setting, DMI triggers a higher enhancement of macrophage responsiveness when compared with itaconate, however the mechanisms that contribute to this discrepancy were not elucidated.

Trained immunity was firstly described as heavily relying on glycolysis potentiation, while the contribution of oxidative respiration was underscored. More recently the role of mitochondrial function in trained immunity has gained more emphasis. In addition to the potentiation of oxidative phosphorylation of trained macrophages [26], cells trained with oxLDL showed increased mitochondrial mass, which points to a relevant role of mitochondrial biogenesis for macrophage responsiveness [30]. Although DMI trained macrophages did not present higher mitochondrial mass, our study showed that DMI-trained cells consumed more oxygen and exhibited a hyperpolarized mitochondrial membrane potential. Taken together, mitochondrial biogenesis and function appears to play an important role in different trained immunity programs, but the underlying mechanisms remain to be fully explored.

Itaconate has not only been identified as a regulator of macrophage phenotype and function, but it also has direct bacteriostatic activity. Itaconate inhibits isocitrate lyase, involved in the glyoxylate shunt pathway, which is present in most prokaryotes, namely *M. tuberculosis*, *C. albicans* or *S. aureus* [31–33], but absent in mammals. Indeed, itaconate was shown to blunt the growth of *Mycobacterium* [29,32,34] and *Salmonella* [32,35]. The dichotomic features of acute anti-inflammation and long-term trained immunity, combined with this potential direct antimicrobial action, make DMI an attractive molecule for therapeutic use in infectious diseases. Specifically, in infections by itaconate-sensitive

pathogens and in which an over-stimulation of the immune system is deleterious. It is also enticing to firstly consider DMI for topical application, as DMI was seen to reduce skin thickness in a mouse model of chemically induced psoriasis. In addition, mouse models of skin inflammation have shown that previously challenged skin presents a higher capacity for wound healing after a second trigger [36], thus possibly displaying features of memory.

The studies performed in this thesis have also a number of limitations. It is important to highlight that different trained immunity inducers might trigger distinct metabolic programs. For example, the increase of lipid mediators by BCG and β -glucan exposed monocytes might not be present in oxLDL trained immunity. Also, the insights gained *in vitro* might not be translated to an *in vivo* context. To minimize this possibility, we have corroborated some of our findings with the use of *in vivo* models and one human cohort of approximately 300 participants. The 300BGC cohort provided suggestive associations between variations in genes of the glutathione and LOX pathways and trained immunity features. However, genome wide associations studies with such a relatively small cohort make the identification of significant effects challenging. Plasma glutathione and itaconate concentrations were also associated with *ex vivo* stimulated pro-inflammatory cytokine production. It would be interesting to validate these findings in a separate cohort, especially in a cohort with a non-caucasian ancestry. For example, the variant Asn152Ser of the *IRG1* gene is enriched in the African ethnicity (20% allele frequency when compared to 0.2% in European population). This variant confers an increase in IRG1 activity and consequently enhanced itaconate production [37].

In conclusion, the induction of trained immunity represents an interesting tool to harness the potential of innate immunity in immune-impaired patients, while the dampening of trained immunity programs might contribute to alleviate hyperinflammatory conditions. In this thesis, we have identified the glutathione and LOX pathways as crucial for trained immunity. The study of the pathways that promote trained immunity may suggest new therapeutic targets. For example, we propose that the use of aspirin may promote BCG-trained responses, while FADS2 inhibition possibly decreases colitis morbidity. Here, we have also identified a new inducer of trained immunity, the itaconate derivative DMI. Due to its multifaceted immunomodulatory effects and possible direct antimicrobial characteristics, DMI is an attractive candidate for future research. The present thesis focused on monocyte and macrophage responses but the future approach to the study of non-immune cells within

a trained immunity conceptual framework is very intriguing. It could give rise to more therapies that rely on the potentiation of the cell-intrinsic mechanisms, or conversely which dampen their response to control inflammation.

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APPENDIX

- I DUTCH ABSTRACT (NEDERLANDSE SAMENVATTING)**
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- VIII CURRICULUM VITÆ**
- XIX FINANCIAL SUPPORT**

I. DUTCH ABSTRACT (NEDERLANDSE SAMENVATTING)

Het aangeboren immuunsysteem kan langdurige functionele veranderingen vertonen die verhoogde inflammatoire reacties bevorderen. Dit fenomeen, het geheugen van het aangeboren immuunsysteem, staat bekend als 'getrainde immuniteit'. De mechanismen die een rol spelen in het induceren en controleren van getrainde immuniteit zijn de afgelopen jaren het onderwerp van veel onderzoek geweest. Desondanks is ons begrip van de metabole netwerken die getrainde immuniteit kunnen beïnvloeden nog niet compleet. In dit proefschrift hebben we de bijdrage van verschillende metabole processen aan het induceren van getrainde immuniteit door het vaccin Bacille Calmette-Guérin (BCG) of β -glucan onderzocht.

Reactieve zuurstofcomponenten, in het Engels 'reactive oxygen species' (ROS), vormen een belangrijk verdedigingsmechanisme tegen pathogenen, en spelen daarnaast een rol als signaalstoffen. In dit onderzoek laten we zien dat glutathion, een molecuul dat ROS onschadelijk kan maken, bijdraagt aan de verhoogde reactiviteit van getrainde macrofagen. Getrainde macrofagen hebben een verhoogd glutathiongehalte en farmacologische modulatie van glutathionniveaus verminderde de productie van pro-inflammatoire (ontstekingsbevorderende) cytokinen. Ook laten we zien dat in een cohort van met BCG gevaccineerde individuen de basale glutathionspiegels in het bloedplasma samenhangen met de productie van pro-inflammatoire cytokinen. Verschillende onderzoeken hebben het belang van lipiden voor het induceren van getrainde immuniteit gesuggereerd. Wij hebben waargenomen dat een verrijking in lipide-mediators belangrijk is voor de inductie van getrainde immuniteit door BCG in monocyten. Met name de van lipoxygenase afgeleide mediators zijn in staat de reactiviteit van macrofagen te verhogen. Dit komt overeen met onze bevinding dat monocyten die afkomstig zijn van met BCG gevaccineerde individuen verrijkt zijn met 12-lipoxygenase producten.

Getrainde immuniteit wordt gekarakteriseerd door metabole herprogrammering van zowel de perifere immuuncellen, als ook de hematopoëtische stamcellen of bloedstamcellen (HSC's). De milt, een orgaan waar extramedullaire hematopoëse plaatsvindt, vormt een belangrijke bron van ongedifferentieerde monocyten, en zou dus kunnen bijdragen aan getrainde immuniteit. We evalueerden de perifere immunologische kenmerken van β -

glucan-getrainde muizen waarin de milt verwijderd was en observeerden dat verwijdering van de milt voorkwam dat neutrofilie optrad. Dit wijst op de minimale rol van de milt bij de inductie en instandhouding van getrainde immuniteit *in vivo*.

Kennis over de mechanismen die getrainde immuniteitsprogramma's reguleren, dragen bij aan de potentiële therapeutische rol ervan. Hier rapporteren we dat dimethylitaconaat (DMI), een stof afgeleid van de immuun-modulerende metabool itaconaat (itaconzuur), getrainde immuniteit kan induceren. Mechanistisch gezien verandert DMI de redoxtoestand en de glutathionniveaus van monocyten, vergelijkbaar met onze eerdere bevindingen voor andere stoffen die getrainde immuniteit induceren.

Bij elkaar genomen biedt dit proefschrift relevante inzichten in de regulatie van getrainde immuniteit. Op systemisch niveau toonden we aan dat de milt cruciaal is voor de toename van circulerende neutrofielen, terwijl we op cellulair niveau de relevantie van glutathion- en lipide mediator-mechanismen hebben aangetoond. We identificeerden ook DMI, een nieuwe stof die getrainde immuniteit kan induceren, welke in de toekomst zou kunnen worden overwogen als een therapeutisch hulpmiddel om de functies van het aangeboren immuunsysteem te verbeteren.

II. PORTUGUESE ABSTRACT (RESUMO EM PORTUGUÊS)

O sistema imune inato pode possuir alterações funcionais de longo prazo que potenciam a resposta imunitária, conhecidas como imunidade treinada ou memória do sistema imune inato. Os mecanismos que induzem e controlam os programas de imunidade treinada têm sido um foco de pesquisa nos últimos anos. No entanto, o nosso conhecimento sobre as redes metabólicas que governam a imunidade treinada é incompleto. Nos presentes estudos, explorámos a contribuição de vias metabólicas distintas na indução da imunidade treinada induzida pela vacina Bacille Calmette-Guérin (BCG) ou β -glucano.

As espécies reativas de oxigénio (reactive oxygen species - ROS) promovem a eliminação de patógenos e medeiam a transdução de vias de sinalização. Aqui, mostramos que a glutathione, molécula eliminadora de ROS, contribui para o aumento da capacidade de resposta de macrófagos treinados. Macrófagos treinados *in vitro* têm tendencialmente mais glutathione intracelular e a modulação farmacológica da quantidade de glutathione reduziu a produção de citocinas pró-inflamatórias, aquando de restimulação. Recorrendo ao uso de uma coorte de indivíduos saudáveis vacinados com BCG, a concentração basal de glutathione plasmática foi positivamente associada à produção de citocinas pró-inflamatórias por células mononucleares do sangue periférico estimuladas *ex vivo*. Em paralelo, estudos sugerem a importância dos lipídios para a indução da imunidade treinada. Nós observámos que o enriquecimento de mediadores lipídicos em monócitos é crucial para a indução da imunidade treinada por BCG. Especificamente, mediadores derivados de lipoxigenases foram suficientes para aumentar a responsividade de macrófagos. Em concordância, monócitos de indivíduos vacinados com BCG apresentam um enriquecimento em produtos da enzima 12-lipoxigenase.

A imunidade treinada é conferida pela reprogramação metabólica, não apenas das células imunes inatas periféricas, mas também das células progenitoras hematopoiéticas (hematopoietic stem cells - HSCs). O baço é um local de hematopoiese extramedular e um reservatório de monócitos imaturos, podendo contribuir para a manutenção da imunidade sistémica treinada. Para responder a esta questão, avaliamos as características de imunidade periférica treinada de murganhos treinados com β -glucano e sujeitos a esplenectomia. A remoção do baço preveniu a neutrofilia característica de imunidade

treinada, enquanto outras propriedades da imunidade treinada não foram moduladas, apontando para o papel limitado do baço na imunidade treinada.

A exploração dos mecanismos que regulam os programas de imunidade treinada podem contribuir para estabelecer o seu potencial papel terapêutico. Aqui, nós reportamos que o dimetil itaconato (dimethyl itaconate -DMI), um derivado do metabolito imunomodulador itaconato, induz imunidade treinada. O DMI altera o equilíbrio de oxidação-redução de monócitos e a concentração de glutathione, de forma semelhante ao que também observamos para outros indutores de imunidade treinada.

Concluindo, a presente tese demonstra novas e relevantes formas de regulamentação da imunidade treinada. A nível sistêmico, mostramos que o baço é fundamental para o aumento de neutrófilos circulantes, enquanto em nível celular, demonstramos a relevância das vias da glutathione e dos lipídios. Identificamos ainda o DMI como um novo indutor de imunidade treinada, que poderá futuramente ser considerado como uma ferramenta terapêutica para aumentar a função do sistema imune inato.

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To my fellow GABBA friends Ana Filipa, Ana Maria, Catarina, Inês, Joana, Manuel, Marta and Sílvia, for all the weekly *brindes* that made that first PhD year bearable. For all the shared stress in organizing a symposium and the help in navigating frustrating bureaucracies. To my university tribe Ana, Clara, Fred, Gonçalo, João, Lia and Pi for reminding me that there is life outside of a PhD. For all the consistent fun and support you gave directly and even through international borders.

To Francisco, for proving that 2000km distance is okay but that 0km is better. I am grateful that we shared the bitter realities and the great joys of doing a PhD. Thank you for peeling the tangerines. Thank you for always being supportive of my crazy plans and dreams, and for taking me along for yours.

To my family, that have accompanied and supported me through this journey. For all the words of encouragement, worry and strength. Mãe, tia, Joana and Lia for showing me that *As musas do Sado* can do it all. Specially to my mom, who learned to really enjoy the Dutch sweets, this thesis is dedicated to you.

IV. LIST OF PUBLICATIONS

The following works were included in this thesis:

AV Ferreira, J Domiguéz-Andrés and MG Netea. The Role of Cell Metabolism in Innate Immune Memory. *J Innate Immun* 2022;14:42–50

AV Ferreira, VACM Koeken, V Matzaraki, S Kostidis, JC Alarcon-Barrera, LCJd Bree, SJCFM Moorlag, VP Mourits, B Novakovic, MA Giera, MG Netea and J Domínguez-Andrés. Glutathione Metabolism Contributes to the Induction of Trained Immunity. *Cells* 2021; 10:971

AV Ferreira*, JC Alarcon-Barrera*, J Domínguez-Andrés , Ö Bulut, G Kilic, HN Özhan, A Ziogas, S Kostidis, V Matzaraki, MG Netea and M Giera. Polyunsaturated fatty acids and lipoxigenase pathways support trained immunity. *Submitted*

AV Ferreira, RF Uijen, Ö Bulut, Mld Jonge, J Domínguez-Andrés and MG Netea. Limited role of the spleen in a mouse model of trained immunity: Impact on neutrophilia. *J Leukoc Biol.* 2022;111:9–17

AV Ferreira, MG Netea and J Domínguez-Andrés. Itaconate as an immune modulator. *Aging (Albany NY).* 2019 24;11(12):3898–9

AV Ferreira, LA Groh, VACM Koeken, M Bruno, G Kilic, Ö Bulut, KD Synodinou, T Gkavogianni, B Novakovic, WJH Koopman, FLvd Veerdonk, G Renieris, EJ Giamarellos-Bourboulis, J Domínguez-Andrés. Itaconate and its derivatives induce long-term innate immune responses and confer protection against infections through trained immunity. *In preparation*

In the context of the present thesis, the following studies were also performed:

SM Gonçalves*, **AV Ferreira***, C Cunha, A Carvalho. Targeting immunometabolism in host-directed therapies to fungal disease. *Clinical and Experimental Immunology.* 2021

AV Ferreira, C Duarte-Oliveira, CF Campos, D Antunes, C Cunha, A Carvalho. The role of AMPK in host response against *Aspergillus fumigatus*. *In preparation*

M Bruno, B Zhang, **AV. Ferreira**, CE Kröger, R Liu, A Ziogas, L Groh, RJ. Röring, V Klück, SJCFM Moorlag, P Debisarun, NAF Janssen, D Rosati, J Saiz, O Gaal, HJ. Ferreira, S Warnat-Herresthal, M Becker, L Holsten, K Händler, JL Schultze, C Cunha, C Barbas, LAB Joosten, MG Netea, Y Li, AC Aschenbrenner, A Carvalho and FLvd Veerdonk. Dysregulated immunometabolism in Chronic Granulomatous Disease is amenable to interferon gamma treatment. *Submitted*

Z Liu*, G Kilic*, W Li*, O Bulut, MK Gupta, B Zhang, C Qi, P He, J T-Say, CF Soon, YA Mekonnen, **AV Ferreira**, Cvd Made, Bv Cranenbroek, HJPM Koenen, E Simonetti, D Diavatopoulos, Md Jonge, L Müller, H Schaal, PN Ostermann, M Cornberg, B Eiz-Vesper, Avd Ven, Fvd Veerdonk, Rv Crevel, LAB Joosten, J Domínguez-Andrés, C-J Xu#, MG Netea#, Y Li#. Multi-omics integration reveals only minor long-term molecular and functional sequelae in immune cells of individuals recovered from COVID-19. *Submitted*

LA Groh, **AV Ferreira**, L Helder, CDCCvd Heijden, B Novakovic, Evd Westerlo, V Matzaraki, SJCFM Moorlag, LCd Bree, VACM Koeken, VP Mourits, ST Keating, JHv Puffelen, A Hoischen, LAB Joosten, Mihai G. Netea, Werner J. H. Koopman and Niels P. Riksen. oxLDL-Induced Trained Immunity Is Dependent on Mitochondrial Metabolic Reprogramming. *Immunometabolism*. 2021 3(3)

J Domínguez-Andrés, RJW Arts, S Bekkering, H Bahrar, BA Blok, LCJd Bree, M Bruno, Ö Bulut, PA Debisarun, H Dijkstra, JCd Santos, **AV Ferreira**, D Flores-Gomez, LA Groh, I Grondman, L Helder, C Jacobs, L Jacobs, T Jansen, G Kilic, V Klück, VACM Koeken, H Lemmers, SJCFM Moorlag, VP Mourits, JHv Puffelen, K Rabold, RJ Röring, D Rosati, H Tercan, Jv Tuijl, J Quintin, Rv Crevel, NP. Riksen, LAB Joosten and MG Netea. In vitro induction of trained immunity in adherent human monocytes. *STAR Protoc*. 2021 19;2(1):100365

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R Tarancón, J Domínguez-Andrés, S Uranga, **AV Ferreira**, LA Groh, M Domenech, F González-Camacho, NP Riksen, N Aguilo, J Yuste, C Martín and MG Netea. New live attenuated tuberculosis vaccine MTBVAC induces trained immunity and confers protection against experimental lethal pneumonia. *PLoS Pathog*. 2020 16(4)

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V. COPYRIGHT LICENSES

AV Ferreira, J Domiguéz-Andrés and MG Netea. The Role of Cell Metabolism in Innate Immune Memory. *J Innate Immun* 2022;14:42–50

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AV Ferreira, VACM Koeken, V Matzaraki, S Kostidis, JC Alarcon-Barrera, LCJd Bree, SJCFM Moorlag, VP Mourits, B Novakovic, MA Giera, MG Netea and J Domínguez-Andrés. Glutathione Metabolism Contributes to the Induction of Trained Immunity. *Cells* 2021 21 10(5):971

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AV Ferreira, RF Uijen, Ö Bulut, Mld Jonge, J Domínguez-Andrés and MG Netea. Limited role of the spleen in a mouse model of trained immunity: Impact on neutrophilia. *J Leukoc Biol.* 2022;111:9–17

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AV Ferreira, MG Netea and J Domínguez-Andrés. Itaconate as an immune modulator. *Aging (Albany NY)*. 2019 24;11(12):3898–9

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VI. RESEARCH DATA MANAGEMENT

Data obtained in this thesis are archived according to the Findable, Accessible, Interoperable and Reusable (FAIR) principles [1]. All data produced at the Radboudumc is stored in lab journals, which will be kept at the department. Additionally, data was stored on the Radboudumc server of the department of Internal Medicine which is backed-up daily.

Use of human material was conducted in accordance with the principles of the Declaration of Helsinki [2]. All participants gave written informed consent before participation, and where applicable studies were approved by the Medical Ethics Committee of the Radboudumc.

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- 2 World Medical Association. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *JAMA*. 2013 Nov 27;310(20):2191–4. DOI:10.1001/JAMA.2013.281053

VII. PHD PORTFOLIO

Institute for Molecular Life Sciences
Radboudumc

PhD student:	Anaísa V. Ferreira	PhD period:	01.01.2018-31.01.2022
Department:	Internal Medicine	Promotor(s):	Prof. Dr. Netea; Dr. Carvalho
Graduate School:	Radboud Institute for Molecular Life Sciences	Co-promotor(s):	Prof. Dr. Vilanova; Dr. Domigúez-Andrés;

Training Activities	Year(s)	ECTS
Courses & Workshops		
Introduction day – Radboudumc	2019	0.2
“In the Lead of my PhD” Course – RIMLS	2019	0.5
Introduction to Laboratory Animal Science Course – Radboudumc	2019	3
Panel Design Workshop – BD	2020	0.2
Scientific Integrity Course – Radboudumc	2020	0.75
Congresses and Symposia		
GABBA PhD meeting – ICBAS *, #	2018-2021	3
RIMLS PhD retreat – RIMLS *, #	2019-2021	2.25
4 th International Conference on Innate Immune Memory *, &	2019	1
1 st Dutch Translational Metabolism Conference	2021	0.75
XLVI Annual Meeting of the Portuguese Society for Immunology *, &	2021	1
Seminars and Lectures		
Pathology Lectures – ICBAS #	2018	4
Principles of Biology Lectures – ICBAS #	2018	4
Complex Systems in Biology Lectures – ICBAS #	2018	7
Project Lectures – ICBAS	2018	1.75
Other		
Organization of the GABBA Symposium 2018 – ICBAS \$	2018	3
Department Meetings (weekly) – Radboudumc #	2018-2020	3
Department Meetings (biweekly) – ICVS #	2021	0.5

Teaching Activities		
Internship supervision (1 month) – International Medicine student	2019	0.5
Internship supervision (1 year) – MSc Molec Mech of Disease student	2020	2
Lecture on Immunometabolism for Host-Microbe Interactions Course	2020-2021	0.75
Total ECTS		39.15
* Poster presentation, # Oral presentation		
& Best poster presentation award, \$ https://gabbasymposium2018.wordpress.com/		

VIII. CURRICULUM VITÆ

ANAÍSA VALIDO FERREIRA |DoB: 28.05.1992| Naturality: Setúbal, Portugal

2018 - 2022 **PhD student** – Immunometabolic pathways in the regulation of trained immunity

Joint PhD Graduate Program in Basic and Applied Sciences (GABBA) & Radboud Institute for Molecular Life Sciences (RIMLS)
Supervisors: Prof. M. Netea, Dr. A. Carvalho, Dr. J. Domínguez-Andrés, Prof. M. Vilanova
University of Porto, Portugal & University Nijmegen, The Netherlands

2016 -2017 **Research Assistant** – Iron promotes articular cartilage degeneration

Supervisor: Dr. G. Porto, Basic & Clinical Research Iron Biology Group
Instituto de Investigação e Inovação em Saúde (i3S), Porto, Portugal

2014 - 2015 **Student Assistant** – The role of Del-1 in the resolution of inflammation

Supervisors: Prof. T. Chavakis and Dr. I. Kourtzelis, Regulation of Vascular Inflammation in Immunity and Metabolic Disease Group
Department of Clinical Pathobiochemistry, Technische Universität Dresden, Germany

2014 **ERASMUS Internship** – Del-1 plays a role in islet transplantation

Supervisors: Prof. T. Chavakis and Dr. I. Kourtzelis, Regulation of Vascular Inflammation in Immunity and Metabolic Disease Group
Department of Clinical Pathobiochemistry, Technische Universität Dresden, Germany

2013 **Curricular Internship** – S100A8/A9 in Hereditary Hemochromatosis

Supervisor: Dr. G. Porto, Basic & Clinical Research Iron Biology Group
Instituto de Biologia Molecular e Celular (IBMC), Porto, Portugal

2010 - 2015 **Msc in Bioengineering – Molecular Biotechnology** (17/20, A)

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