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The sirtuin inhibitor cambinol impairs MAPK signaling, inhibits inflammatory and innate immune responses and protects from septic shock

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ABSTRACT

Sirtuins (SIRT1-7) are NAD^+-dependent histone deacetylases (HDACs) that play an important role in the control of metabolism and proliferation and the development of age-associated diseases like oncologic, cardiovascular and neurodegenerative diseases. Cambinol was originally described as a compound inhibiting the activity of SIRT1 and SIRT2, with efficient anti-tumor activity in vivo. Here, we studied the effects of cambinol on microbial sensing by mouse and human immune cells and on host innate immune responses in vivo. Cambinol inhibited the expression of cytokines (TNF, IL-1β, IL-6, IL-12p40, and IFN-γ), NO and CD40 by macrophages, dendritic cells, splenocytes and whole blood stimulated with a broad range of microbial and inflammasome stimuli. Sirtinol, an inhibitor of SIRT1 and SIRT2 structurally related to cambinol, also decreased macrophage response to TLR stimulation. On the contrary, selective inhibitors of SIRT1 (EX-527 and CHIC-35) and SIRT2 (AGK2 and AK-7) used alone or in combination had no inhibitory effect, suggesting that cambinol and sirtinol act by targeting more than just SIRT1 and SIRT2. Cambinol and sirtinol at anti-inflammatory concentrations also did not inhibit SIRT6 activity in in vitro assay. At the molecular level, cambinol impaired stimulus-induced phosphorylation of MAPKs and upstream MEKs. Going well along with its powerful anti-inflammatory activity, cambinol reduced TNF blood levels and bacteremia and improved survival in preclinical models of endotoxic shock and septic shock. Altogether, our data suggest that pharmacological inhibitors of sirtuins structurally related to cambinol may be of clinical interest to treat inflammatory diseases.

1. Introduction

The innate immune system provides the first line of defense against microbial infections. Macrophages and dendritic cells (DCs) use pattern-recognition receptors (PRRs) to sense molecular structures broadly shared by microorganisms (collectively called microbial-associated molecular patterns or MAMPs) such as LPS, lipopeptides, peptydoglycans, glucans, flagellin and nucleic acids. PRRs include TLRs, NOD-like receptors (NLRs), C-type lectin receptors, RIG-I like receptors and the cytosolic DNA sensors [1]. Recognition of microbial ligands by PRRs triggers intracellular signaling pathways, including the NF-κB, ERK1/2, p38 and JNK MAPK and IFN-regulatory factor (IRF) signaling pathways [1]. These events control the expression of cytokines and adhesion, major histocompatibility and co-stimulatory molecules that play a crucial role in the initiation, amplification and regulation of the inflammatory response and in the coordination of cellular and humoral responses aimed at the eradication or containment of invasive pathogens [2].

There are eighteen histone deacetylases (HDACs) in mammals, the "classical" Zn-dependent HDAC1-11, and the NAD^+-dependent sirtuins (SIRT) 1–7. HDACs are sub-grouped into class I (HDAC1, 2, 3, and 8), class II (HDAC4-7, 9, 10), class III (SIRT1-7) and class IV (HDAC11) [3–5]. HDACs catalyze the cleavage of acetyl groups from lysine residues. Deacetylation of histones by HDACs is associated with chromatin compaction and gene repression. HDACs also target numerous non-histones proteins, among which are tubulin, transcriptional regulators and enzymes. Accordingly, HDACs affect numerous biological and pathological processes, and have been implicated in the pathogenesis of oncologic, metabolic, cardiovascular, neurodegenerative and autoimmune diseases. Small compound inhibitors of classical HDACs are developed as potent anticancer drugs with remarkable tumor specificity. Beside their anticancer activity, inhibitors of classical HDACs exert powerful immuno-modulatory and anti-inflammatory activities [5,6].

Sirtuins have been proposed to promote longevity in several organisms, albeit this topic is of intense debates [7]. Sirtuins require NAD^+ for

Abbreviations: BMDCs, bone marrow-derived dendritic cells; BMDMs, bone marrow-derived macrophages; CpG, CpG motif containing oligonucleotide 1826; DC, dendritic cell; *E. coli*, *Escherichia coli* O18; HDAC, histone deacetylase; LPS, lipopolysaccharide; MKP, MAPK phosphatase; *K. pneumonia*, *Klebsiella pneumoniae* Caroli; Pam_3CSK_4, N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine; *S. aureus*, *Staphylococcus aureus* AW7; SIRT, sirtuin

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their enzymatic activity and thus are connected to metabolism. Sirtuins have been implicated in age related pathologies including metabolic, neurodegenerative, cardiovascular and oncologic diseases [8–10]. The picture is not so clear concerning the influence of sirtuins on immune responses. Indeed, both pro-inflammatory and anti-inflammatory activities have been attributed to SIRT1 and SIRT6 [11–22]. Pharmacological inhibitors of sirtuins are developed for their potential curative usage in metabolic and oncologic diseases. Most of sirtuin inhibitors available today target SIRT1, the best characterized sirtuin, and SIRT2. Recently, cambinol was identified as a β-naphthol compound that inhibits SIRT1 and SIRT2 with similar IC_{50}s [23]. Cambinol competes with substrate but not with NAD^+ binding to SIRT1 and SIRT2, which may explain its selectivity and low toxicity. Interestingly, cambinol possesses antitumor activity in vivo [23].

Considering that sirtuins share targets with HDAC1-11, and considering that we and others have described the powerful anti-inflammatory activity of inhibitors of classical HDACs in vitro and in vivo [6,24,25], we questioned whether the sirtuin inhibitor cambinol modulates inflammatory and innate immune responses. Here, we report that cambinol, but not selective SIRT1 and SIRT2 inhibitors, strongly affects the response of macrophages and DCs to a large panel of pathogen- and danger-associated molecular patterns. Cambinol inhibits MEK activation thereby impairing phosphorylation-induced MAPK signaling and immune gene expression. Most importantly, cambinol protects animals from endotoxic and septic shock. Our data support the concept of cambinol-like pharmacological inhibitors as promising drugs to treat inflammatory diseases.

2. Materials and methods

2.1. Ethics statement

Animal experimentations were approved by the Office Vétérinaire du Canton de Vaud (authorizations n° 876.7 and 877.7) and performed according to our institution guidelines.

2.2. Mice, cells and reagents

BALB/c mice (8–12 week females; Charles River Laboratories) were housed under specific pathogen-free conditions. Bone marrow cells were cultured in IMDM containing 50 μM 2-ME and M-CSF to generate bone marrow-derived macrophages (BMDMs), or GM-CSF to generate bone marrow-derived dendritic cells (BMDCs). Splenocytes were cultured in RPMI 1640 medium containing 2 mM glutamine and 50 μM 2-ME [25,26]. Mouse RAW 264.7 macrophages (ATCC) were cultured in RPMI 1640 medium containing 2 mM glutamine [27]. Culture media (Invitrogen) were supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), 100 UI/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Human whole blood assay was performed as previously described [28].

Cells were exposed to *Salmonella minnesota* ultra pure lipopolysaccharide (LPS, List Biologicals Laboratories), N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine (Pam_3CSK_4, EMC microcollections), CpG motif containing oligonucleotide 1826 (CpG) (Invivogen), or heat-inactivated (56 °C for 2 h) *Escherichia coli* (*E. coli*) O18 and *Staphylococcus aureus* (*S. aureus*) AW7. Octacalcium phosphate crystals (OCT), a gift of Dr N. Busso (CHUV, Lausanne, Switzerland) were prepared as described [29]. The sirtuin pharmacological inhibitors cambinol [23], sirtinol [30], EX-527 [31], AGK2 [32], AK-7 [32] and CHIC-35 [31] and DMSO (Sigma-Aldrich) were used at concentrations giving <20% cell death after 18 h of culture to exclude nonspecific toxic effect. Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell proliferation and viability assay [33].

2.3. Cytokine and NO measurements

TNF, IL-6, IL-1β and IFN-γ concentrations in cell culture supernatants or plasma were measured using DuoSet ELISA kits (R&D Systems). TNF and IL-6 produced by human whole blood and PBMCs were measured using WEHI 164 cells (TNF) and 7TD1 cells (IL-6) [34]. NO concentrations were measured using the Griess reagent [35]. Serial dilutions of $NaNO_2$ were used as a standard.

2.4. RNA analyses by real-time polymerase chain reaction

Total RNA was isolated with the RNeasy kit (QIAGEN), reverse transcribed using the QuantiTect reverse transcription kit (QIAGEN) and used for real-time PCR analyses using a 7500 FAST Real-Time PCR System (Applied Biosystems) as described [36]. Primer pairs are listed in Supplementary Table 1. Amplifications with the 7500 Fast mode consisted of forty cycles of 3 second denaturation step at 95 °C and 30 second annealing/extension step at 60 °C. Samples were tested in duplicates. Gene specific expression was normalized to an endogenous control (GAPDH or hypoxanthine guanine phosphoribosyl transferase, HPRT) and expressed in arbitrary units relative to the expression in untreated cells.

2.5. Flow cytometry

Cells were incubated 30 min at 4 °C in PBS containing 5 mM EDTA, 5% FCS, 2.4G2 monoclonal antibody (Fc-Block™, BD Biosciences) and anti-mouse CD40 antibody (3/23-biotin revealed with CyChrome-conjugated streptavidin, BD Biosciences). Flow cytometric analyses were performed using a FACSCalibur™ flow cytometer (BD Biosciences) and data analyzed using FlowJo 8.8.6 software (FlowJow) [25,37].

2.6. Proliferation assay

The proliferation of splenocytes (1.5×10^5) cultured for 48 h in 96-well plates was assessed by measuring ^{3}H-thymidine incorporation over 18 h [25].

2.7. Impact of cambinol and sirtinol on the deacetylase activity of SIRT6

The deacetylase activity of recombinant SIRT6 incubated with increasing concentrations of cambinol and sirtinol was determined using the SIRT6 Screening Assay Kit from Abcam, according to the manufacturer's recommendations. Briefly, recombinant sirtuin 6 was incubated for 45 min at 37 °C with cambinol or sirtinol, NAD^+ and a peptide substrate corresponding to amino acids 379–382 of human p53 conjugated to aminomethylcoumarin. The reaction was stopped with the stop solution containing nicotinamide. Fluorescence was read with excitation wavelength of 355 nm and measure wavelength of 460 nm. Background fluorescence was substracted to values. Percent activity was calculated using the formula: % activity = (activity with inhibitor / activity without inhibitor) × 100.

2.8. Western blot analyses

Nuclear and cytosolic protein extracts and total histones were electrophoresed through SDS-polyacrylamide gels, transferred onto nitrocellulose membranes and revealed as described previously [38–40]. Membranes were incubated with primary antibodies specific for total and acetylated histone H4, ERK1/2, phospho-ERK1/2, p38, phospho-p38, JNK, phospho-JNK, phospho-MEK1/2, phospho-MEK4 (Cell Signaling Technology), actin, NF-κB p65 (Santa Cruz Biotechnology) and tubulin (Sigma-Aldrich), then with a secondary horseradish peroxidase-conjugated antibody (Sigma-Aldrich) [41]. Blots were revealed with the enhanced chemiluminescence (ECL) Western blotting system (GE Healthcare). Images were recorded using a LAS4000 system (Fujifilm).

2.9. Luciferase assay

RAW 264.7 macrophages (at 30% confluence in 24-well plates) were transfected with 600 ng of a AP-1 luciferase reporter vector together with 60 ng of the *Renilla* pRL-TK vector (Promega) using the Fugene 6 transfection reagent (Roche Applied Sciences). Cells were pre-incubated 1 h with or without cambinol (50 μM) and then exposed for 18 h to LPS (10 ng/ml). Luciferase and *Renilla* luciferase activities were quantified using the Dual-Luciferase Reporter Assay System (Promega). Results were expressed as the ratio of luciferase activity to *Renilla* luciferase activity [42,43].

2.10. Growth of Klebsiella pneumoniae in vitro

Klebsiella pneumoniae Caroli (*K. pneumoniae*) was cultured overnight at 37 °C in Luria–Bertani (LB) broth (BD biosciences). The following day, 40 μl of the bacterial suspension was diluted 100-fold in LB broth containing 0, 3.1, 12.5, 50 or 200 μM cambinol and incubated at 37 °C under agitation. Optical density was recorded at 640 nm.

2.11. In vivo models

To induce endotoxemia, mice were injected i.p. with 350 μg LPS. Cambinol (10 mg/kg i.p.) was injected 1 h before and 24 h after LPS challenge. To induce lethal sepsis, mice were injected intra-nasally with 20–60 CFU of *K. pneumoniae*. Mice were treated with cambinol (10 mg/kg i.p.) or vehicle three consecutive days starting 24 h before bacterial challenge. Blood was collected to quantify TNF and circulating bacteria. Body weight, severity scores and survival were registered at least once daily [26].

2.12. Statistical analyses

Comparisons between the different groups were performed by analysis of variance followed by the Fisher's exact test for categorical data and the Mann–Whitney tests for continuous variables. The Kaplan–Meier method was used for survival and differences were analyzed by the log-rank sum test. All analyses were performed using PRISM (GraphPad Software). *P* values are two-sided, and values < 0.05 were considered to indicate statistical significance.

3. Results

3.1. Cambinol inhibits cell response to microbial stimulation

Bone marrow-derived macrophages (BMDMs) stimulated with microbial products recognized through TLR4 (LPS), TLR1/TLR2 (Pam_3CSK_4) and TLR9 (CpG) or with bacteria sensed primarily through TLR4 (*E. coli*) and TLR2 (*S. aureus*) produced large amounts of TNF, IL-6 and IL-12p40 (Fig. 1A–B and data not shown). Cambinol inhibited TNF, IL-6 and IL-12p40 secretion in a dose-dependent manner. Percent inhibition of cytokine release ranged from 75% to 98% using 50 μM cambinol, which is close to cambinol's IC_{50} for SIRT1 (56 μM) and SIRT2 (59 μM) [23]. The inhibition of cytokine production was unlikely dependent from a toxic effect of cambinol, as cell viability measured at 4 and 18 h was $>95\%$ and $>80\%$, respectively. Moreover, real-time PCR analyses revealed that cambinol interfered with LPS- and Pam_3CSK_4-induced *Tnf*, *Il6* and *Il12b* gene expression (1, 4 and 18 h after stimulation; Fig. 1C–D and data not shown), suggesting that cambinol inhibits cytokine gene transcription. Confirming these results, cambinol also inhibited LPS-induced *Il1b* mRNA upregulation and octacalcium phosphate crystals-mediated IL-1β secretion (Fig. 1E–F). Upon infection, macrophages produce reactive nitrogen species that are highly toxic for microorganisms and upregulate CD40 co-stimulatory molecules involved in cell–cell interactions. NO is produced during the nitrosative burst by the inducible NO synthase (iNOS, encoded by *Nos2*) [44]. In BMDMs, cambinol dose-dependently inhibited *Nos2* induction and NO production in response to LPS, Pam_3CSK_4, CpG, *E. coli* and *S. aureus* (Fig. 2A–B). Furthermore, real-time PCR and flow cytometry analyses showed that cambinol inhibited the upregulation of Cd40 mRNA and CD40 receptor (Fig. 2C–D).

We then analyzed the effects of cambinol on the response of bone marrow-derived dendritic cells (BMDCs), splenocytes and human whole blood and PBMCs to extend our observations beyond macrophages. As observed with BMDMs, cambinol decreased the secretion of TNF, IL-6 and IL-12p40 and the upregulation of CD40 by BMDCs (Fig. 3A–B and data not shown). Moreover, cambinol dose-dependently inhibited the proliferation of splenocytes induced by toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxin B (SEB), LPS and Pam_3CSK_4 (Fig. 3C), and inhibited the production of IFN-γ by splenocytes exposed to concanavalin A (Fig. 3D). Finally, cambinol markedly reduced TNF and IL-6 levels in human whole blood (71–85% and 52–66% reduction of TNF and IL-6 using 50 μM cambinol in two donors; Fig. 3E) and PBMCs (data not shown) stimulated with LPS. Overall, these results indicated that cambinol has powerful anti-inflammatory and immunosuppressive activity, interfering with the response of master regulators of innate immune responses like macrophages and DCs.

3.2. Sirtinol inhibits cytokine production by macrophages; selective SIRT1 and SIRT2 inhibitors do not

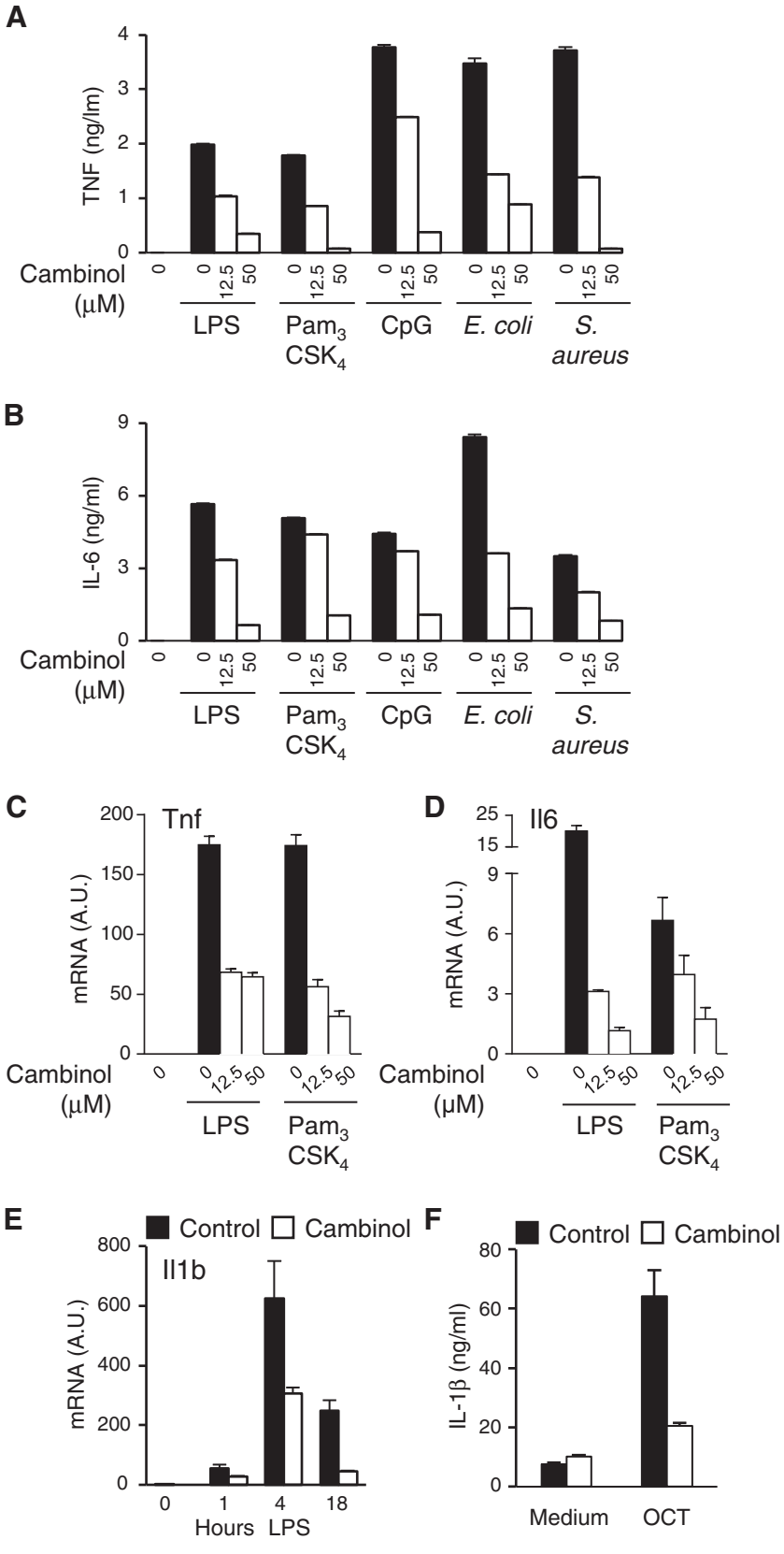
To substantiate cambinol-mediated inhibition of cytokine production by macrophages, we tested sirtinol, an inhibitor of SIRT1 and SIRT2 ($IC_{50\ SIRT1} = 131$ μM; $IC_{50\ SIRT2} = 38$ μM; Table 1) that shares with cambinol a hydroxynaphthaldehyde type of structure [30,45,46]. Like cambinol, sirtinol impaired TNF and IL-6 production by BMDMs stimulated with LPS, Pam_3CSK_4, CpG ODN, *E. coli* and *S. aureus* (Fig. 4A–B and data not shown). Moreover, sirtinol, as well as cambinol, inhibited the production of TNF and IL-6 by RAW 264.7 macrophages, peritoneal macrophages and human whole blood stimulated with LPS and Pam_3CSK_4 (Fig. 4C–D and data not shown).

To define whether inhibition of SIRT1 or SIRT2 alone have anti-inflammatory effects, we used the selective SIRT1 inhibitors EX-527 ($IC_{50\ SIRT1} = 0.1$–1 μM; $IC_{50\ SIRT2} = 20$–33 μM) and CHIC-35 ($IC_{50\ SIRT1} = 0.06$–0.12 μM; $IC_{50\ SIRT2} = 2.8$ μM), and the selective SIRT2 inhibitors AGK2 ($IC_{50\ SIRT2} = 3.5$ μM; $IC_{50\ SIRT1} > 50$ μM) and AK-7 ($IC_{50\ SIRT2} = 15.5$ μM; $IC_{50\ SIRT1} > 50$ μM) (Table 1) [31,32,45,47]. None of these inhibitors used over a large range of concentrations (around 1/4, 1 and 4-fold the IC_{50}s) inhibited LPS-induced upregulation of Tnf, Il6 and Il12b mRNA (Fig. 5A) and TNF and IL-6 release by BMDMs (Fig. 5B) and RAW 264.7 macrophages (data not shown).

Next, we thought to test whether dual inhibition of SIRT1 and SIRT2 was required to recapitulate the anti-inflammatory effects of cambinol and sirtinol. For that purpose, we tested all possible combinations of EX-527 or CHIC-35 with AGK2 or AK-7 at the concentrations reported above (1/4, 1 and 4-fold IC_{50}s). Unexpectedly, combined treatment

Fig. 1. Cambinol inhibits cytokine production by macrophages exposed to microbial products and bacteria. (A–D) BMDMs were pre-incubated for 1 h with or without cambinol before exposure for 4 h (A, C and D) or 18 h (B) to LPS (10 ng/ml), Pam_3CSK_4 (10 ng/ml), CpG (2 μg/ml), *E. coli* (10^6 CFU/ml) and *S. aureus* ($5x10^6$ CFU/ml). TNF and IL-6 concentrations in cell culture supernatants and Tnf and Il6 mRNA levels were quantified by ELISA (A, B) and real time-PCR (C, D). Tnf and Il6 mRNA levels were normalized to GAPDH mRNA levels. Data are means ± SD of triplicate samples from one experiment representative of 2 to 4 experiments. $P < 0.05$ for all concentrations of cambinol versus control. A.U.: arbitrary units. (E) BMDMs were pre-incubated for 1 h with or without cambinol (50 μM) before exposure to LPS (10 ng/ml). Il1b mRNA levels were quantified by real time-PCR and normalized to GAPDH mRNA levels. (F) BMDMs were primed 14 h with LPS (100 ng/ml), washed, pre-incubated for 1 h with or without cambinol (50 μM) and exposed for 6 h with octacalcium phosphate crystals (OCP, 500 μg/ml). IL-1β concentrations in cell culture supernatants were quantified by ELISA. Data are means ± SD of triplicate samples from one experiment. $P < 0.05$ for OCT plus cambinol versus OCT. A.U.: arbitrary units.

with SIRT1 and SIRT2 inhibitors did not inhibit TNF and IL-6 release by BMDMs and RAW 264.7 macrophages. Indeed, as illustrated in Fig. 6A–B, LPS-stimulated BMDMs exposed to EX-527 plus AGK2 or CHIC-35 plus AK-7 produced normal or supra-normal levels of TNF. In line with these observations, EX-527 at concentrations inhibiting both SIRT1 and SIRT2 (7.5, 30 and 120 μM = 8, 32 and 120-fold $IC_{50\ SIRT1}$ and 1/4, 1 and 4-fold $IC_{50\ SIRT2}$) did not reduce LPS-induced IL-6 and IL-12p40 mRNA and protein expression by BMDMs, and even slightly enhanced TNF production (Fig. 6C–D). Nonetheless, the SIRT1 and SIRT2 inhibitors were active in primary macrophages, as demonstrated



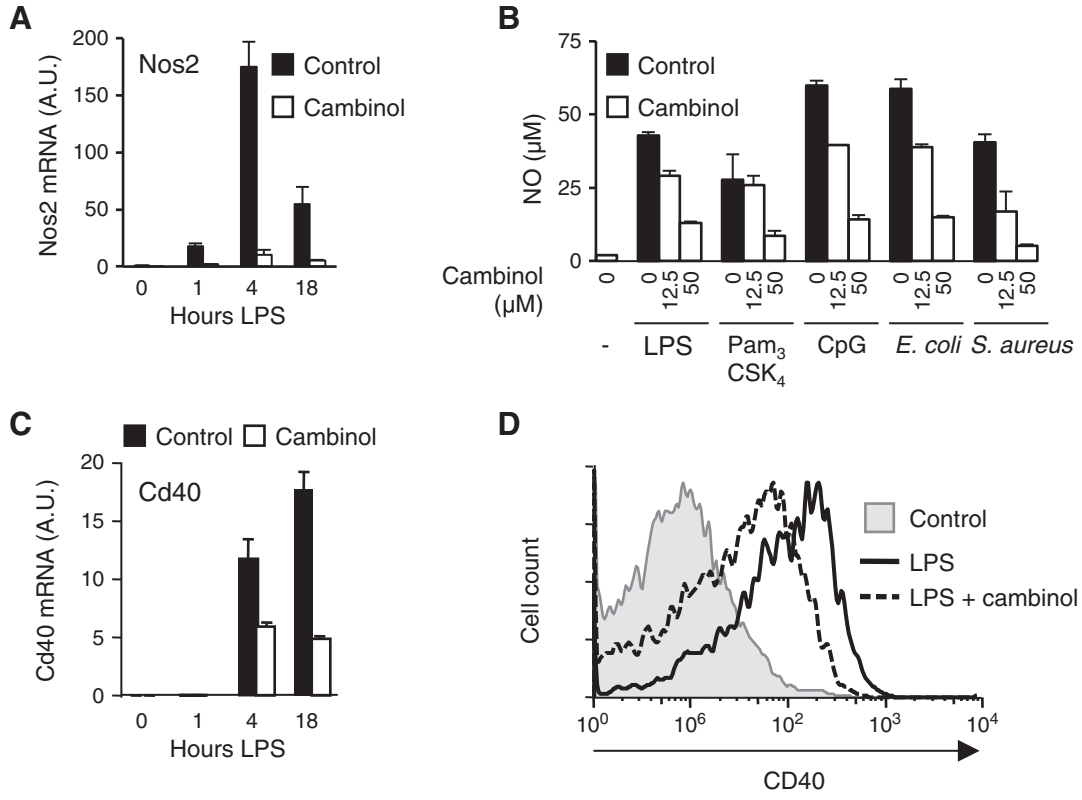


Fig. 2. Cambinol inhibits the response of macrophages to Toll-like receptor stimuli. (A–B) BMDMs were primed 18 h with IFN-γ (100 U/ml), washed, pre-incubated for 1 h with or without cambinol (50 μM in A) and exposed to stimuli as mentioned in Fig. 1. (A) Nos2 mRNA levels were quantified by real time-PCR and normalized to GAPDH mRNA levels. (B) Nitrites/nitrates in cell culture supernatants collected after 18 h were quantified using the Griess reagent. Data are means ± SD of triplicate samples from one experiment representative of 2 experiments. $P < 0.05$ for cambinol versus control, except for 12.5 μM cambinol + Pam_3CSK_4. (C–D) BMDMs were pre-incubated for 1 h with or without cambinol (50 μM) before exposure to LPS (10 ng/ml). (C) Cd40 mRNA levels were quantified by real time-PCR and normalized to GAPDH mRNA levels. Data are means ± SD of triplicate samples from one experiment representative of 2 experiments. (D) Flow cytometry analysis of CD40 expression by cells collected after 18 h. Data are representative of 2 experiments.

by the fact that CHIC-35, EX-527, AGK2 and AK-7 increased acetylation of histone H4 similar to cambinol (Fig. 6E).

Considering that SIRT6 has been reported to promote cytokine production by innate immune cells [16,21], we questioned whether cambinol and sirtinol mediated their effects by targeting SIRT6 activity. Therefore, we quantified the deacetylase activity of recombinant SIRT6 incubated in the presence of increasing concentrations of cambinol and sirtinol. As shown in Fig. 6F, cambinol and sirtinol used at concentrations able to inhibit cytokine production by macrophages (Fig. 1) did not reduce the enzymatic activity of SIRT6. Thus, cambinol and sirtinol inhibit SIRT1, SIRT2 and other targets that are neither SIRT3 nor SIRT6 ([48] and present data).

Altogether, these data suggested that cambinol and sirtinol inhibit microbial product-induced cytokine production through targeting more than just SIRT1 and SIRT2. They also strongly advocated pursuing the development of hydroxynaphthaldehyde type of inhibitors as efficient anti-inflammatory compounds.

3.3. Influence of cambinol on the expression of sirtuins and pattern recognition receptors

Considering the above results, we analyzed whether cambinol influenced sirtuin expression. We first measured by real-time PCR the expression of Sirt1-7 in BMDMs exposed for 0, 1, 4 and 18 h to LPS. Resting BMDMs expressed predominantly Sirt2, 4-6-fold less Sirt1 and Sirt7, and 15-85-fold less Sirt4-Sirt6 (Fig. 7A). LPS increased 2-fold Sirt1 mRNA at 4 h and 2-7-fold Sirt2-Sirt7 mRNA at 18 h (Fig. 7B). Cambinol modestly impaired LPS-induced Sirt1 (4 h) and Sirt5 (18 h) mRNA upregulation, and slightly increased Sirt3 (18 h) and Sirt7 (4 h) mRNA expression (Fig. 7B). Western blot analyses confirmed these results, showing that LPS faintly increased SIRT1 and SIRT2 protein levels (SIRT1: 1.3-fold, SIRT2: 3.0-fold, t = 18 h) and that cambinol had almost no additional effect on the expression of SIRT1 and SIRT2 (Fig. 7C). Thus, cambinol does not mediate its effects by modulating sirtuin expression.

We then questioned whether cambinol impaired innate immune responses by targeting the expression of pattern recognition receptors involved in the sensing of LPS (Tlr4, Cd14, and Md2), Pam_3CSK_4 (Tlr1, Tlr2, and Cd36), CpG ODN (Tlr9), OCT crystals (Nlrp3: NLR family, pyrin domain containing 3) and bacteria (Msr1: macrophage scavenger receptor 1/CD204; and Itgax: $integrin_{\alpha X}$/CD11c). Real-time PCR analyses revealed that cambinol (50 μM) inhibited LPS-mediated early upregulation of Nlrp3 (79% reduction at 4 h) and late upregulation of Tlr1, Tlr2 and Md2 (30–59% reduction at 18 h). In contrast, cambinol did not modify the expression of Tlr4, Tlr6, Cd36, Clec7a (encoding for dectin-1), Msr1, Itgax and Itgb2 ($integrin_{\beta 2}$/CD18) and increased Tlr9 and Cd14 (Fig. 7D and data not shown). Hence, the early on inhibition of *Tnf*, *Il1b*, *Il6*, *Il12b*, *Cd40* and *iNos* gene expression by cambinol (Figs. 1 and 2) unlikely resulted from an effect on the expression of pattern recognition receptors. Considering that Nlrp3 has to be induced to generate a functional inflammasome in macrophages [49], inhibition of Nlrp3 expression by cambinol may play a role in blunting maturation and secretion of IL-1β (Fig. 1F).

3.4. Cambinol impairs MAPK signaling

The sensing of microbial products through TLRs initiates intracellular signaling leading to the activation of ERK1/2, p38 and JNK MAPKs and

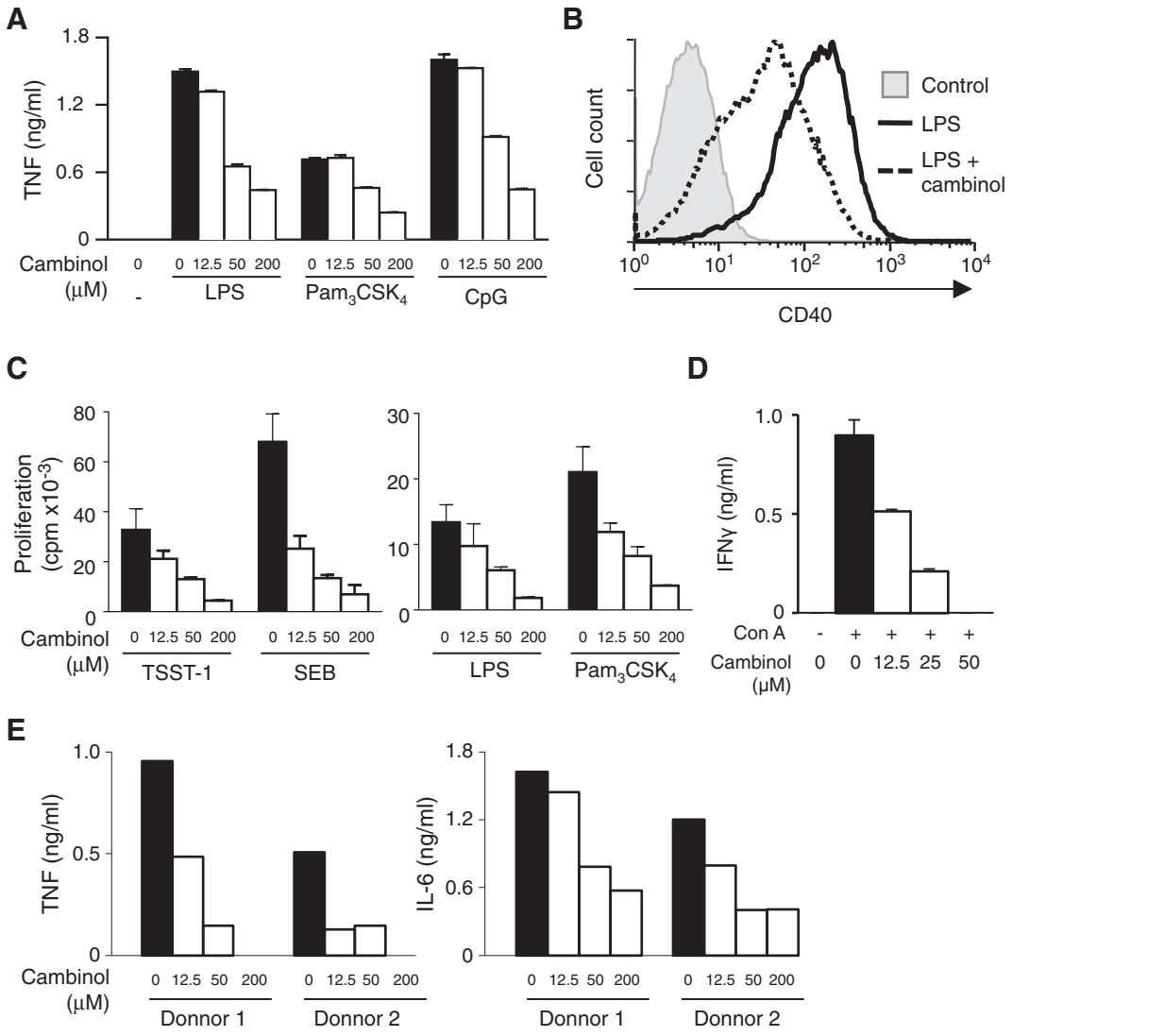


Fig. 3. Cambinol inhibits the response of dendritic cells, splenocytes and whole blood. BMDCs were pre-incubated for 1 h with or without cambinol before exposure for 4 h (A) or 18 h (B) to LPS (10 ng/ml), Pam_3CSK_4 (10 ng/ml) and CpG (2 μg/ml). (A) TNF concentrations in cell culture supernatants were determined by ELISA. Data are means ± SD of triplicate samples from one experiment representative of 2 experiments. $P < 0.05$ for 50 μM and 200 μM cambinol versus control. (B) CD40 expression analyzed by flow cytometry. Data are representative of 2 experiments. (C–D) Splenocytes were incubated 48 h with cambinol and toxic shock syndrome toxin-1 (TSST-1, 2 μg/ml), staphylococcal enterotoxin B (SEB, 1 μg/ml), LPS (100 ng/ml), Pam_3CSK_4 (100 ng/ml) and concanavalin A (Con A; 5 μg/ml). (C) Proliferation was measured by ^{3}H-thymidine incorporation. $P < 0.05$ for cambinol versus control, except 12.5 μM cambinol with TSST-1 and LPS. (D) IFN-γ concentrations in cell culture supernatants were quantified by ELISA. Data are means ± SD of triplicate samples from one experiment representative of 2 experiments. $P < 0.05$ for cambinol versus control. (E) Whole blood from two healthy volunteers was incubated for 18 h with cambinol and LPS (100 ng/ml). TNF and IL-6 concentrations in cell culture supernatants were quantified by bioassay. Data are means of duplicate samples from one experiment.

NF-κB and AP-1 transcription factors which control the transcriptional activation of immune genes [1]. We examined whether cambinol affected signal transduction in macrophages. Interestingly, cambinol strongly interfered with LPS-induced phosphorylation of ERK1/2, p38 and JNK MAPKs, whereas it barely affected NF-κB p65 nuclear translocation (Fig. 8A). In agreement, cambinol impaired LPS-induced AP-1-mediated transcriptional activity in macrophages transfected with an AP1-responsive luciferase construct (Fig. 8B). The phosphorylation

Table 1
IC_{50} (in μM) of the inhibitors used in this study.

	SIRT1	SIRT2	SIRT3	SIRT4	SIRT5	SIRT6	SIRT7
Cambinol	56	59	No inhibition	Not tested	42% inhibition at 300 μM	No inhibition up to 250 μM*	Not tested
Sirtinol	37.6–131	38–58	>50	?	?	No inhibition up to 62.5 μM*	?
EX-527	0.1–1	20–33	49	?	No inhibition	?	?
AGK2	>50	3.5	>50	Not tested	Not tested	Not tested	Not tested
AK-7	>50	15.5	>50	Not tested	Not tested	Not tested	Not tested
CHIC-35	0.06–0.12	2.8	No inhibition	?	?	?	?

IC_{50}s defined in vitro are expressed in μM [30–32,45–47].
?: Not mentioned in studies testing these drugs (probably not tested).
* Data obtained in this study.

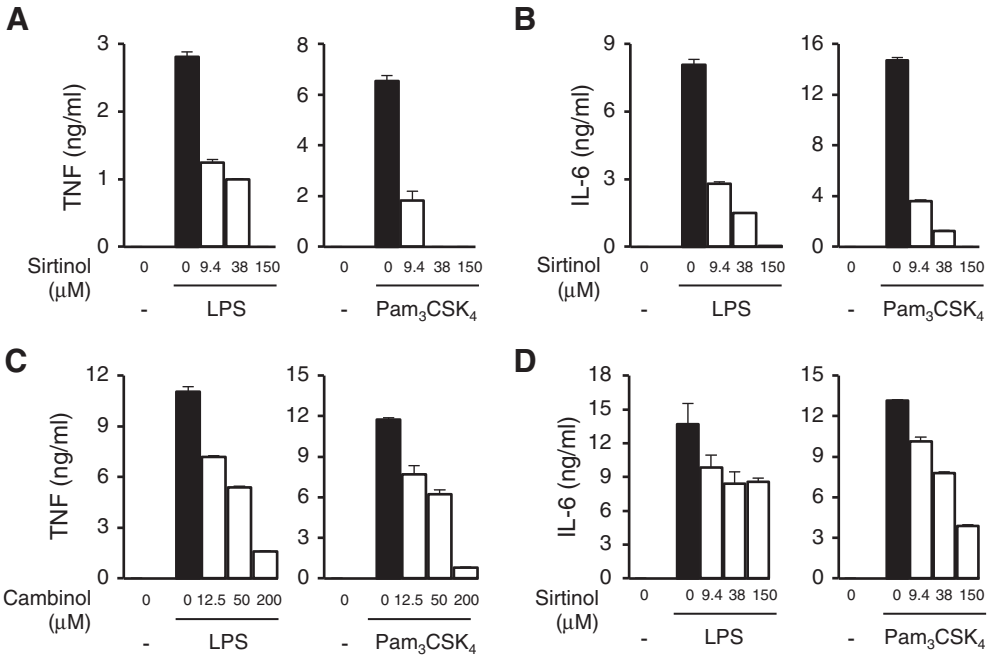


Fig. 4. Sirtinol inhibits cytokine production by macrophages. BMDMs (A–B) and RAW 264.7 macrophages (C–D) were pre-incubated for 1 h with or without sirtinol and cambinol before exposure for 4 h (A, C) or 18 h (B, D) to LPS (10 ng/ml) and Pam_3CSK_4 (10 ng/ml). Concentrations of TNF and IL-6 in cell culture supernatants were quantified by ELISA. Data are means ± SD of triplicate samples from one experiment representative of 2 experiments. $P < 0.05$ for all concentrations of sirtinol and cambinol versus control.

of MAPKs is catalyzed by MEKs (MAP2Ks) that are quickly activated following microbial sensing. Western blot analyses revealed that cambinol inhibited LPS-mediated phosphorylation of MEK1/2 (upstream ERK1/2) at 15 min and phosphorylation of MEK4 (upstream JNK and p38) at 15–60 min (Fig. 8C). These observations suggested that cambinol interferes with inflammatory responses at least in part through inhibiting MAPK and MEK activation.

3.5. Cambinol protects from lethal endotoxemia and septic shock

Severe sepsis and septic shock are characterized by an early overwhelming inflammatory response, and interfering with the release or the activity of pro-inflammatory mediators conferred protection in pre-clinical models of sepsis [50,51]. To investigate in vivo the relevance of our in vitro data, we first analyzed the possible protective

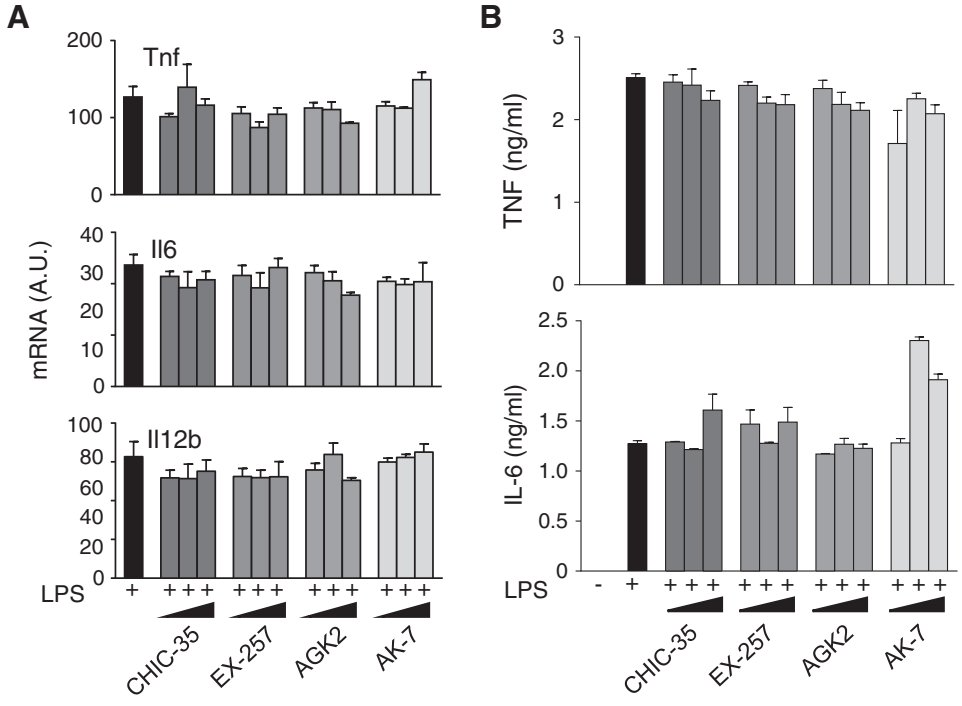


Fig. 5. Selective inhibitors of Sirt1 or Sirt2 used alone or in combination do not inhibit cytokine production by macrophages. BMDMs were pre-incubated for 1 h with or without EX-527 (0.25, 1, and 4 μM), AGK2 (0.8, 3.5, and 14 μM), AK-7 (6, 24, and 96 μM), CHIC-35 (30, 120, and 480 nM) before exposure for 4 h (A and B upper panel) or 18 h (B lower panel) to LPS (10 ng/ml). Tnf, Il6 and Il12b mRNA levels were quantified by real time-PCR and normalized to GAPDH mRNA levels (A). Tnf, Il6 and Il12b mRNA were not detected in not stimulated cells (see Fig. 1). Data are means ± SD of triplicate samples from one experiment. Concentrations of TNF and IL-6 in cell culture supernatants were quantified by ELISA (B). Data are means ± SD of triplicate samples from one experiment representative of 2 experiments.

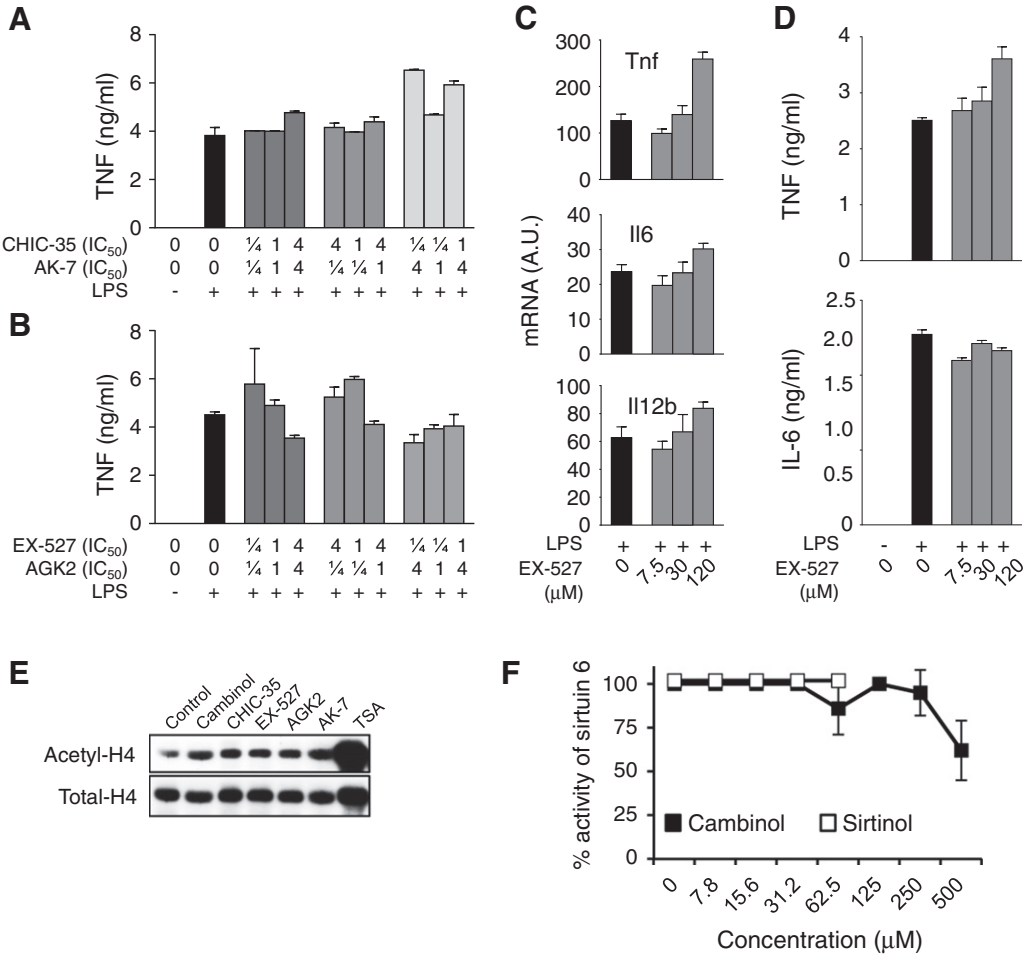


Fig. 6. Dual inhibition of Sirt1 or Sirt2 does not reduce cytokine production by macrophages. BMDMs were pre-incubated for 1 h with combination of CHIC-35 (1/4, 1 and 4: 30, 120 and 480 nM) and AK-7 (1/4, 1 and 4: 6, 24, and 96 μM) (A) or EX-527 (1/4, 1 and 4: 0.25, 1, and 4 μM) and AGK2 (1/4, 1 and 4: 0.8, 3.5, and 14 μM) (B) before exposure for 4 h to LPS (10 ng/ml). Concentrations of TNF in cell culture supernatants were quantified by ELISA. Data are means ± SD of triplicate samples from one experiment representative of 2 experiments. BMDMs were pre-incubated for 1 h with or without EX-527 at concentrations inhibiting SIRT1 and SIRT2 (7.5, 30, and 120 μM) before exposure for 4 h (C and D upper panel) or 18 h (D lower panel) to LPS (10 ng/ml). Tnf, Il6 and Il12b mRNA levels were quantified by real time-PCR and normalized to GAPDH mRNA levels (C). Tnf, Il6 and Il12b mRNA were not detected in not stimulated cells (see Fig. 1). Concentrations of TNF and IL-6 in cell culture supernatants were quantified by ELISA (D). Data are means ± SD of triplicate samples from one experiment. (E) BMDMs were incubated for 16 h with cambinol (200 μM), CHIC-35 (0.5 μM), EX-527 (1 μM), AGK2 (10 μM), AK7 (100 μM) and TSA (40 nM). Expression levels of acetylated and total histone 4 (H4) were analyzed by Western blotting. Results are representative of two independent experiments. (F) Activity of recombinant SIRT6 incubated for 45 min at 37°C with cambinol (0–500 μM) or sirtinol (0–62.5 μM, higher concentrations interfered with measurement). SIRT6 activity was measured using the SIRT6 Screening Assay Kit according to the manufacturer's recommendations. Percent activity was calculated using the formula: % activity = (activity with inhibitor / activity without inhibitor) × 100. Data represent the average of two independent measurements.

effect of cambinol in a model of endotoxemia (17.5 mg/kg LPS intraperitoneally). Administration of cambinol significantly reduced TNF circulating levels (1.5-fold, $P = 0.04$, Fig. 9A) and remarkably increased survival from 8% to 46% ($P < 0.001$, Fig. 9B). To further substantiate the concept of immunomodulation by cambinol as a treatment strategy for severe sepsis, we tested cambinol in an experimental model of lethal, acute *K. pneumoniae* pneumonia. Cambinol increased survival from 13% to 60% ($P = 0.013$, Fig. 9C), which was associated with a trend towards reduced bacterial burden in the lungs (median counts: 170 versus 5800 CFU/lung in cambinol versus DMSO treated mice; $P = 0.3$). Importantly, cambinol had no direct toxic effect on *K. pneumonia* in vitro (Fig. 9D).

4. Discussion

HDAC inhibitors have been widely studied in the field of cancer; their influence on the innate immune system is less well characterized. This is particularly true for inhibitors of sirtuins, which were developed more recently than inhibitors of classical HDACs (HDAC1-11). Here, we report that cambinol, a compound that inhibits the deacetylase activity of SIRT1 and SIRT2 [23], powerfully inhibits inflammatory and innate immune responses in vitro and in vivo. Importantly, cambinol protects mice from lethal endotoxic and septic shock. In line with our results, reducing the bioavailability of the sirtuin's co-factor NAD^+ through inhibition or deletion of the nicotinamide phosphoribosyltransferase (Nampt) decreased TNF and IFN-γ secretion by PHA-stimulated peripheral blood lymphocytes and TNF production in mice challenged with LPS [16,21,52]. These observations could argue for a role of sirtuins in the regulation of innate immune gene expression and in host defensive responses against microbial infections. Yet, as discussed below, cambinol acts probably by targeting more than just SIRT1 and SIRT2.

Inhibitors of classical HDACs impair numerous facets of inflammatory and innate immune responses and have shown great efficacy in models of inflammatory and autoimmune diseases such as colitis, arthritis, graft-versus host disease, lupus and atherosclerosis to cite only a few (reviewed in [6]). More recently, we reported a proof of concept study showing that such inhibitors also protect from toxic shock and septic shock [25,53]. To which extent members of different HDAC subfamilies (i.e. HDACs versus SIRTs) play a redundant or a complementary role in the regulation of immune responses is still

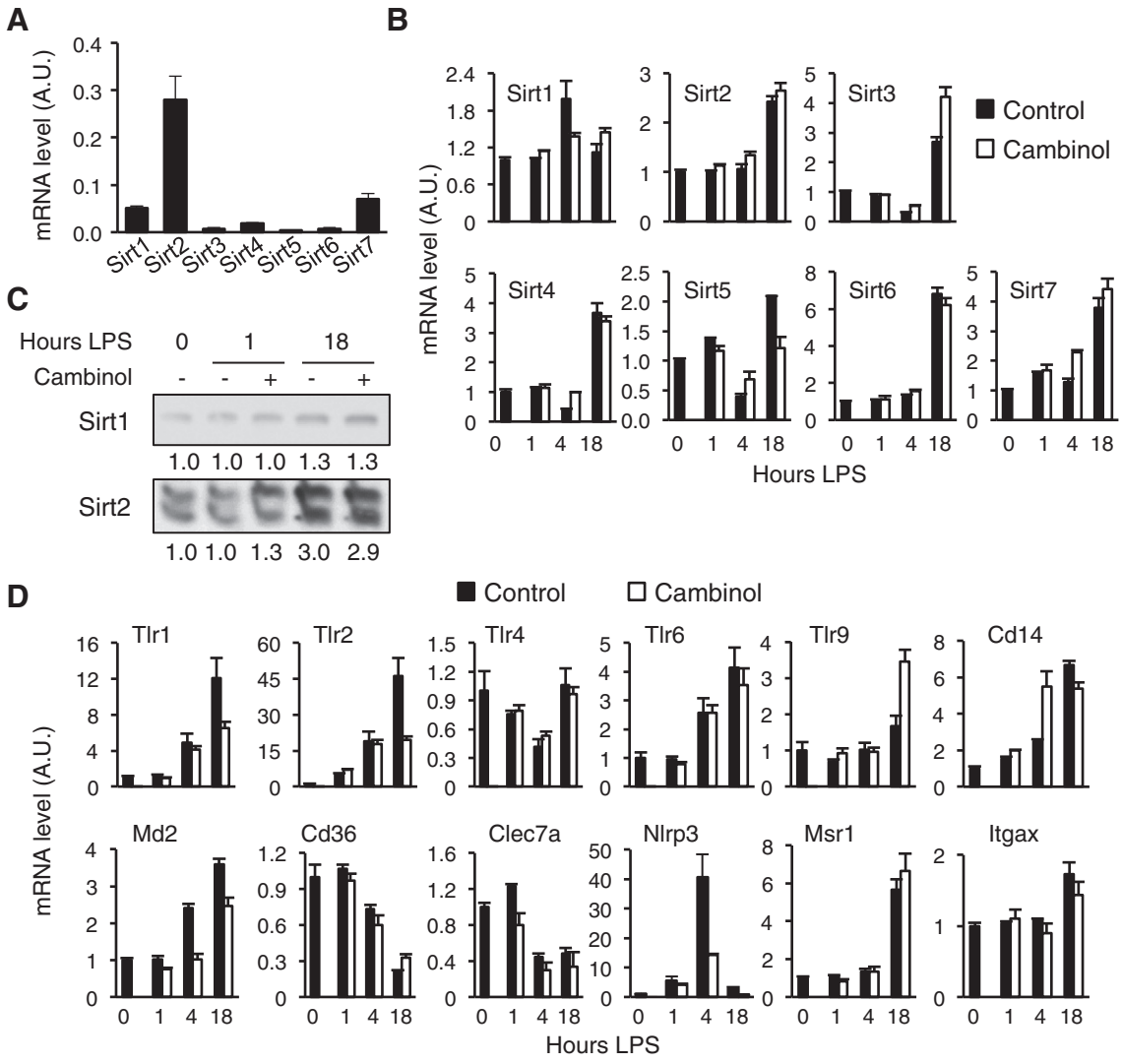


Fig. 7. Effect of cambinol on the expression of sirtuins and pattern recognition receptors. (A) Sirt1-7 mRNA levels in resting BMDMs were quantified by real time-PCR and normalized to GAPDH mRNA levels. Data are means ± SD of triplicate samples from one experiment representative of three experiments. (B–D) BMDMs were pre-incubated for 1 h with or without cambinol (50 μM) and exposed for 0, 1, 4 and 18 h to LPS (10 ng/ml). Sirt1-7 mRNA (B), SIRT1 and SIRT2 protein (C) and Tlr1, Tlr2, Tlr4, Tlr6, Tlr9, Cd14, Md2, Cd36, Clec7a, Nlrp3, Msr1 and Itgax mRNA (D) were analyzed by real time-PCR (B, D) and Western blotting (C). mRNA levels were normalized to GAPDH mRNA levels. In B, C and D expression levels in resting cells were set at 1. Data are means ± SD of triplicate samples from one experiment (A, B, D) and are representative of two independent experiments. A.U.: arbitrary units.

largely unknown. The observation that HDAC and SIRT knockout animals display specific phenotypes strongly advocates non-redundant roles of these enzymes in vivo (reviewed in [4,54]). Furthermore, a common phenotype may arise from multiple mechanisms. For example, HDAC6, HDAC9 and SIRT1 all target forkhead box P3 (Foxp3), and therapeutic inhibition of HDAC6, HDAC9 or SIRT1 increased *Foxp3* gene expression and the function of regulatory T cells [55,56]. Whereas HDAC6 inhibition enhanced Treg function through the induction of the heat shock response, loss of HDAC9 and SIRT1 increased Foxp3 expression through the acetylation and the stabilization of STAT5 and p65, respectively [57]. From pharmacologic and biological points of view, it will be interesting to define whether inhibitors of HDAC1-11 and sirtuins have additional or synergistic effects on cytokine production and immune responses, as recently reported for the pro-apoptotic activity of HDAC1-11 and sirtuin inhibitors towards human leukemia cells [58].

In agreement with strong inhibition of cytokine production, cambinol impaired the phosphorylation of ERK1/2, p38 and JNK, and of the upstream MAPK kinases in LPS-stimulated macrophages. Our results are in line with studies showing that sirtinol blocked epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I)-induced phosphorylation of ERK1/2, p38 and JNK in human breast and lung cancer cells [59] and reduced Ras activation as well as ERK1/2 phosphorylation in rat neurons [60]. Further supporting the concept of MAPK targeting by sirtuin inhibitors, the pan-sirtuin inhibitor nicotinamide impaired MAPK activation (primarily ERK1/2) upon BCR engagement in primary B cells [61]. On the contrary, there was no discernible reduction of MAPK phosphorylation in TNF-stimulated macrophages from myeloid conditional SIRT1 knockout mice [11]. Sirtuin inhibition also did not affect MAPK phosphorylation in LPS-treated J774 macrophages, which may be due to the fact that LPS poorly increased p38 MAPK only in these cells [17]. Moreover, SIRT1 has been shown to deacetylate c-Jun and c-Fos that are the principal components of the AP-1 transcription factor [62–64]. Differences in strategies used to target sirtuins (inhibitors versus knockout), stimuli and cell types may underlie these discrepancies.

MAPK phosphatases (MKPs) belong to the family of dual-specificity phosphatases (Dusps). The ten functional MKPs (Dusp1, Dusp2, Dusp4-10 and Dusp16) are integral components of the negative feedback loop regulating MAPK activity [65,66], opening the possibility that cambinol inhibited the MAPK pathway via MKPs. Indeed, pharmacological inhibition of classical HDACs enhanced MKP1 (Dusp1) acetylation

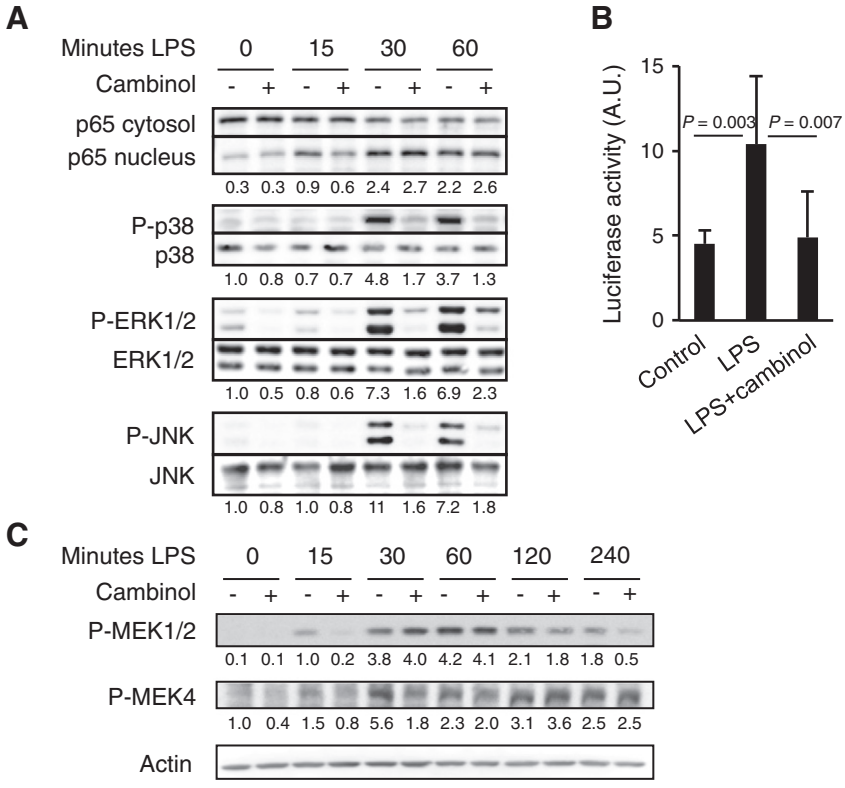


Fig. 8. Cambinol inhibits the MAPK signaling pathway. RAW 264.7 macrophages, transfected with AP-1-reporter luciferase vectors in (B), were pre-incubated for 1 h with (+) or without (−) cambinol (50 μM) and exposed to LPS (10 ng/ml). (A, C) Expression levels of nuclear and cytosolic NF-κB p65, phospho (P-) and total p38, ERK1/2, JNK, MEK1/2 and MEK4 were analyzed by Western blotting. Results are representative of two independent experiments. (B) AP-1-mediated transcriptional activity. Results are expressed as the ratio of luciferase activity to *Renilla* luciferase activity. Data are means ± SD of 5 determinations from two independent experiments. A.U.: arbitrary units.

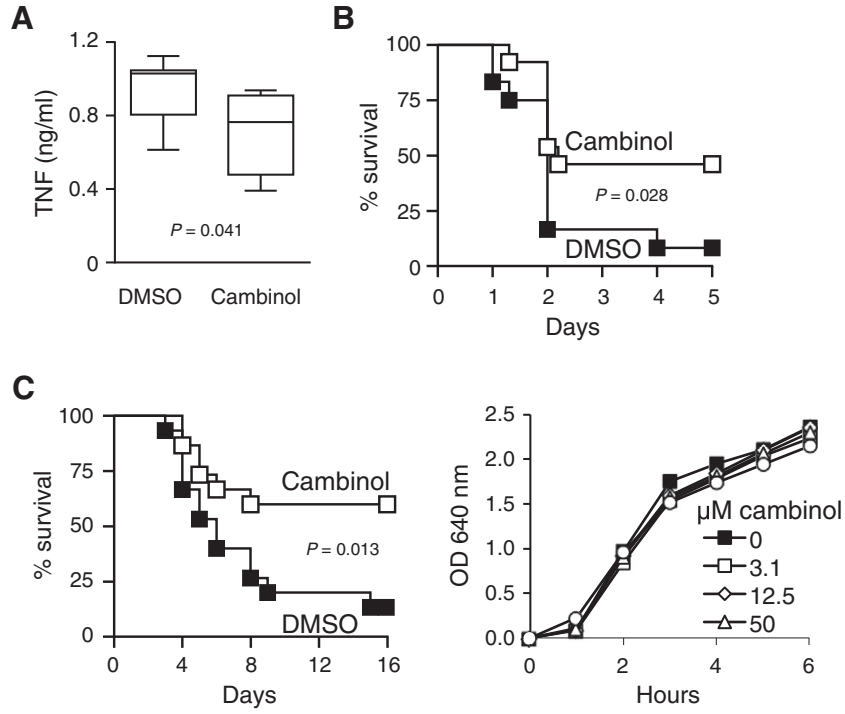


Fig. 9. Cambinol protects from endotoxemia and septic shock. (A–B) BALB/c mice were injected i.p. with LPS (17.5 mg/kg). Cambinol (10 mg/kg) and vehicle (DMSO) were administrated i.p. 1 h before and 24 h after LPS challenge. (A) TNF concentrations in blood collected 1 h after LPS challenge (n = 7–8 per group). (B) Survival of mice (n = 12–13 per group). (C) BALB/c mice (n = 15 per group) were challenged intranasally with 18–63 CFU *K. pneumonia* with or without cambinol (10 mg/kg) or vehicle (DMSO) treatment (q24 hours for 3 days starting 1 day before infection). (D) Growth curves of *K. pneumoniae* cultured in LB broth containing 0, 3.1, 12.5, 50 and 200 μM cambinol.

and MKP1-mediated de-phosphorylation of p38, thus interrupting p38 signaling in macrophages [67]. Moreover, SIRT1 was shown to repress MKP3 (Dusp6) expression, thereby increasing N-Myc phosphorylation and activity in neuroblastoma [68], and to inhibit protein tyrosine phosphatase 1B (PTP1B) in chondrocytes, myoblasts and liver [69,70]. Yet, we have not detected an influence of cambinol on MKP expression in macrophages (data not shown).

The role of sirtuins in inflammatory and immune responses has been addressed mainly for SIRT1, more rarely for SIRT6, leading to controversial conclusions [11–22]. Numerous studies have dissected at the molecular level the negative regulation of AP-1 and NF-κB activities by SIRT1 (reviewed in [71,72]). In agreement, SIRT1 was shown to protect from lung inflammation, chronic obstructive pulmonary disease, experimental autoimmune encephalomyelitis, hepatic steatosis, insulin insensitivity, and inflammatory function of macrophages [11,12,14,63,73,74]. Yet, and in marked contrast, SIRT1 has also been involved in the pathogenesis of lupus, experimental autoimmune encephalomyelitis, collagen-induced arthritis, allergic airway disease, trauma–hemorrhage, allograft rejection and psoriasis [18–20,74–78]. Other studies suggested that SIRT1 and SIRT6 promote rather than inhibit inflammatory responses. For instance, SIRT1 stimulated HIF-2α transactivation capacity during hypoxia [79], antagonized the development and the function of Treg cells [55–57] and regulated autophagy, an important arm of innate and adaptive immune responses [80,81]. Likewise, SIRT6 was required for optimal cytokine synthesis by macrophages, DCs and lymphocytes [16,21]. As mentioned above, differences in experimental approaches, especially the usage of sirtuin activators and inhibitors or the inhibition of sirtuin expression using small interfering RNA, short hairpin RNA and knockout animals, may explain these divergences. Importantly, targeting several sirtuins (and maybe other uncharacterized targets) with inhibitors such as cambinol, sirtinol or nicotinamide may have different outcomes than targeting one specific sirtuin using isoform-specific inhibitors, siRNA/shRNA or knockout approaches. As a good example, targeting SIRT1 and SIRT2, but not SIRT1 or SIRT2, was necessary to induce p53 acetylation and cell death [82], indicating that sirtuins may work in concert to develop some of their biological effects. Selective inhibitors of SIRT1 (EX-527 and CHIC-35) and SIRT2 (AGK2 and AK-7) used either alone or in combination and EX-527 used at concentrations inhibiting both SIRT1 and SIRT2 had no inhibitory effect on cytokine production by macrophages. SIRT6 has been shown to promote cytokine production by innate immune cells [16,21], and was thus a potential target of cambinol and sirtinol. Yet, neither cambinol nor sirtinol inhibited SIRT6 deacetylase activity in vitro at concentrations reported to inhibit cytokine production by macrophages. Thus, we speculate that, apart from SIRT1 and SIRT2, cambinol and sirtinol target enzymes which are not SIRT3 and SIRT6 [48], and possibly not sirtuins.

Our observations that cambinol powerfully inhibits the production of pro-inflammatory cytokines induced by microbial products and bacteria in macrophages, DCs and splenocytes are in full agreement with the efficient protection afforded by cambinol during toxic shock and septic shock. Our results strengthen the development of inhibitors with increased specificity and activity in order to minimize possible undesirable side effects of the drug. Overall, our data suggest that pharmacological inhibitors structurally related to cambinol have promising therapeutic potential for the treatment of pathologies characterized by acute and chronic inflammatory responses.

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Author contributions

Conceived and designed the experiments: JL, EC, AS, GG, IDS, DLR, and TR. Performed the experiments: JL, EC, AS, GG, IDS, and DLR. Analyzed the data: JL, EC, and TR. Contributed reagents/materials/analysis tools: TR. Wrote the paper: TR.

Conflict of interest statement

All authors declare no conflict of interest.

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