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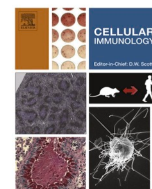

Research paper

The role of sirtuin 1 on the induction of trained immunity

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ABSTRACT

Sirtuin 1 (SIRT1) has been described to modify immune responses by modulation of gene transcription. As transcriptional reprogramming is the molecular substrate of trained immunity, a *de facto* innate immune memory, we investigated the role of SIRT1 in the induction of trained immunity. We identified various *SIRT1* genetic single nucleotide polymorphisms affecting innate and adaptive cytokine production of human peripheral blood mononuclear cells (PBMCs) in response to various stimuli on the one hand, and *in vitro* induction of trained immunity on the other hand. Furthermore, inhibition of SIRT1 upregulated pro-inflammatory innate cytokine production upon stimulation of PBMCs. However, inhibition of SIRT1 *in vitro* had no effect on cytokine responses upon induction of trained immunity, while activation of SIRT1 mildly modified trained immunity responses. In conclusion, SIRT1 modifies innate cytokine production by PBMCs in response to various microbes, but has only a secondary role for BCG and β-glucan-induced trained immunity responses.

1. Introduction

Sirtuins are a family of highly conserved nicotinamide adenine dinucleotide $(NAD)^+$-dependent protein deacetylases. The mammalian sirtuin family consists of seven proteins (SIRT1-7), which are involved in a variety of cellular processes including cell differentiation, metabolism, and stress responses [1,2]. Sirtuins deacetylate lysine residues of both histone proteins and nonhistone substrates, including transcription factors [1]. An increasing body of evidence demonstrates that SIRT1 modifies immune responses and inflammation [3]. On one hand, most studies show that acute inflammation decreases the expression level of SIRT1, which leads to a pro-inflammatory response [4–7]. This can occur via the deacetylation of NF-κB subunit RelA/p65, or indirectly by inducing repressive transcriptional complexes [3,8]. On the other hand, prolonged microbial exposure led to an increase in SIRT1 levels and caused immunosuppression [9]. Additionally, SIRT1 has been described to support the switch from glycolysis to fatty acid oxidation in a THP-1 monocyte cell line during adaptation to acute inflammation, in conjunction with SIRT6, through an epigenetic-based mechanism [10]. SIRT1 is able to deacetylate H1 histones at lysine (K) 26, as well as H3 histones at lysine 9 (H3K9), lysine 14 (H3K14), and H4 histones at lysine 16 (H4K16) [9,11,12], this results in pleiotropic effects.

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The term *trained immunity* describes the process by which innate immune cells undergo functional reprogramming after certain stimulations/infections, to mount a *de facto* immune memory that supports long-term altered immune responses to secondary non-specific stimulation [13,14]. Both β-glucan, a cell wall component of many fungal species, and the bacillus Calmette-Guérin (BCG) vaccine, which is currently used for prevention of tuberculosis, have been extensively studied the last years for their ability to induce trained immunity [13]. This non-specific innate immune memory is characterized by epigenetic and metabolic rewiring and results in enhanced pro-inflammatory cytokine production. β-glucan-induced trained immunity is mediated by binding to the Dectin-1 receptor, subsequent activation of mTOR/HIF-1α [15], and upregulation of both glycolysis and oxidative phosphorylation [16]. Furthermore, H3K4me3 and H3K27ac enrichment at promoters of pro-inflammatory genes is associated with chromatin accessibility in trained cells, resulting in increased transcription of pro-inflammatory genes [17].

In contrast, lipopolysaccharide (LPS) from gram-negative bacteria can induce a tolerant macrophage phenotype, which is refractory to immune stimulation and characterized by decreased pro-inflammatory cytokine production [18]. SIRT1 has been shown to play a role during endotoxin tolerance [9], as its inhibition significantly improved survival of sepsis in rodents [3,19]. A low SIRT1 activity is observed in chronic inflammatory diseases, therefore increasing SIRT1 activity would be beneficial in this state [3]. Interestingly, *SIRT1* expression was found to be decreased upon β-glucan-induced trained immunity in monocytes [15].

Trained immunity is pivotal for the beneficial heterologous effects of some vaccines, but also in mediating deleterious effects in inflammatory diseases in which it is inappropriately activated [13,20]. It is thus essential to identify the mechanisms that regulate trained immunity responses [13,21] in order to design novel immunomodulatory strategies. Given that SIRT1 affects the immune response by metabolic and epigenetic mechanisms, we sought to investigate the role of SIRT1 in innate immune memory using genetic and pharmacological approaches.

2. Results

2.1. SIRT1 genetic polymorphisms are associated with cytokine responses upon PBMC stimulation

To explore the involvement of SIRT1 in immune responses, we first assessed the effect of *SIRT1* single nucleotide polymorphisms (SNPs) on cytokine responses of healthy individuals in response to different microbial stimuli. This was investigated in a cohort of 534 healthy individuals (500FG study), in which isolated peripheral blood mononuclear cells (PBMCs) were stimulated *ex vivo* with various microorganisms or (non-)microbial products, and cytokine production was subsequently measured [22,23]. Both innate (IL-6, TNF-α, IL-1β after stimulation for 24 h) and adaptive (IL-22, IL-17, IFN-γ after stimulation for 7 days) cytokine responses were influenced by SNPs in the *SIRT1* gene known as expression quantitative trait loci (eQTLs), in response to bacteria (*Bacteroides (fragilis), Borrelia (burgdorferi), Coxiella burnetti, Escherichia coli, Mycobacterium tuberculosis, Staphylococcus aureus*), fungi (*Aspergillus fumigatus* conidia), yeasts (*Cryptococcus, Candida albicans* and hyphae), TLR ligands (CpG oligodeoxynucleotides, LPS, Pam3Cys) and non-microbial stimuli (monosodium urate crystals (MSU) + palmitic acid (C16.0)) (Table 1). For a minority of the stimuli (heat-killed *C. albicans*, influenza virus, MSU alone, phytohemagglutinin, or poly I:C), no effects of *SIRT1* genetic variants were observed.

2.2. SIRT1 inhibition increases innate pro-inflammatory cytokine production in PBMCs

This prompted us to investigate whether inhibition of SIRT1 affects inflammatory cytokine production in PBMCs isolated from healthy individuals *in vitro*. PBMCs were exposed to the synthetic SIRT1 inhibitor EX-527 at various concentrations (1–100 μM), and stimulated for 24 h with TLR4 ligand LPS or TLR1/2 ligand Pam3Cys. These concentrations of EX-527 were not toxic to the cells (Supplementary Fig. 1). Transcription of *IL6*, *TNFA*, and *IL1B* was increased upon SIRT1 inhibition, although this was not statistically significant (Fig. 1A). Accordingly, PBMC stimulation with LPS together with inhibition of SIRT1 resulted in a 1.2–1.4 fold increased production of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α (Fig. 1B). A similar, though less pronounced, significant increase in IL-6 and IL-1β production was observed in response to SIRT1 inhibition and Pam3Cys stimulation (Fig. 1B). In contrast, SIRT1 inhibition in *Candida albicans*-stimulated PBMCs did not affect production of adaptive cytokines IFN-γ, IL-22, and IL-17 after 7 days (Fig. 2). Additionally, inhibition of SIRT1 directly affected surface expression of human monocyte markers CD14, CD11b, and HLA-DR. Our data indicate increased CD14 expression after 24 h exposure to 10 μM EX-527. Similarly, a minor but significant increase in CD11b expression on CD14 + monocytes was observed in 100 μM EX-527 treated cells. Though not significant, we observed a trend towards decreased expression of HLA-DR on CD14 + monocytes (Supplementary Fig. 2).

Table 1
SIRT1 genetic polymorphisms are associated with cytokine responses upon PBMC stimulation. QTL mapping of *SIRT1* genetic variants from healthy individuals of the 500FG cohort and cytokine production of PBMCs in response to stimulation *in vitro* with various stimuli (see Methods). The strongest significantly associated genetic variants for a specific cytokine-stimuli combination are shown.

18S FW	GATGGGCGGCGGAAAATAG
18s RV	GCGTGGATTCTGCATAATGGT
TNFA FW	AACGGAGCTGAACAATAGGC
TNFA RV	TCTCGCCACTGAATAGTAGGG
IL1B FW	ATCACTGAACTGCACGCTCC
IL1B RV	TGGAGAACACCACTTGTTGC
IL6 FW	AGCCCACCGGGAACGA
IL6 RV	GGACCGAAGGCGCTTGT

2.3. SIRT1 genetic polymorphisms are associated with trained immunity in vitro

After confirming the importance of SIRT1 for modulating direct cytokine production, we sought to investigate whether genetic variation in the *SIRT1* gene influences trained immunity. The effect of *SIRT1* SNPs on cytokine production upon induction of trained immunity *in vitro* was investigated in PBMCs isolated from 267 healthy individuals of the 300BCG cohort [24]. Monocytes were stimulated with β-glucan, BCG, or RPMI medium as control, for 24 h. Thereafter, cells were washed, rested for 5 days, and on day 6 restimulated with the non-specific stimulus LPS for 24 h. Subsequently, cytokine production (IL-6 and TNF-α) was measured in the supernatant to assess trained immunity responses. The impact of *SIRT1* gene polymorphisms on trained immunity was assessed by correlating individual SNPs with the magnitude of trained immunity responses, measured by cytokine production in trained cells compared to untrained cells (RPMI control). As shown in Fig. 3, a genetic variant (rs10740283 at chromosome 10) in close proximity to the *SIRT1* gene (described as *SIRT1* eQTL in whole blood [25]), influenced IL-6 production in cells trained with BCG ($P = 0.004$ and $P = 0.01$). SNP rs2485679 (also known as *SIRT1* eQTL in whole blood [25]), influenced the fold change of TNF-α production upon β-glucan training (borderline significance of $P = 0.05$ and $P = 0.07$). Next, we investigated the impact of *SIRT1* polymorphisms on *in vivo* trained immunity responses. This was assessed by QTL mapping of SNP genotypes and *S. aureus*-induced cytokine responses *ex vivo* of BCG-vaccinated healthy individuals from the 300BCG cohort. However, no significant impact of *SIRT1* polymorphisms on *in vivo* induction of trained immunity was observed ($P >$

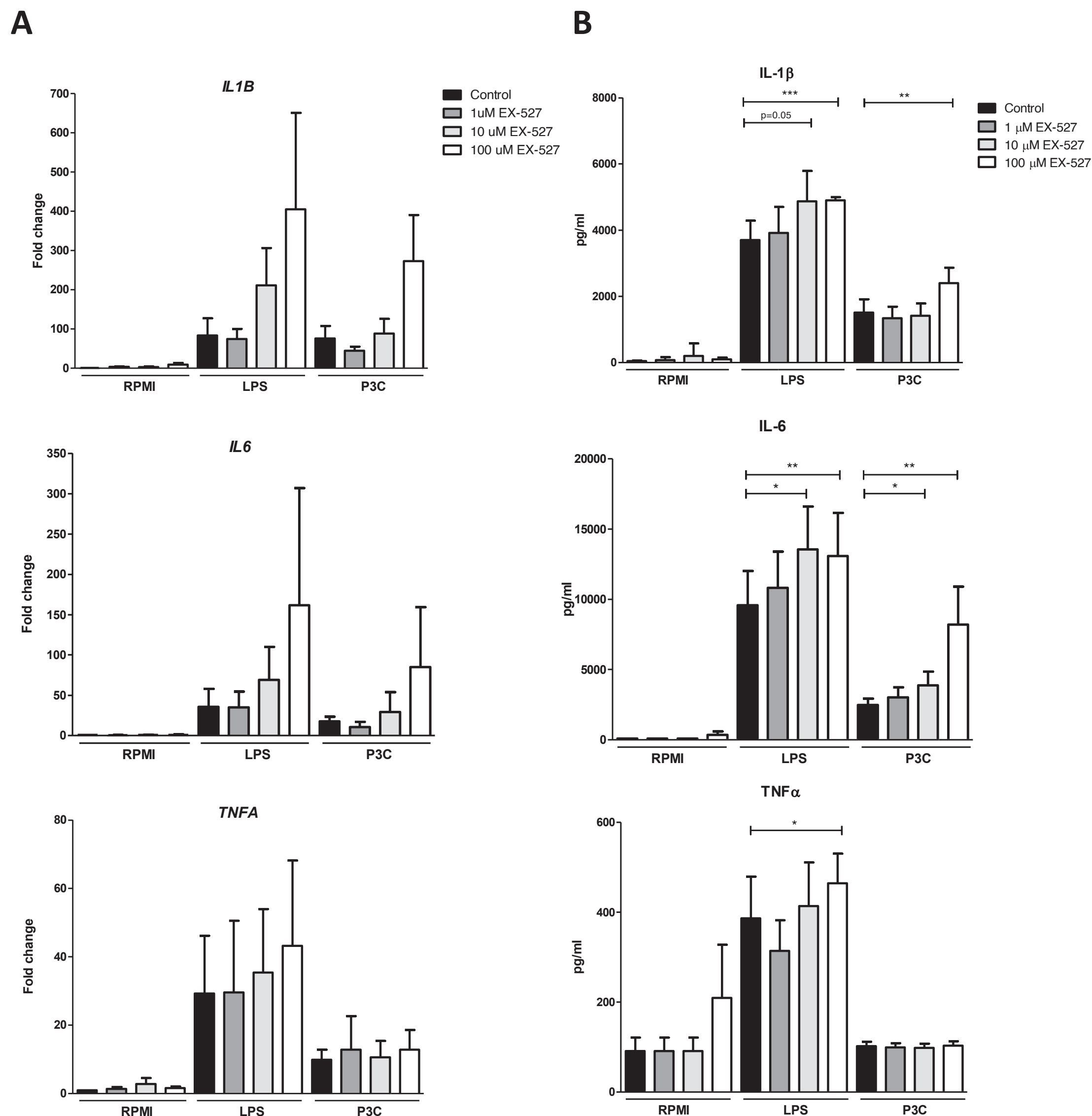

Fig. 1. SIRT1 inhibition increases innate cytokine production of PBMCs. (A) RT-qPCR results of *IL1B* and *TNFA* (n = 7), *IL6* (n = 5), and (B) cytokine production of IL-1β, IL-6, TNF-α in supernatant (n ≥ 10), of PBMCs isolated from healthy individuals were stimulated with LPS (10 ng/mL) or Pam3Cys (10 μg/mL), or non-stimulated (RPMI control), for 24 h in combination with EX-527 (1–100 μM). Mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001, Wilcoxon signed-rank test.

0.05).

2.4. *The effect of SIRT1 on induction of trained immunity*

In a following set of experiments, we assessed the effects of pharmacological inhibition of SIRT1 by EX-527 on trained immunity induced by β-glucan or BCG, or tolerance induced by LPS. The addition of EX-527 did not affect either trained immunity or tolerance in terms of IL-6 and TNF-α production after restimulation with LPS (Fig. 4A). To validate these results, we investigated the effect of SIRT1 activator SRT1720 [26]. Cells trained with BCG in the presence of SRT1720 exhibited elevated production of IL-6 and TNF-α. On the other hand, cells trained with β-glucan in the presence of SRT1720 did not produce more TNF-α than cells trained with β-glucan under normal conditions, but induced a small, yet significant decrease in IL-6 production (Fig. 4B). In addition to cytokine production, the effects of EX-527 on metabolic changes induced by trained immunity were investigated by means of lactate measurement prior to and after restimulation with LPS. Lower levels of lactate were measured in the supernatants of β-glucan trained cells in the presence of 100 μM EX-527, indicating lower glycolytic activity in these cells (Fig. 4C).

3. Discussion

In the present study, we show that genetic variation in *SIRT1* influences the induction of inflammation as reflected by cytokine production upon stimulation of PBMCs, as well as the induction of trained immunity in an *in vitro* experimental model. In contrast, *SIRT1* polymorphisms did not affect the *in vivo* induction of trained immunity by BCG vaccination. Pharmacological inhibition of SIRT1 influenced acute pro-inflammatory innate cytokine production, whereas it did not modulate the induction of trained immunity by BCG or β-glucan. On the other hand, pharmacological SIRT1 activation affected trained immunity responses *in vitro*.

SIRT1 has previously been described to modulate inflammation, with either inhibitory [3,27,28] or stimulatory effects [29], depending on the experimental model. For example, SIRT1 overexpression in arthritis patients is associated with increased pro-inflammatory cytokine production [30,31], whereas SIRT1 activation did not affect PBMCs derived from healthy individuals in the same study [30]. In the current study, we

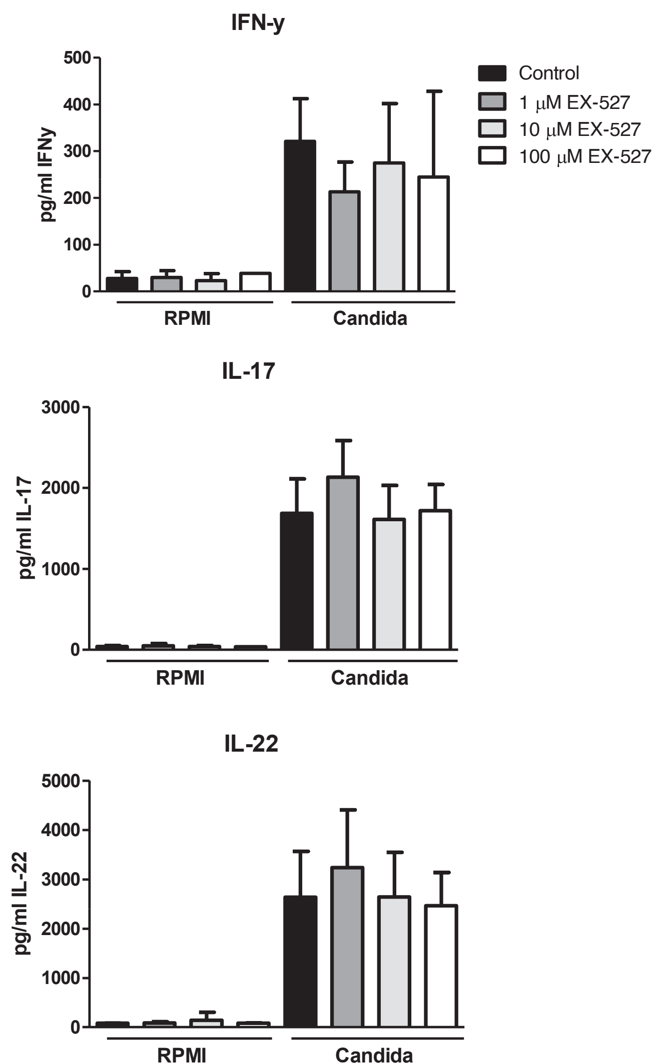

Fig. 2. SIRT1 inhibition does not affect adaptive cytokine production of PBMCs. Cytokine assessment in supernatant of PBMCs isolated from healthy individuals which were stimulated with *C. albicans* (1×10^6 cells/mL), or non-stimulated (RPMI control), for 7 days in combination with EX-527 (1–100 μM). Mean ± SEM, n = n ≥ 3, n.s., Wilcoxon signed-rank test.

validated the role of SIRT1 in the modulation of the inflammatory response by identifying *SIRT1* SNPs that impact cytokine responses upon stimulation with various microorganisms, TLR ligands, and non-microbial stimuli. Some of these SNPs were most strongly associated with cytokine production induced by up to four distinct stimuli (rs12360310 and rs7083505), whereas other SNPs most significantly affected cytokines only after specific stimulations.

We confirmed the anti-inflammatory role of SIRT1 in acute stimulation of human PBMCs by pharmacological inhibition. Interestingly, SIRT1 seems to be specifically involved in the modulation of the cytokines that are mainly produced by innate immune cells, but much less in the regulation of cytokines produced mainly by the adaptive immune cells (IFN-γ, IL-17, IL-22). More studies are needed to unravel the mechanisms by which SIRT1 affects cytokine responses to different stimuli, and in specific cell types. Because EX-527 inhibits SIRT1 enzymes by exploiting their NAD^+-dependent deacetylation mechanism [32], this effect may include modified deacetylation of NF-κB subunit RelA/p65, which mainly regulates the expression of inflammatory genes by myeloid cells [5,6].

To identify possible mechanisms regulating trained immunity responses, we interrogated the role of SIRT1 in the induction of trained immunity using genetic and pharmacological approaches. First, we showed that *SIRT1* SNP rs10740283 influences BCG-induced trained immunity responses in PBMCs of healthy individuals *in vitro*. This SNP has previously been shown to affect *SIRT1* expression in human whole blood [25]. *SIRT1* SNP rs2485679, which is also a *SIRT1* eQTL in human whole blood, influenced β-glucan-induced trained immunity borderline significant. To identify whether SIRT1 also plays a role in induction of trained immunity *in vivo*, we assessed the effect of these polymorphisms on trained immunity responses induced by BCG vaccination in healthy individuals. However, we did not observe significant associations between *SIRT1* SNPs and BCG-induced trained immunity response, suggesting that *SIRT1* has a limited contribution to the process of trained immunity *in vivo*. The sample size of the 300BCG cohort might contribute to the fact that *SIRT1* genetic variants do not show a significant effect on cytokine responses upon induction of trained immunity.

To further assess the effect of SIRT1 on trained immunity, we used pharmacological modulators of SIRT1. We did not observe an effect of SIRT1 inhibitor EX-527 on trained immunity responses, and we observed only limited alterations in trained immunity responses by using the SIRT1 activator SRT1720. Unexpectedly, a small but significant decrease in IL-6 production (but not TNF-α) was observed upon β-glucan-induced trained immunity in combination with SRT1720. In contrast, BCG-induced trained immunity in combination with SRT1720 increased IL-6 and TNF-α production. Because SRT1720 activates SIRT1 by an unknown mechanism [33], it is impossible to speculate on the cause of the discrepancy compared to the results using SIRT1 inhibitor EX-527. Liu et al. identified SIRT1 to play a role in generating endotoxin tolerance [9]. Here, we could not recapitulate the influence of SIRT1 on tolerance in human monocytes. This apparent inconsistency may be due to the different model and cells used: in contrast to the study of Liu et al. which assessed the effect on SIRT1 shortly after LPS stimulation (up to 4 h) in THP-1 cells, we studied the effect of SIRT1 upon non-specific

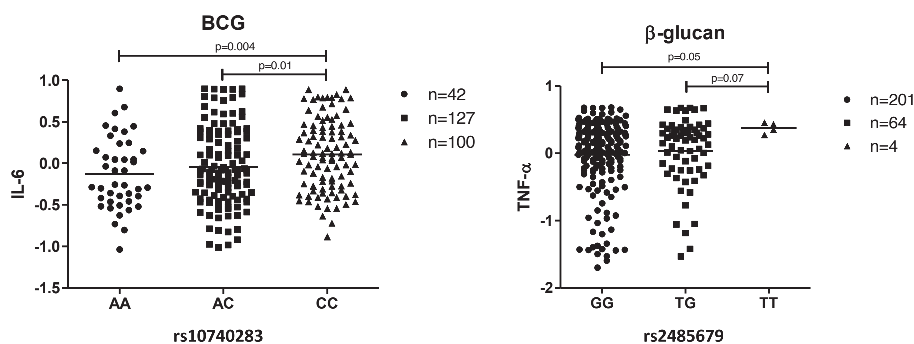

Fig. 3. *SIRT1* genetic polymorphisms are associated with *in vitro* trained immunity responses. Adherent cells of the PBMC fraction derived from healthy individuals of the 300BCG cohort were stimulated *in vitro* with BCG (5 μg/mL) and β-glucan (2 μg/mL) for 24 h, subsequently washed, rested for 5 days, and at day 6 restimulated for 24 h with 10 ng/mL LPS. IL-6 and TNF-α cytokine production was measured in supernatant by ELISA (Mean, Mann Whitney *U* test).

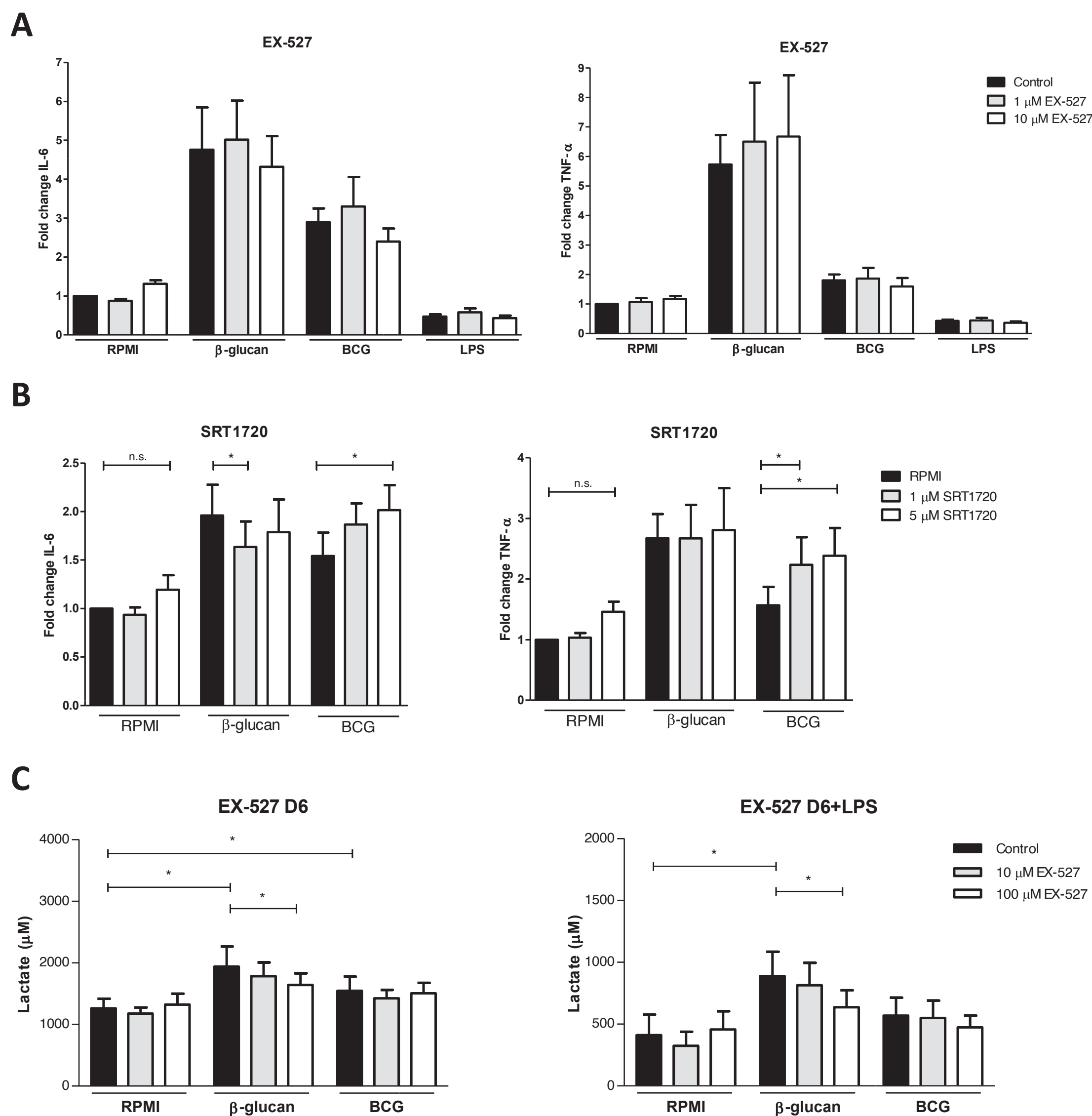

Fig. 4. Effect of SIRT1 modification on *in vitro* trained immunity. Monocytes derived from healthy individuals were stimulated *in vitro* with β-glucan (2 μg/mL), BCG (5 μg/mL), LPS (1 ng/mL), or non-stimulated (RPMI control), for 24 h, in combination with (A, C) EX-527, and (B) SRT1720, subsequently washed, rested for 5 days, and at day 6 restimulated for 24 h with LPS. IL-6 and TNF-α cytokine production and lactate concentrations were measured in supernatant. Mean ± SEM, *P < 0.05, Wilcoxon signed rank test.

restimulation in human macrophages 6 days after 24 h EX-527 treatment.

To conclude, genetic variation in SIRT1 is associated with cytokine responses of PBMCs to stimulation with various microbial and non-microbial stimuli, where SIRT1 inhibition results in increased innate pro-inflammatory cytokine production of PBMCs *in vitro*. Although SIRT1 genetic variants had a moderate effect on *in vitro* BCG- and β-glucan-induced trained immunity, this effect was not validated in *in vivo* models of BCG vaccination. Inhibition of SIRT1 function did not influence the induction of trained immunity in monocytes, whereas activation of SIRT1 only mildly modified trained immunity responses *in vitro*. The impact of SIRT1 inhibition or activation on the function of other immune cells is remains to be investigated. Taken together, despite its regulatory role in the acute induction of inflammation, SIRT1 does not play an important role in BCG- and β-glucan-induced trained immunity.

4. Methods

4.1. Reagents

RPMI 1640 (Dutch modified; Gibco, Life Technologies, MA) was used as culture medium supplemented with 5 μg/ml gentamicin (Centrafarm B.V., the Netherlands), 2 mM L-glutamine (Gibco), and 1 mM pyruvate (Gibco). Synthetic Pam3Cys (Pam3Cys) was purchased from EMC Microcollections (Germany), *Escherichia coli* lipopolysaccharide (LPS; serotype 055:B5, Sigma-Aldrich), β-glucan (β −1,3-(D)-glucan) was kindly provided by Professor David Williams (East Tennessee State University, TN), and bacillus Calmette Guérin (BCG) vaccine was purchased from InterVax (Markham, ON, Canada). SIRT1 inhibitor EX-527 was purchased from Sigma-Aldrich, SIRT1 activator SRT1720 was purchased from Selleckchem. *Candida albicans* UC820 (ATCC MYA-3573) was heat-killed at 95 °C for 30 min.

4.2. Blood samples

Peripheral blood mononuclear cells (PBMCs) were isolated from

buffy coats of healthy blood donors (Sanquin, Nijmegen, The Netherlands). Inclusion of volunteers and experiments were conducted according to the principles expressed in the Declaration of Helsinki. All volunteers gave written informed consent before any material was taken.

4.3. Population cohorts

QTL mapping using genotype data and cytokine production upon stimulation and induction of trained immunity was performed in a cohort of approximately 500 and 300 healthy individuals of Western European ancestry, respectively from the Human Functional Genomics Project (500FG and 300BCG, see www.humanfunctionalgenomics.org). The 500FG cohort comprises 534 adults from Nijmegen, the Netherlands (44% males and 56% females, age range 18–75 years). PBMCs were isolated and stimulated *in vitro* with various stimuli, and cytokines upon stimulation were measured, as previously described [34]. The 300BCG cohort consists of 325 adults from the Netherlands (43% males and 57% females, age range 18–71 years). PBMCs were isolated and seed in 96-wells plates (Corning, USA). After washing away the non-adherent cells with PBS the adherent cells were subsequently stimulated *in vitro* with BCG or β-glucan, and restimulated after 6 days with LPS, and cytokine production was subsequently measured. Furthermore, individuals from the 300BCG cohort were vaccinated with 0.1 mL of BCG (BCG vaccine strain Bulgaria; InterVax, Canada), and PBMCs were isolated, and stimulated *ex vivo* with 5×10^6 CFU/mL heat-killed *S. aureus* before vaccination, and 2 weeks and 3 months after vaccination. IL-1β, IL-6, and TNFα production was measured after 24 h in supernatants. The 500FG and 300BCG study were approved by the ethical committee of the Radboud University and Radboudumc Nijmegen (no. 42561.091.12 and NL58553.091.16).

4.4. PBMC isolation and stimulation

Cells were isolated by density centrifugation on Ficoll-Paque (GE Healthcare, UK), washed three times in PBS and resuspended in culture medium. After isolation, 5×10^5 PBMCs were added to a round bottom 96-wells plate (Greiner, Austria). Pam3Cys (10 μg/mL) or LPS (10 ng/mL) were added for 24 h at 37 °C and 5% CO_2, *Candida albicans* was added for 7 days (1×10^6 cells/mL), with or without addition of EX-527 (1–100 μM).

4.5. Monocyte isolation and stimulation

Percoll monocytes were isolated by layering hyper-osmotic Percoll solution (48,5% Percoll (Sigma-Aldrich), 41,5% sterile H_2O, 0.16 M filter-sterilized NaCl) on PBMCs. After 15 min centrifugation at 580 × g, the interphase layer was isolated, cells were washed with PBS, and resuspended in culture medium. To increase the purity of Percoll-isolated monocytes, the monocytes were adhered to polystyrene flat bottom plates (Corning, USA) or Petri dishes (Falcon, Merck) for 1 h at 37 °C followed by washing with warm PBS. Next, cells were pre-incubated with culture medium supplemented with 10% human pooled serum as control, or together with EX-527 (1–10 μM) or SRT1720 (1–5 μM). Next, culture medium supplemented with 10% human pooled serum was added as a control, or together with either 2 μg/mL β-glucan, 5 μg/mL BCG InterVax, or LPS (1 ng/mL). After 24 h, cells were washed with warm PBS and culture medium was added. Culture medium was refreshed after 3 days of incubation. On day 6, cells were restimulated with RPMI, LPS (10 ng/mL), or Pam3Cys (10 μg/mL). After 24 h, supernatants were collected and stored at −20 °C until further use.

4.6. Cytokine and lactate measurements

Cytokine concentrations were measured in supernatants using commercial sandwich ELISA kits for human IL-6, TNF-α, IL-1β, IL-17, IL-22 (R&D systems) and IFN-γ (Sanquin Research) in accordance with the manufacturer's instructions. Lactate concentration was measured in supernatants of macrophages using an enzymatic assay as described previously [35]. In brief, supernatants containing serum (day 6) were pre-treated with perchloric acid and NaOH to prevent potential protein interference with the assay. Lactate concentration was determined by enzymatic reaction with lactate oxidase (Sigma-Aldrich), Amplex Red reagent (Life Technologies) and horseradish peroxidase (Sigma-Aldrich). After 20 min incubation, fluorescence of resorufin (570/585 nm) was measured.

4.7. Quantitative RT-PCR

At baseline, after 4 h, 24 h, and 6 days, RNA was isolated from trained monocytes by using TRIzol reagent according to manufacturer's instructions. First-strand cDNA synthesis was performed using SuperScript III, followed by synthesis of the second cDNA strand (Thermo Fisher Scientific) according to the manufacturer's protocol. Quantitative PCR was performed using StepOne PLUS machine (Applied Biosciences) using SYBR Green (Invitrogen). The values are expressed as log2 fold increase in mRNA levels relative to those in non-trained cells. *18S* was used as a housekeeping gene. The primer sequences are listed below:

4.8. Viability assay

Cytotoxicity was analyzed by detecting lactate dehydrogenase (LDH) directly in fresh supernatant using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, the Netherlands), in accordance with the manufacturer's instructions.

4.9. Genetic analysis

Isolated DNA of the 500FG individuals was genotyped using the commercially available SNP chip, Illumina HumanOmniExpressExome-8 v1.0. Cytokine QTL mapping was conducted using the genotypes and cytokine measurements upon *in vitro* stimulation of isolated PBMCs, as previously described [23]. The most significantly associated SNP for a particular cytokine-stimuli combination is shown in Table 1. DNA samples from individuals of the 300BCG cohort 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 [36]. 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) ≤ 0.1. Strands of variants were aligned and identified against the 1000 Genome reference panel using Genotype Harmonizer [37]. We then imputed the samples on the Michigan imputation server using the human reference consortium (HRC r1.1 2016) as a reference panel [38], and we filtered out genetic variants with an $R^2 < 0.3$ for imputation quality. We identified and excluded 17 genetic outliers, and selected 4,296,841 SNPs with MAF 5% for follow-up QTL mapping. Both genotype and cytokine data on *in vitro* trained immunity responses induced by BCG or β-glucan in monocytes was obtained for a total of 267 individuals. Raw cytokine levels were log-transformed and the fold change of cytokine production between trained and non-trained cells was taken as a measurement for the magnitude of the *in vitro* trained immunity response. The cytokine changes were mapped to genotype data using a linear regression model with age and sex as covariates to correct the distributions of fold change of cytokine production. Similar approach was followed to identify QTLs using the ex vivo cytokine production from PBMCs after BCG vaccination in healthy volunteers. Both genotype and cytokine data on ex vivo trained immunity responses were obtained for a total of 296 individuals. The fold change in cytokine production (after vaccination compared to baseline) was used as a measurement of the magnitude of the trained immunity response. The fold change of cytokine production was log-transformed, and were mapped to genotype data using a linear

regression model with age and sex as covariates. R-package Matrix-eQTL was used for QTL mapping.

4.10. Flow cytometry

Isolated Percoll monocytes were exposed to EX-527 or vehicle control for 24 h. For cell surface marker analysis, cells were washed in PBS containing 1% BSA and stained with anti-CD14 APC (M5E2 clone), anti-HLA-DR PE-Cy5 (L243 clone), and anti-CD11b BV785 (ICRF44 clone; all Biolegend). Flow cytometry experiments were performed using the Beckman Coulter CytoFLEX. CD14 MFI was determined after exclusion of doublets. During analysis of HLA-DR and CD11b MFI, cells were gated on the CD14 + gate to eliminate debris and lymphocyte populations.

4.11. Statistics

Data was analyzed using a Wilcoxon signed-rank test for paired samples, or a Mann-Whitney *U* test for unpaired samples, using GraphPad Prism software (GraphPad Inc. version 8). Data are expressed as mean ± SEM, and values of $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered statistically significant.

CRediT authorship contribution statement

Vera P. Mourits: Project administration, Conceptualization, Methodology, Formal analysis, Writing - original draft, Validation, Visualization. **Leonie S. Helder:** Conceptualization, Investigation, Formal analysis, Writing - original draft, Visualization. **Vasiliki Matzaraki:** Data curation, Formal analysis, Investigation. **Valerie A.C.M. Koeken:** Data curation, Formal analysis, Investigation. **Laszlo Groh:** Data curation, Investigation. **L. Charlotte J. de Bree:** Data curation, Investigation. **Simone J.C.F.M. Moorlag:** Data curation, Investigation. **Charlotte D.C.C. van der Heijden:** Investigation. **Samuel T. Keating:** Investigation. **Jelmer H. van Puffelen:** Investigation. **Martin Jaeger:** Resources, Supervision. **Leo A.B. Joosten:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **Mihai G. Netea:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declaration of Interest

MGN and LABJ are scientific founders of Trained Therapeutics Discovery.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2021.104393.

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