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IMPACT OF CLASSICAL AND NAD$^+$-DEPENDENT (I.E. SIRTUINS) HISTONE DEACETYLASES (HDACS) ON INNATE IMMUNE RESPONSES: LESSONS FROM THE USAGE OF HDAC INHIBITORS AND SIRTUIN KNOCKOUT MICE

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pour le Doyen
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Prof. Jacqueline Schoumans Pouw

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SUMMARY

The innate immune system provides the first line of defense against microbial infections. Innate immune responses have to be tightly regulated to eradicate or contain invasive pathogens without causing collateral damages to the host. In mammals, histone deacetylases (HDACs) are split into 2 main subfamilies: Zn-dependent HDACs (HDAC1-11) and NAD^+-dependent sirtuins (SIRT1-7). HDACs regulate gene expression by deacetylating histone and non-histone proteins and impact on numerous biological processes. Dysregulated expression or activity of HDACs has been associated with oncologic, cardiovascular, neurodegenerative, metabolic and autoimmune diseases. Several compounds inhibiting HDACs have been clinically approved for oncologic diseases.

In the first part of this thesis, we review our knowledge about the impact of inhibitors of HDAC1-11 (HDACi) on innate immune responses with a focus on sepsis, a particularly deletary complication of infection. HDACi inhibit innate immune responses and improve the outcome of lethal sepsis, but sensitize to non-severe infections. Thus, HDACi represent attractive drugs for treating inflammatory diseases. We also propose a close survey of the immunological and infectious status in order to prevent opportunistic infections in immunocompromised patients treated with HDACi.

Short chain fatty acids (SCFAs) are metabolites produced by gut commensals, with propionate one of the most abundantly produced SCFA. SCFAs act as inhibitors of HDAC1-11. Therefore, we questioned whether propionate impacts on innate immune responses. In the second part of this work, we report that propionate inhibits cytokine production and proliferation by mouse and/or human monocytes/macrophages, splenocytes and, less efficiently, dendritic cells. Surprisingly, and in contrast with other HDACi, propionate neither sensitizes nor protects from infection and sepsis. These data support the development of therapies using propionate or directed at the microbiota for treating non-infectious inflammatory diseases.

The expression and the impact on innate immune responses of sirtuins are barely characterized. In the third section of this thesis, we describe the expression pattern of SIRT1-7 in macrophages exposed to immunomodulatory and microbial triggers and in liver, spleen and kidney of normal and endotoxemic mice. In the fourth part, we report that cambinol, a newly developed inhibitor of SIRT1 and SIRT2 with anti-tumor activity, powerfully inhibits antimicrobial responses by innate immune cells and protects mice from sepsis and septic shock. In the fifth part, we provide a straightforward approach based on our routinely used protocols to study the impact of drugs directed at sirtuins on innate immune responses *in vitro* and *in vivo*. Finally, in the last part of this work, we characterize the immunological status of SIRT2 knockout mice. SIRT2 deficiency does not impact on cytokine production but enhances bacterial phagocytosis by macrophages and reduces morbidity and mortality in a model of chronic infection by *Staphylococcus aureus*.

Overall, our data support the development of pharmacological inhibitors of HDACs as promising drugs to treat non-infectious inflammatory diseases and possibly as adjunctive therapies for chronic bacterial infections and sepsis.

RÉSUMÉ

Le système immunitaire inné représente la première ligne de défense contre les infections. La réponse immunitaire innée doit être finement régulée afin d'éradiquer les agents pathogènes sans causer de dommages à l'hôte. Chez les mammifères, la famille des déacétylases d'histones (HDACs) est subdivisée en 2 sous-groupes: les HDAC dépendantes du Zn (HDAC1-11) et les sirtuines dépendantes du NAD^+ (SIRT1-7). Les HDACs régulent l'expression génique en déacétylant les histones et d'autres protéines. Ainsi, les HDACs impactent sur de nombreux processus biologiques. Une expression ou une activité dérégulée des HDACs est associée aux maladies oncologiques, cardiovasculaires, neuro-dégénératives, métaboliques et auto-immunes. Plusieurs inhibiteurs d'HDACs ont été cliniquement approuvés pour combattre certains cancers.

Dans la première partie de cette thèse, nous résumons nos connaissances sur l'impact des inhibiteurs des HDAC1-11 (HDACi) sur la réponse immunitaire innée avec un intérêt particulier pour le sepsis, une complication particulièrement délétère d'infections. Les HDACi inhibent la réponse immunitaire innée et protègent de sepsis mortel. Par contre, les HDACi sensibilisent aux infections non létales. Les HDACi pourraient donc être utilisés pour traiter les maladies inflammatoires. Nous proposons également un suivi étroit de l'état immunologique et infectieux des patients immunodéprimés traités avec des HDACi afin de prévenir la survenue d'infections opportunistes.

Les acides gras à chaîne courte (SCFAs), parmi lesquels le propionate, sont produits par les bactéries de l'intestin. Certains SCFAs se comportant comme des HDACi, nous avons testé si le propionate influence la réponse immunitaire innée. Dans la deuxième partie de ce travail, nous montrons que le propionate inhibe la production de cytokines et la prolifération de monocytes/macrophages, splénocytes et, dans une moindre mesure, cellules dendritiques chez la souris et/ou l'homme. De manière surprenante, le propionate ne sensibilise pas aux infections et ne protège pas du sepsis. Ces données supportent le développement de thérapies utilisant le propionate ou dirigées envers le microbiote pour traiter des maladies inflammatoires non-infectieuses.

Dans la troisième partie de cette thèse, nous présentons l'analyse de l'expression des SIRT1-7 dans les macrophages et les organes de souris. Dans la quatrième partie, nous rapportons que le cambinol, un inhibiteur de SIRT1 et SIRT2 à activité anti-tumorale, inhibe la réponse antimicrobienne par les cellules immunitaires innées et protège d'infections mortelles dans des modèles animaux. Dans la cinquième partie, nous décrivons une approche simple pour tester *in vitro* et *in vivo* l'impact sur la réponse immunitaire innée de drogues dirigées contre les sirtuines. Enfin, dans la dernière partie de ce travail, nous caractérisons le statut immunologique de souris déficientes en SIRT2. Cette déficience n'a pas d'effet sur la production des cytokines, mais augmente la phagocytose par les macrophages et réduit la morbidité et la mortalité dans un modèle d'infection chronique à *Staphylococcus aureus*.

Globalement, nos données étayent le développement d'inhibiteurs pharmacologiques des HDACs pour traiter des maladies inflammatoires stériles et éventuellement comme thérapie d'appoint des infections bactériennes chroniques et du sepsis.

RÉSUMÉ DESTINÉ À UN LARGE PUBLIC

Le système immunitaire inné représente la première ligne de défense contre les infections. Il regroupe les barrières physiques (peau, muqueuses), des composants solubles du sang (notamment le système du complément) et des cellules spécialisées telles que les macrophages et les cellules dendritiques. La réponse de ces différents acteurs doit être coordonnée et finement régulée afin d'éradiquer les agents pathogènes sans causer de dommages à l'hôte.

Les enzymes appelées déacétylases d'histones (ou HDACs) jouent un rôle important dans la régulation de l'expression des gènes et, en conséquence, influencent de nombreux processus biologiques. Par ailleurs, une production ou une activité dérégulée des HDACs est associée au développement de maladies oncologiques, cardiovasculaires, neuro-dégénératives, métaboliques et auto-immunes. Chez les mammifères, la famille des HDACs est subdivisée en 2 sous-groupes: les HDAC classiques dépendantes du Zn (HDAC1-11) et les sirtuines dépendantes du NAD^+ (SIRT1-7).

Dans cette thèse, nous avons étudié l'impact des HDAC1-11 et des sirtuines sur le développement de la réponse immunitaire innée et des infections, avec un intérêt tout particulier pour le sepsis. Le sepsis est un syndrome clinique extrêmement sévère résultant d'une réponse immunitaire inappropriée de l'hôte à l'infection. L'approche scientifique utilisée consistait dans un premier temps à étudier l'effet de drogues inhibant l'activité des HDAC1-11 ou des sirtuines, et dans un second temps à étudier la réponse de cellules et de souris n'exprimant pas la sirtuine 2 (i.e. déficientes en SIRT2).

Les résultats obtenus démontrent que le propionate, un inhibiteur des HDAC1-11 qui est produit par les bactéries de l'intestin, inhibe la réponse inflammatoire des cellules immunitaires innées de la souris et de l'homme. De plus, une drogue anticancéreuse appelée cambinol inhibe les sirtuines 1 et 2 et protège de la mortalité induite par le sepsis chez la souris. Enfin, la déficience en SIRT2 favorise l'ingestion des bactéries par les macrophages, une fonction biologique dénommée phagocytose et qui joue un rôle important dans l'élimination des microbes et l'amplification de la réponse immunitaire. En accord avec ces données, les souris déficiente en SIRT2 sont plus résistantes que les souris normales lors d'une infection chronique à une bactérie appelée *Staphylococcus aureus*.

Globalement, ces observations démontrent les propriétés immuno-régulatrices des inhibiteurs des HDAC1-11 et des sirtuines. Elles suggèrent par ailleurs que des drogues inhibant les HDACs pourraient être utilisées pour traiter des maladies inflammatoires et éventuellement comme thérapie d'appoint des infections bactériennes chroniques et du sepsis.

MAIN ABBREVIATIONS

ADP	Adenosine diphosphate
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
CARD	Caspase-recruiting domain
CD	Cluster of differentiation
CDS	Cytosolic DNA sensor
CLR	C-type lectin receptor
CpG	Cytosine-phosphate-guanosine
CR	Calorie restriction
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
ELISA	Enzyme-linked imunosorbent assay
ERK	Extracelluar signal regulated kinase
ETs	Extracellular traps
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi/HDI	Inhibitor of histone deacetylase
IC_{50}	Half maximal inhibitory concentration
IFN	Interferon
IL	Interleukin
IL-1R	Interleukin-1 receptor
ILC	Innate lymphoid cell
iNOS	Inducible NO synthase
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal Kinase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAMP	Microbial-associated molecular pattern
MAPK	Mitogen activated protein kinase
MHC-II	Major histocompatibility complex class II molecule
MIF	Macrophage migration inhibitory factor
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88

NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NALP	NACHT domain-, leucine-rich repeat-, and PYD-containing protein
NAM	Nicotinamide
NAMPT	Nicotinamide phosphoribosyltransferase
NETs	Neutrophil ETs
NF-κB	Nuclear factor-κB
NK	Natural killer
NLR	NOD-like receptor
NO	Nitric oxide
p38	p38 mitogen-activated protein kinase
Pam_3CSK_4	N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine
PBMC	Peripheral blood mononuclear cell
PRR	Pattern recognition receptor
RIG-I	Retinoic acid-inducible gene-I
RLR	RIG-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
SCFA	Short chain fatty acid
SIRT	sirtuin
ssRNA	Single-stranded RNA
Th	Helper T cell
TIR	Toll/Interleukin-1 receptor
TIRAP	Toll/Interleukin-1 receptor domain containing adaptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	Tumor necrosis factor receptor-associated factor
Tregs	Regulatory T cells
TRIF	TIR-domain-containing adaptor inducing interferon β

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1 INTRODUCTION

1.1 Innate immunity

We are constantly exposed to a multiplicity of potentially pathogenic microorganisms. The innate immune system provides the very first line of defense against microbial infections through skin and mucosal physical barriers. Once these barriers are crossed, microorganisms are sensed by the coordinated actions of soluble molecules, comprising components of the complement system and acute phase proteins (collectins and complement molecules, pentraxins, ficolins, lipopolysaccharide (LPS) binding protein (LBP), bactericidal/permeability-increasing protein (BPI)), and innate immune cells. Innate immune cells (monocytes/macrophages, dendritic cells (DCs), granulocytes, natural killer (NK) cells, innate lymphoid cells (ILCs)), localized in tissues or patrolling into the body, sense the presence of microorganisms through pattern recognition receptors (PRRs). PRRs recognize conserved microbial motifs, common to groups of microbes and not present in mammals, collectively called microbial-associated molecular patterns (MAMPs). PRRs also bind to endogenous molecules like nucleic acids, histones, uric acid crystals, ATP, cytochrome c, S100 molecules and high-mobility group protein 1 (HMGB1), released by injured or stressed cells, called danger-associated molecular patterns (DAMPs) (1).

The best-characterized families of PRRs are Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs) and cytosolic dsDNA sensors (CDSs) (2). TLRs and CLRs are membrane-bound PRRs, whereas NLRs, RLRs and CDSs are cytoplasmic PRRs (**Figure 1**). TLR4 is the archetypal PRR that has driven the most attention, the identification and characterization of which representing cornerstone discoveries for all subsequent progresses in the field of innate immune sensing (3).

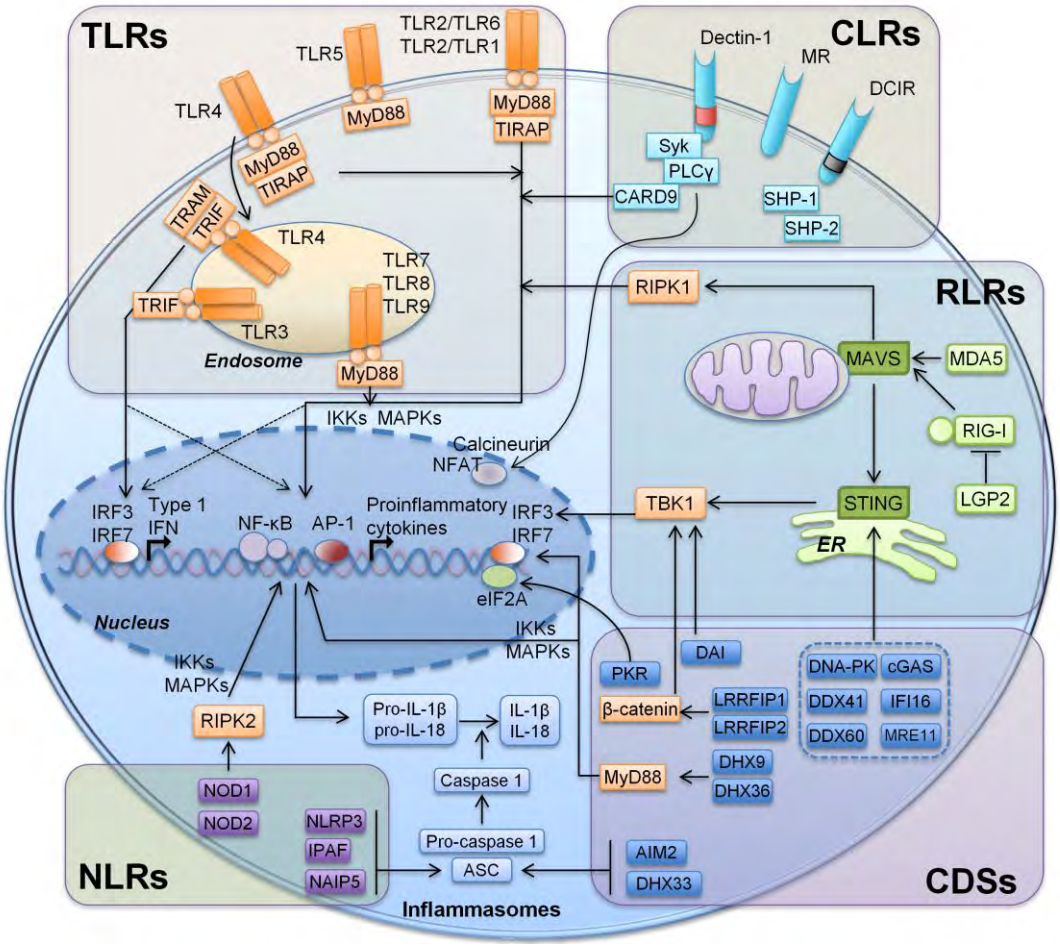

Figure 1: Schematic representation of the five main families of pattern recognition receptors. Members of the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs) and cytosolic dsDNA sensors (CDSs) are grouped according to structural similarity, subcellular localization and microbial specificity. The main adaptor molecules and signaling pathways activated by each of the families are depicted. Adapted from (2).

1.1.1 Toll-like receptors (TLRs)

Ten functional TLRs (TLR1-10) are expressed in humans, and 12 functional TLRs (TLR1-9, TLR11-13) are expressed in mice. TLRs are type I transmembrane glycoproteins composed of an extracellular leucine-rich repeat (LRR) domain ensuring ligand recognition, a transmembrane domain, and an intracellular toll/interleukin-1 receptor (IL-1R) (TIR) signaling domain. TLRs are located at the cell surface, except TLR3 and TLR7-9 that reside in endosomes.

TLRs recognize a broad range of bacterial, fungal and viral MAMPs, but also some DAMPs (4-6). TLR4 was the first TLR for which a microbial ligand was definitely identified (7). In collaboration with CD14 and MD-2 (Ly96), TLR4 detects LPS, the main proinflammatory compound of the outer-membrane of gram-negative

bacteria. Upon LPS sensing, TLR4 shuttles to late endosome. TLR2 forms heterodimers with either TLR1 or TLR6, and possibly homodimers, to detect a wide range of microbial products among which glycoproteins, glycosylphosphatidylinositol (GPI) anchors, lipopeptides, lipoproteins, peptidoglycan (PGN), porins and β-glucan from gram-positive and gram-negative bacteria, mycoplasma, mycobacteria, fungi, parasites and viruses. TLR5 recognizes flagellin of motile flagellated bacteria. The endosomal TLRs, TLR3 and TLR7-9, recognize nucleic acids: double-stranded RNA (dsRNA) by TLR3, single-stranded RNA (ssRNA) by TLR7 and TLR8 and unmethylated CpG motif containing DNA by TLR9. TLR10, upon dimerization with TLR2, inhibits TLR2-mediated inflammatory response (8).

Going well along with these patterns of ligand specificity, membrane bound TLRs are primarily involved in host responses to bacteria, fungi and parasites, whereas endosomal TLRs are involved in host responses against viral infections. A non-exhaustive list of the main natural or synthetic MAMPs recognized by TLRs is presented in **Table 1**.

Upon ligand binding, TLRs engage intracellular adaptor molecules recruited through TIR-TIR homotypic interactions to initiate intracellular signaling and gene expression. Five adaptor molecules have been identified: myeloid differentiation primary response gene (MyD88), TIR domain-containing adaptor inducing interferon (IFN) β (TRIF), TIR domain-containing adaptor protein (TIRAP/MAL), TRIF related adaptor molecule (TRAM) and sterile α-, and armadillo-motif containing protein (SARM). All TLRs except TLR3 use MyD88 to initiate early activation of the nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) pathways involved in the production of inflammatory cytokines and chemokines. TIRAP/MAL is recruited with MyD88 to TLR2 and TLR4. TLR3 and TLR4 that have shuttled to endosomes use TRIF to initiate IFN regulatory factor 3 (IRF3) and late NF-κB activation that control the production of type I IFNs and IFN-inducible genes. TRAM bridges TRIF to TLR4 in endosomes, whereas SARM negatively regulates TLR3 and TLR4 TRIF-dependent signaling (9).

Table 1. TLR subcellular localization, adaptor proteins and important microbial ligands/mimics.

TLR	Cellular localisation	Adaptor molecule	MAMPs	Origin of MAMPs
TLR1/2	Cell surface	MyD88/TIRAP	Lipoproteins/peptides (Pam_3CSK_4)	Bacteria Synthetic
TLR2	Cell surface	MyD88/TIRAP	Peptidoglycan, Lipoteichoic acid	Gram-pos bacteria
			Lipomannan	Mycobacterium
			Zymosan/β-glucan	Fungi
			LPS	*P. gingivalis*
TLR2/6	Cell surface	MyD88/TIRAP	Lipoproteins/peptides Pam_2CSK_4	Bacteria Synthetic
TLR3	Endosome	TRIF	ssRNA	Virus
			dsRNA	Virus
			poly(I:C), poly(A:U)	Synthetic
TLR4	Cell surface (endosome)	MyD88/TIRAP (TRIF/TRAM)	LPS	Gram-neg bacteria
			Mannans	Fungi
			Glycoinositol phospholipids	Trypanosoma
			F-protein	Virus
			Monophosphoryl lipid A	Synthetic
TLR5	Cell surface	MyD88	Flagellin	Bacteria
TLR7	Endosome	MyD88	ssRNA	Virus
TLR8	Endosome	MyD88	ssRNA	Virus
TLR9	Endosome	MyD88	DNA containing unmeth. CpG	Bacteria, virus, fungi

Poly(I:C): polyinosine-polycytidylic acid; poly(A:U): polyadenylic-polyuridylic acid.

1.1.2 C-type lectin receptors (CLRs)

The CLR family comprises more than 1'000 members organized into 17 subgroups based on phylogenetic and structural hallmarks. CLRs are characterized by one or more extracellular carbohydrate recognition domains (CRDs) (10). Unfortunately, only few CLRs have been studied in the context of innate immunity. Among the best known CLRs are the myeloid CLRs dectin-1, dectin-2, mincle, DC-SIGN, mannose receptor (MR, CD206), DEC-205 (CD205) and galectin-3. CLRs are transmembrane proteins specialized in the sensing of fungal carbohydrates: β-glucans and chitin, the major and second-most abundant polysaccharide of the fungal cell wall, mannose and fucose (11). CLRs also mediate the recognition of bacteria, virus and parasit carbohydrates (mannose, mannans, N-acetyl-glucosamine) (12-14). Myeloid CLRs induce signalling through 3 main pathways based on the presence of specific intracellular domains: 1) CLRs with an immunoreceptor tyrosine-based activation motif (ITAM) domain that activates the Syk tyrosine kinase pathway (dectin-1, dectin-2, mincle), 2) CLRs with an immunoreceptor tyrosine-based inhibition motif (ITIM) domain (DCIR, Ly49Q) that modulates signaling pathways induced by co-receptor engagement, and 3) CLRs without evident ITAM or ITIM domains (MR, DEC-205, DC-SIGN) (10). Syk mediates the activation of the NF-κB, MAPK and nuclear factor of activated T cell (NFAT) signaling pathways.

1.1.3 NOD-like receptors (NLRs)

NLRs form a family of 22 members in humans: NLRA (class II, major histocompatibility complex, transactivator, CIITA), NLRB (NLR family, apoptosis inhibitory protein, NAIP), NLRC1-5 (NLR family, CARD domain containing 1-5), NLRP1-14 (NLR family, pyrin domain-containing 1-14) and NLRX1 (NLR family member X1). NLRs are classified according to their structural domains. All NLRs contain NACHT (or NAIP, neuronal apoptosis inhibitor protein) and LRR domains, and specific CARD (Caspase activation and recruitment domain; in all NLRs except NLRP1-14), PYD (pyrin domain; in NLRP1-14) or BIR (Baculovirus Inhibitor of apoptosis protein Repeat; in NLRB) domains. NLRs are cytoplasmic receptors able to recognize MAMPs and DAMPs from exogenous and endogenous origin (15, 16).

The founding members of the NLR family are NOD1 (NLRC1) and NOD2 (NLRC2). NODs drove much attention following the early discovery that polymorphisms in *NOD* genes are the strongest genetic risk factors associated with Crohn's disease development. Both NOD1 and NOD2 were initially characterized by their ability to sense cytosolic peptidoglycan fragments and to activate the NF-κB pathway (17). Nowadays, the most well-known and studied NLR is NLRP3 (also known as NALP3). Activation of NLRP3, for example by uric acid crystals, ATP, cytochrome c, muramyl dipeptide (MDP, a subcomponent of peptidoglycan) cholera toxin B, pneumolysin and streptolysin O, leads to the assembly and activation of the NLRP3 inflammasome, a cytosolic multiprotein complex that also comprises the adaptor protein ASC (Apoptosis-associated speck-like protein containing a CARD) and procaspase-1. NLRP3 inflammasome activation leads to the generation of active caspase-1 that cleaves pro-IL-1β and pro-IL-18 into mature, secreted, IL-1β and IL-18. Inflammasome activation also drives pyroptosis, an inflammatory cell death program (18, 19). Other NLRs form inflammasome. For instance, NLRC4 forms an inflammasome that senses flagellin and PrgJ-like proteins delivered through bacterial type III and type IV secretion systems (T3SS and T4SS) by gram-negative bacteria including *Legionella pneumophila*, *Pseudomonas aeruginosa* and *Salmonella enterica*. This sensing involves the actions of Naip2, Naip5 or Naip6 (20, 21).

1.1.4 Retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs)

RLRs are RNA helicases. The RLR family comprises two functional members, RIG-I (encoded by DDX58) and MDA5 (Melanoma differentiation-associated antigen 5, encoded by IFIH1) that sense ssRNA and dsRNA of viral origin. A third member called LGP2 (Laboratory of genetics and physiology 2, encoded by

DHX58) is commonly believed non-functional. RLRs are composed of three domains: an N-terminal CARD domain, involved in signal transduction and absent in LGP2, a central DExD/H box RNA helicase domain, and a C-terminal RNA binding domain (CTD). Recognition of viral RNA by RLRs leads to their interaction, through CARD/CARD homotypic interactions, with MAVS (mitochondrial antiviral-signaling protein also known as IPS-1/IFNB-promoter stimulator and VISA (virus-induced signaling adapter)) anchored to mitochondria, and subsequent activation of the IRF and NF-κB pathways involved in the production of type I and type III IFNs (22). The function of LGP2 is not fully understood, but could be to dampen RIG-I and MDA5-mediated responses. Yet, some recent works suggest that LGP2 can also, in certain circumstances, assist RIG-I and MDA5-mediated response to viral RNA (23).

1.1.5 Cytosolic dsDNA sensors (CDSs)

The family of CDSs has been characterized more recently, with a growing number of members identified such as DNA-dependent activator of IRFs (DAI), AIM2 (absent in melanoma 2), cGAS (cyclic GMP–AMP synthase), DDX41, DHX9, DNA-PK, DHX36, IFI16, Ku70, LRRFIP1, LRRFIP2, MRE11, STING and Rad50 (24, 25). CDSs sense the presence of DNA in the cytoplasm of cells. Upon viral DNA sensing, CDSs usually initiate signaling through the adaptor protein STING (also called MITA, MPYS and ERIS) that is localized to the endoplasmic reticulum, and that relays IRF and NF-κB pathway activation. As such, CDSs play an essential role in antiviral immunity to DNA viruses (12, 25, 26). Of note, CDSs can also form inflammasomes. A well known example is AIM2 that forms an AIM2 inflammasome once activated by the presence of cytosolic dsDNA in cells infected by cytomegalovirus and vaccinia virus, but also by bacteria such as *Francisella tularensis* and *Listeria monocytogenes* (*L. monocytogenes*) (20).

As mentioned above, the recognition of MAMPs/DAMPs through PRRs initiates multiple intracellular signaling pathways in innate immune cells. This leads to the activation and nuclear translocation of transcription factors like NF-κB, AP-1 and IRFs, modulation of gene transcription and production or upregulation of receptors, adhesion molecules, cytokines and soluble factors important for cell activation, cell recruitment, phagocytosis and killing of pathogens and initiation of the adaptive immune response. These processes have to be perfectly orchestrated to mount an appropriate inflammatory response able to clear or contain the infectious agents and rescue homeostasis without causing collateral damages to the host.

1.2 Importance and mechanisms of macrophage defense against infections

Macrophages are innate immune cells of central importance to fight infections (13, 27, 28). Tissue resident macrophages derive from the yolk sac and fetal liver during embryogenesis and have a certain level of self-renewal capacity (14, 29, 30). Later in the adult life, monocytes generated in the bone marrow egress into the blood and can be recruited to sites of infection/inflammation where they differentiate into macrophages (31, 32).

Macrophages form a complex population of cells that dynamically respond to environmental signals. Two extreme phenotypes have been described: the classically activated M1 macrophage with proinflammatory and microbicidal properties through the production of high levels of IL-12, IL-23, tumor necrosis factor (TNF), reactive oxygen species (ROS) and nitric oxide (NO), and the alternatively activated M2 macrophages which have regulatory and tissue repair functions through the production of high levels of IL-10 and transforming growth factor-β (TGF-β), IL-1R antagonist (IL-1Ra). It is now admitted that macrophages are a continuum between the two extremes, rather than existing as either M1 or M2 polarized cells (33, 34).

Macrophages express a broad range of PRRs that allow them to sense all kind of invading pathogens. When a microbe is recognized by macrophages, they produce a number of mediators including cytokines, chemokines and lipids. These molecules act on neighboring cells and attract to the site of infection additional immune cells, mainly monocytes, polymorphonuclear cells and lymphocytes. Macrophages, together with DCs and neutrophils are professional phagocytes equipped with a broad range of antimicrobial effectors to engulf and kill pathogens. Phagocytosis happens as a consequence of the recognition of a pathogen through phagocytic and opsonic phagocytic PRRs including macrophage scavenger receptor 1 (Msr1/SR-AI/CD204), mannose receptor, CD14, CD36, C-type lectins (dectin-1) and members of the integrin superfamily (integrin α5/Itga5/CD49e, integrin αM/Itgam/CD11b, integrin αX/Itgax/CD11c, integrin β2/Itgb2/CD18) forming complement receptor 3 (CR3; i.e. CD11b/CD18) and CR4 (CD11c/CD18). The process of phagocytosis is enhanced when microbes are opsonized by soluble PRRs or molecules among which collectins, ficollins, pentraxins and complement component C3b, iC3b and C4b. Moreover, in case of previous exposure of the host to a specific pathogen, opsonization is efficiently performed by anti-microbial antibodies produced during the initial infection and during the recall response by memory B cells. In this case, Fc receptors (FcR) on macrophages and other specialized phagocytic cells will be engaged to facilitate the phagocytosis of

opsonized pathogens. Mechanistically, the binding of complement receptor or FcR to their ligands provokes their clusterization and initiate intracellular signaling leading to local actin polymerization to form pseudopodia that envelop the target. While actin is polymerizing around the target, it depolymerizes underneath allowing the formation of a phagocytic cup from the plasma membrane (35). The subsequent closure of the cup leads to the formation of the phagosome that has no microbicidal properties at this stage. The phagosome then undergoes to an articulated and complex maturation process carried on by fission and fusion with *trans*-Golgi, endosome and lysosome vesicles. The final result is a compartment called phagolysosome that allows pathogen killing and degradation through acidification and delivery of antimicrobial enzymes (acid hydrolases, lysozyme) and peptides and of toxic ROS generated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and NO generated by the inducible NO synthase (iNOS) (35). Phagocytosis by macrophages has two functions: on the one hand it allows eliminating the engulfed pathogens, and on the other hand it generates microbial-derived antigenic peptides that will be presented at the cell surface by major histocompatibility complex (MHC) molecules. Macrophages and DCs may then migrate to secondary lymphoid organs to present antigens to T-cells and initiate the development of the adaptive immune response (36).

Another mechanism by which macrophages can fight extracellular pathogens is through the formation of extracellular traps (ETs), although this has been studied in much more detail for neutrophils than for macrophages (37). ETs were described in 2004 for neutrophils and called NETs (neutrophil ETs) by the group of Zychlinsky (38). NETs are produced during NETosis that may represent, although not everybody agrees on that, a distinct form of cell death from apoptosis and necrosis. Indeed, it has been suggested that neutrophils may retain their viability and several essential functions such as phagocytosis and chemotaxis, while undergoing NETosis (39). NETosis is stimulated when neutrophils encounter pathogens (bacteria, fungi), microbial products (LPS) or are exposed to activated platelets, cytokines (IL-8) or stimulatory agents such as phorbol 12-myristate 13-acetate (PMA). NETosis is dependent on ROS production and decondensation of nuclear DNA. NETs consist of DNA and histones (likely in the form of chain of nucleosomes from stretched chromatin), antimicrobial peptides, enzymes and other proteins and serve to “capture” and immobilize microbes, directly kill them and/or promote their phagocytosis and killing in the phagolysosome.

1.3 Infection and sepsis

During infection a multiplicity of processes are put in place with the aim to eliminate the invading pathogen. Host response must be tightly regulated to mount an appropriate inflammatory response able to clear the infection and rescue homeostasis without causing collateral damages to the host.

Sepsis is a clinical syndrome characterizing severe complications of infections. The annual incidence of sepsis is around 300 cases per 100'000 people in USA, and sepsis is the second most common cause of death in non-coronary intensive care units. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) have just been released with accompanying reports (40-43). It states that "*sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection*". Sepsis may result from infection by virulent pathogens or from infection under genetic or non-genetic conditions (neutropenic cancer patients under chemotherapy, immunosuppressed transplanted patients…) impairing the mounting of appropriate immune responses. Advances in supportive care and antimicrobial therapy have improved the outcome of sepsis patients. Yet, mortality rate of sepsis remains high, ranging from 20% to 80%. In addition, more than 30% of survivors develop long-term functional disabilities and cognitive impairments. Whereas gram-negative infections were prevailing in the 60s and 70s, gram-positive infections now account for around half of cases of sepsis. Markedly, the proportion of fungal infection has strongly increased over the last 30 years.

Our understanding of the pathogenesis of sepsis has progressed significantly over the last decades. Experimental studies have demonstrated that deficiency or neutralization of cytokines and their receptors and PRRs impair host defenses, and human association studies have linked polymorphisms in innate immune genes with an increased susceptibility to infections (44, 45). An initial assumption was that sepsis results from an overwhelming inflammatory response, commonly known as the "cytokine storm", responsible for tissue injury, vascular collapse and organ dysfunction. It was then proposed that this "systemic inflammatory response syndrome" (SIRS) was followed by a state of immunosuppression called the "compensatory anti-inflammatory response syndrome" (CARS) that favors the reactivation of latent viral infections and the development of secondary and nosocomial infections that impact on long term-survival of sepsis patients. Indeed, septic patients show a state of immunosuppression characterized by increased apoptosis of DCs and T lymphocytes, impaired MHC-II expression by DCs and defective innate and adaptive immune responses. Yet, it is now clear that there is no sharp temporal segregation between the pro- and

anti-inflammatory responses occurring in sepsis (46-49). The transcriptome of circulating leucocytes after severe trauma, burn injury and low-dose endotoxin challenge revealed a “genomic storm” which affected more than 80% of the cellular functions and pathways, simultaneously increasing the expression of proinflammatory and anti-inflammatory genes, as well as suppressing adaptive immunity-related genes (50), and it is now commonly accepted that pro- and anti-inflammatory responses are temporally overlapping.

Studies published in recent weeks confirmed and extended this assumption. Results from the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) program confronting clinical data and blood whole-genome transcriptome from patients in 2 ICUs in the Netherlands indicate that gene expression profile at baseline is not predictive of the risk of secondary infection, but that the genomic response at the onset of secondary infection is consitent with immunosuppression (51). Yet, the UK Genomic Advances in Sepsis (GAinS) study reports that on admission in 29 participating ICUs, the transcriptome of leukocytes from patients with sepsis could be stratified into two signatures: sepsis response signature 1 (SRS1) and SRS2. SRS1 identifies those individuals with features of immunosuppression and metabolic dysregulation associated with higher 14-days mortality than SRS2 bearing patients (52). Finally, the groups of van der Poll (AMC, Amsterdam) and Netea (Radboud University, Nijmegen) have shown that broad leukocyte immunometabolic defects are associated with immunoparalysis in sepsis (53).

For many years, clinical trials have tested single immunomodulating therapies, mainly anti-inflammatory/immunosuppressive ones such as glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDs), inhibitors of TNF, IL-1, PAF (platelet-activating factor), bradykinin, NO or anti-LPS agents, all resulting in no consistent benefit for severely ill patients (54). The context become even grimmer when, in 2011, a phase III clinical study of the efficacy of the TLR4 antagonist Eritoran (Eisai) was stopped because it failed to meet the primary endpoint in the ACCES trial (55). The next year, a phase II-III trial testing recombinant lactoferrin (Talactoferrin alfa, Agennix), a molecule with antimicrobial and DC immunostimulatory activities, was also stopped because lactoferrin failed to improve 28 and 90-days mortality (56).

Unfortunately, there is nowadays no treatment targeting the underlying mechanisms of sepsis. Indeed, recombinant human activated protein C (rhAPC, Xigris®, Eli Lilly), an anti-thrombotic, pro-fibrinolytic and anti-inflammatory drug registered for sepsis in 2002, was withdrawn from the market in 2011 following the negative results from the PROWESS-SHOCK study that did not show reduction in mortality at 28 or 90 days

in patients with septic shock (57). Many reasons may account for the lack of efficacy of anti-sepsis startegies, among others the heterogeneity of the patients recruited and the design of clinical trials with poorly predefined genetic and non-genetic appropriate selection parameters and real-time follow-up using biomarkers to weigh treatment efficacy and allow treatment adjustment (58, 59). Also, targeting one single molecule may not suffice to interfere with the complex series of events that drive sepsis pathogeneis. New directions in the field consider supporting or modulating rather than inhibiting immune responses and preventing apoptosis in sepsis patients, for example using or IL-7, IL-15, anti- programmed cell death 1 (PD1) and PD1-ligand, IFNγ, and drugs improving epithelial barriers functions such as granulocyte macrophage-colony stimulating factor, IL-11, IL-22, hepatocyte growth factor and insulin-like growth factor-1. New avenues are also emerging such as those impacting on epigenetic modifications and the microbiome (60). Therefore, although many advances have been achieved in recent past, great efforts are required in order to better understand the causes and the molecular mechanisms involved in the pathogenesis of sepsis and develop innovative therapeutical strategies.

1.4 Histones and histone deacetylases (HDACs)

Chromatin structure is a key element regulating gene expression. Histones form the basic unit of chromatin: the nucleosome. A nucleosome is an octamer of 2 copies of histones H2A, H2B, H3 and H4 around which DNA is wrapped. When chromatin is tightly compacted, DNA is not accessible to the transcription machinery. Conversely, open chromatin allows transcription regulators and polymerase to reach gene promoters and initiate transcription. The N-terminal tails of histones are subjected to posttranscriptional modification among which acetylation, ubiquitination, sumoylation and ADP-ribosylation of lysine, methylation of arginine and lysine and phosphorylation of serine and threonine. Each modification on histones is thought to affect the structure and the function of the chromatin, thereby modulating gene expression (**Figure 2**). For example, the transcriptionally inactive heterochromatin is characterized by hypoacetylated and highly methylated H3K9, H3K27 and H4K20 histones whereas the transcriptionally active euchromatin is enriched in acetylated and trimethylated H3K4, H3K36, and H3K79 histones. Beside histones, DNA is subjected to intense modifications, with the most studied epigenetic modification being methylation. The reversible covalent modification of DNA occurs at 5'-methyl cytosine residues mainly in the context of CpG dinucleotides. Methylation of DNA is usually associated with histone deacetylation and repressed gene expression.

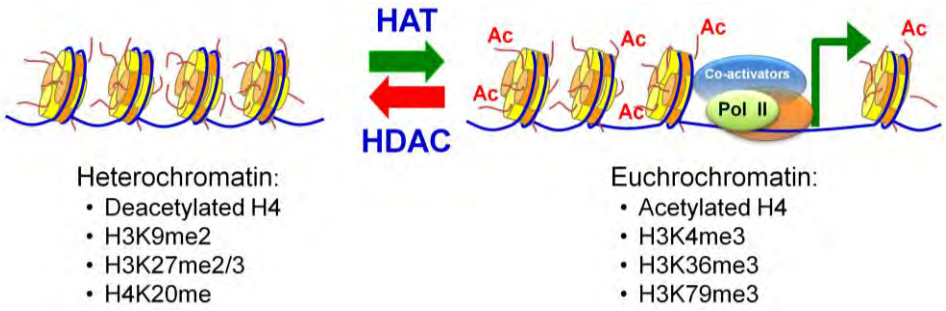

Figure 2. Heterochromatin-euchromatin equilibrium by histone acetyltransferases (HAT) and histone deacetylases (HDACs). Octamers of histones composed of 2 x H2A, H2B, H3 and H4 are wrapped by stretches of 147 DNA base pairs. Protruding histone tails are subjected to covalent modifications. Ac: acetylation; me2: dimethylation, me3: trimethylation. K: lysine (with amino acid position).

The acetylation status of histone tails is determined by the opposite actions of histone acetyltransferases (HATs, such as CREB-binding protein and p300) and histone deacetylases (HDACs) (**Figure 2**). Histone acetylation neutralizes positive charges of lysines and opens the chromatin structure. Moreover, acetylation acts as a tag that attracts additional transcription regulators, hence promoting gene expression (61).

Mammals express 18 HDACs, organized in four classes based on their homology with yeast HDACs, subcellular localization and enzymatic activity (**Figure 3**). Class I HDACs (HDACs 1, 2, 3 and 8) are homologues of yeast RPD3. They share a compact structure and are found exclusively in the nucleus of most cell lines and tissues. Class II HDACs (HDACs 4, 5, 6, 7, 9 and 10) are homologues of yeast Hda 1 and found in both the nucleus and the cytoplasm of a limited number of cell types. Class III HDACs (SIRT1-SIRT7) are homologues of yeast Sir2 and form a structurally distinct class that has an absolute requirement for nicotinamide adenine dinucleotide (NAD^+) for its enzymatic activity. Finally, class IV HDAC11 has conserved residues in the catalytic core region that are shared by both class I and class II enzymes.

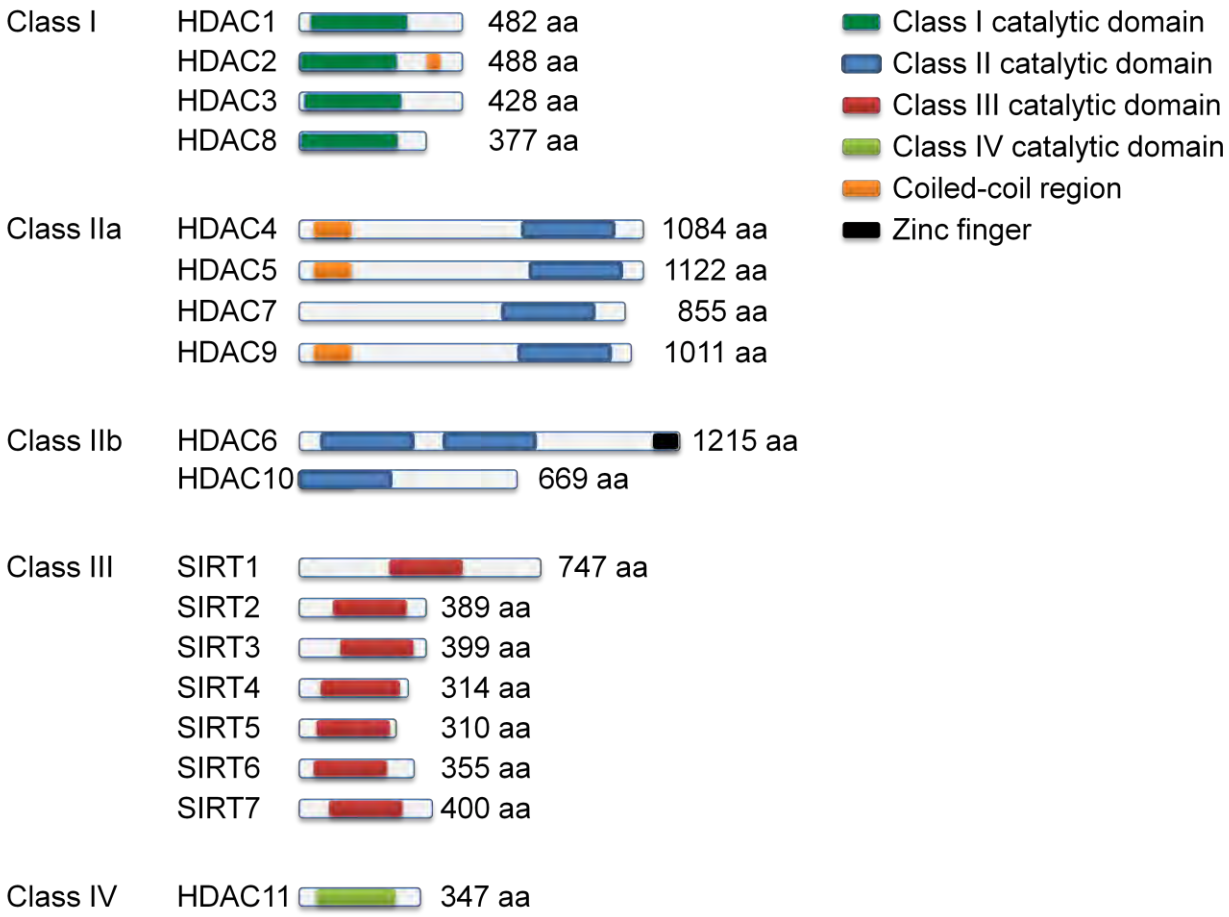

Figure 3. The HDAC superfamily. Schematic organization and classification of mammalian HDAC1-11 and SIRT1-7. The number of amino acids (aa) of full length proteins is given on the right.

HDACs received their name from the original observation that they deacetylate histones. Yet, HDACs control the acetylation of numerous non-histone proteins that regulate gene expression (transcription factors and regulators) and are involved in cell structure (tubulin) and in the control of cell cycle, cell death, circadian rhythm and metabolism (62). In fact, mass spectrometry identified thousands of acetylated proteins (63). As such, HDACs impact on many cellular functions. Moreover, reversible acetylation affects mRNA and protein stability, protein localization, protein-protein interactions and protein enzymatic activity. Although it has been proposed to rename HDACs by lysine deacetylase (KDACs), no consensus arose and the great majority of the scientific community still uses the term HDACs.

1.5 Histone deacetylases inhibitors (HDACi) and the control of immune responses

We have published a review about HDACs inhibitors (HDACi) and their impact on innate immune responses and sepsis (**section 3.1**). Therefore, I will not develop this topic in great details herein.

In the mid-seventies, sodium butyrate (NaB), a short-chain fatty acid (SCFA) that is produced by anaerobical bacterial fermentation of dietary fibers, was reported to stop DNA synthesis and cell proliferation, induce erythroleukemic cell differentiation and to increase the level of acetylated histones (64). These pioneer studies provided a strong impulsion into the development of research programs aimed at identifying new HDACi, deciphering their mode of action, and testing their potential usage as anti-cancer drugs. Overall, HDACi can be classified into four main structural classes: hydroximates, cyclic peptides, aliphatic acids and benzamides (65, 66). Interestingly, HDACi are commonly used to study the role of HDACs in physiological and pathophysiological processes, including those related to immunity. To avoid confusion, the term HDACi will refer here exclusively to drugs targeting HDAC1-11.

Numerous HDACi are tested in clinical trials and some have been approved for cancer therapy. Valproate, used over the last decades as a mood stabilizer and anti-epileptic, suberoylanilide hydroxamic acid (SAHA, vorinostat) and romidepsin (FK228/FR901228) have been registered for the treatment of patients with cutaneous T-cell lymphoma, and panobinostat (LBH-589) for the treatment of patients with multiple myeloma who experienced two prior therapies (67-69).

As epigenetic modifications of chromatin structure accompany every major cell function, it has been proposed that HDACi could also be used to modulate other patho-physiological processes. Moreover, since carcinogenesis is intricately linked to inflammatory processes, the impact of HDACi has been tested in several experimental models of inflammatory and autoimmune diseases (69-72). The first study reporting that HDACi provide immuno-therapeutic benefits *in vivo* concerns the MRL lpr/lpr mouse model that spontaneously develop an autoimmune syndrome closely resembling human systemic lupus erythematosus (SLE). Trichostatin A (TSA, a pan-HDACi) and SAHA down-regulate TNF, IL-6, IL-10, IL-12, IFNγ and NO production by splenocytes and mesangial cells from MRL-lpr/lpr mice and significantly improve mouse health status (73, 74). Since then, and in line with their powerful anti-inflammatory and immunosuppressive activities, HDACi have been reported to improve outcome in a multitude of experimental models of inflammatory and autoimmune diseases: multiple sclerosis (75, 76), rheumatoid arthritis (77-83), systemic

lupus erythematosus (73, 74, 84), graft-versus host disease (85-89), colitis (87, 90, 91) and asthma (92-94). Among other mechanisms of action, HDACi impair the function of DCs and increase the generation and function of $Foxp3^+$ regulatory T-cells (Treg cells) that are important to establish self-tolerance and to prevent occurrence of autoimmune diseases (83, 89, 91, 95-97). Clinical trials are ongoing to test HDACi as novel therapies for autoimmune diseases (http://www.clinicaltrials.gov).

In a pivotal paper published in 2002, Leoni et al. reported that SAHA, at concentrations much lower than those used to inhibit tumor cell proliferation, inhibits cytokine production by PBMCs and decreases cytokine levels in the blood of LPS-treated mice (98), suggesting that HDACi may impact on innate immunity. Indeed, numerous subsequent studies have shown that HDACi inhibit cytokine, chemokine, nitric oxide (NO) and co-stimulatory molecule expression by PBMCs, monocytes, macrophages and DCs. Interestingly, HDACi impair the generation of Th1 and Th17 immune responses, protective against intracellular and extracellular pathogens (72, 99-106).

Our laboratory has participated to characterize the impact of HDACi in innate immune responses. In initial studies, it was shown that HDACi inhibit the production of macrophage migration inhibitory factor (MIF), a cytokine controlling inflammatory and innate immune responses (107), through a local chromatin deacetylation, thus impairing the recruitment of the transcriptional machinery to the *MIF* proximal promoter (108). Subsequently, the laboratory performed the first comprehensive study of the impact of HDACi on innate immune responses (96). Genome wide expression studies revealed that HDACi act as negative regulators of immune receptors and antimicrobial products in mouse and human immune cells, a phenomenon resulting at least in part from an increased activity of the Mi-2/NuRD complex that acts as a transcriptional repressor of cytokine production by macrophages. TSA and valproate also impair the phagocytosis and the killing of bacteria by macrophages (109). Finally, in preclinical mouse models, valproate protects from rapidly lethal Pam_3CSK_4 lipopeptide-induced toxic shock and cecal ligation and puncture, but increases mortality to nonsevere *Klebsiella pneumonia* (*K. pneumonia*) pneumonia and chronic *Candida* infection (96). All these observations fit well with the concept that interfering with inflammatory and innate immune responses may, on the one hand, provide benefits in acute models of infection, but on the other hand increase the risk of infections. As an example, anti-TNF therapies are associated with reactivation of latent tuberculosis and viral infections and increase of opportunistic infections (110). From these studies, it was proposed to have a close monitoring of the infection and immune status of patients

treated with HDACi (72, 96, 109). Supporting this claim, NK cells and DCs from patients treated with romidepsin have impaired function and reduced response to TLR-stimulation (111, 112), and episodes of severe infection have been reported in patients treated with HDACi (113-117).

1.6 Sirtuins, the class III HDACs

Sirtuins are evolutionary conserved from bacteria to humans (118) and are unique because they require NAD^+ for their deacetylase activity. Sirtuins evolved in prokaryotes and subsequently underwent great functional diversification and numerical increase during evolution and growing organism complexity. In particular, while the catalytic domain is well conserved, the N-terminus and C-terminus of sirtuins vary among species and among different sirtuins of a given organism. This diversification is believed to be the basis of functional implications during evolution (119). In mammals there are seven functional *SIRT* genes, all localized on different chromosomes, with different length and complexity ranging from 4 to 16 exons (**Table 2**). *SIRT* genes encode for proteins with different cellular localizations, targets and functions.

Table 2: Characteristics of human *sirtuin* genes.

Sirtuin gene	Chromosome	Start	End	Length (bp)	Nbr of exons
SIRT1	10q21.3	69,644,427	69,678,147	33721	9
SIRT2	19q13.2	39,369,195	39,390,502	21308	16
SIRT3	11p15.5	215,030	236,931	21902	7
SIRT4	12q24.31	120,740,119	120,751,052	10934	4
SIRT5	6p23	13,574,792	13,614,790	39999	10
SIRT6	19p13.3	4,174,106	4,182,596	8491	8
SIRT7	17q25.3	79,869,815	79,879,199	9385	10

1.6.1 Cellular localization of sirtuins

Sirtuins are found in three main compartments: cytosol, nucleus and mitochondria (**Table 3**). SIRT1 carries a nuclear export sequence and can shuttle between the nucleus and the cytoplasm. Its subcellular distribution is tissue dependent (120-122). SIRT2 is mainly cytoplasmic, but translocates to the nucleus during the cell cycle transition from G2 to M phases (123-126). SIRT3, SIRT4 and SIRT5 are mitochondrial sirtuins localized mainly in the matrix thanks to mitochondrial targeting sequences (127, 128). SIRT3 localization into the nucleus is still under debate and contrasting results have been obtained in human and mouse (129-133). SIRT5 exists in two isoforms with slightly different C-terminus. Both possess mitochondrial targeting signals, but one of the two isoforms localizes in both the mitochondria and the cytoplasm (134). SIRT5 is additionally

found in the nuclear compartment (135). SIRT6 is essentially nuclear (136, 137). Yet, a study has linked SIRT6 activity to secretory organelles of the endoplasmic reticulum (138) and another one has localized SIRT6 in the nucleolus (139). Finally, SIRT7 is basically a nucleolar protein (140), but a recent work suggests its redistribution from the nucleolus to the nucleoplasm under stress conditions (141).

Table 3: Enzymatic activity and sub-cellular localization of mammalian sirtuins.

Sirtuin	Enzymatic activity	Sub-cellular localization
SIRT1	Deacetylase, deformylase	Nuclear, cytoplasmic
SIRT2	Deacetylase, demyristoylation	Cytoplasmic, nuclear
SIRT3	Deacetylase	Mitochondrial (nuclear)
SIRT4	ADP-ribosyltransferase (deacetylase)	Mitochondrial
SIRT5	Deacetylase, demalonylase, desuccinylase, deglutarylase	Mitochondrial (cytoplasmic, nuclear)
SIRT6	ADP-ribosyltransferase, deacylase (deacetylase)	Nuclear (cytoplasmic)
SIRT7	Deacetylase	Nucleolar

Weak enzymatic activities and ambiguous sub-cellular localizations are presented in parenthesis.

1.6.2 Deacetylation activity of sirtuins

The deacetylase activity of sirtuins was first described in 2000 (142-144). The catalytic core is formed by 250 amino acids (aa) and two domains: a large conserved Rossman fold-domain that contains the NAD^+-binding site and a smaller and more variable Zn^{2+}-binding domain (**Figure 4**). Multiple interactions between the two domains create a tunnel that can have different levels of hydrophobicity according to the variability of the Zn^{2+}-binding domain. Substrates and NAD^+ are placed at the interface of the two domains during the catalytic reactions (118, 145, 146). Contrary to classical HDACs, sirtuins do not require zinc for their catalytic activity, but to maintain the structure of catalytic core.

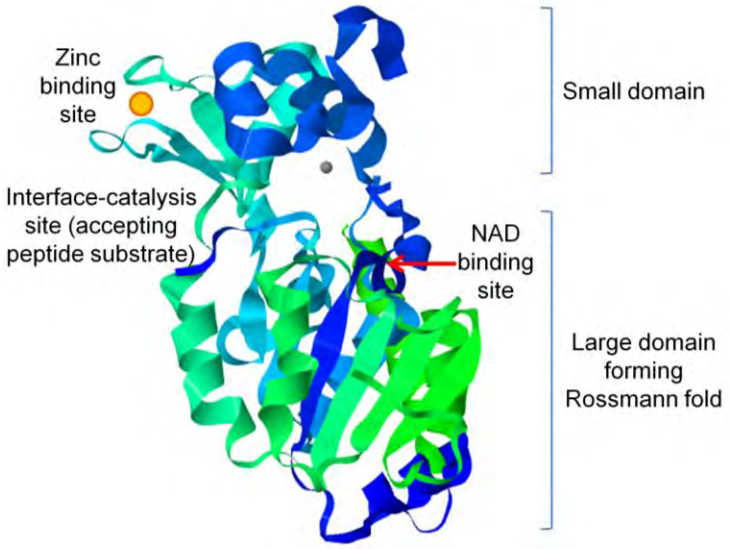

Figure 4. Crystallographic structure of human SIRT2 in complex with the myristoylated peptide H3K9myr. The structure was built from the Protein Data Bank (PDB, # 4Y6L) of the RCSB (Research Collaboratory for Structural Bioinformatics; www.rcsb.org/pdb/). Zinc ion is shown as an orange ball.

The deacetylation reaction of sirtuins occurs in subsequent steps. First, sirtuin binds a substrate, then NAD^+ is cleaved and nicotinamide (NAM) is released. This leads to the formation of the intermediate molecule 1-α-*O*-ADP-ribose-peptidylimidate (or O-alkylamidate). Second, the 2′-OH group of the ribose interacts with substrate's imidate, yielding a deacetylated protein and 2'-*O*-acetyl-ADP-ribose (OAADPr) (147). The stoichiometry of the reaction is represented in **Figure 5.**

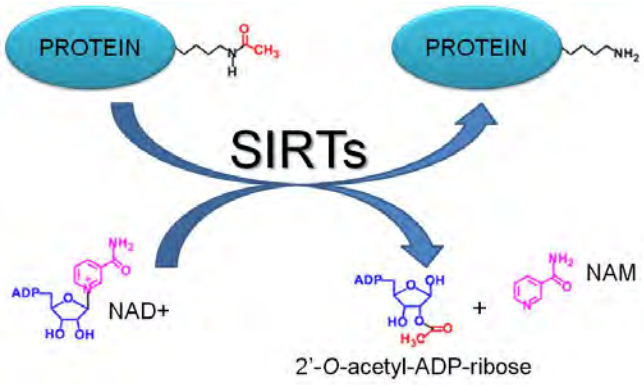

Figure 5. Protein deacetylation by sirtuins (SIRTs). Products formed by the reaction are one deacetylated protein, one nicotinamide (NAM) and one 2'-*O*-acetyl-ADP-ribose.

1.6.3 Other enzymatic activities of sirtuins

SIRT4, 6 and 7 are weak deacetylases, and sirtuins catalyze reactions other than deacetylation (**Table 3**). SIRT4 and SIRT6 work as ADP-ribosyltransferases (148, 149). In that case, they catalyse the transfer of an ADP-ribose to the target protein instead of transferring an acetyl group from a substrate to an ADP-ribose. SIRT6 is probably a stronger deacylase than deacetylase (138). Finally, SIRT1 displays deformylase activity (150), SIRT2 demyristoylase activity (151) and SIRT5 demalonylase, desuccinylase and deglutarylase activities (152, 153).

1.6.4 Targets of sirtuins

The first identified sirtuin, silent information regulator 2 (SIR2), was described as mating-type regulator 1 (MAR1) in yeast and found necessary for silencing at mating-type loci and telomeres (154, 155). This silencing ability was linked to particular lysine residues in the amino-terminal tail of histones H3 and H4 (156-158). These lysines were acetylated in active chromatin and deacetylated in silenced chromatin (156, 159). As overexpression of SIR2 was associated with global deacetylation of yeast histones (159), SIR2 was suggested to be an histone deacetylase. Sirtuins were then established as histone deacetylases in multiple studies. Yet, similar to what happened for HDAC1-11, many non-histone protein substrates of sirtuins have been identified. The first of them was described in 1997 as the tumor suppressor p53 (160). Considering that sirtuin deacetylase activity is not linked to a target consensus sequence, high-throughput and/or unbiased studies of acetylome in human, mouse and lower organisms have contributed considerably to the discovery of sirtuin targets. Some studies are based on the usage of lysine acetylation inhibitors/modulators (161), others use sirtuin mutant/knockout (162-166) and others are based on the analysis of the literature (167). Among known targets, there are chromatin elements and chromatin modifiers, metabolic enzymes, transcription machinery elements, structural cell components and signal transduction actors, and new ones are continuously being discovered. The top 12 substrates of human SIRT1-7 are listed In **Table 4**.

Table 4. Top 12 substrates of SIRT1-7 (extracted from (167)).

Sirtuin	Sequence	Protein name (Lysine position)	Localization
Sirt1	AEEILEKGLKVRE	Ribosomal protein L11 (RPL11) (83;85)	Nucleus
		Cell growth-inhibiting protein 34 (84)	
	DVGRMFKQFNKLT	Tripartite motif-containing 28 (TRIM28) (770)	Nucleus
	GGSVLVKRMFRPM	Grainyhead-like protein 2 homolog (512)	Nucleus
	GPCKMIKPFFHSL	Thioredoxin (39)	Nucleus
	IEIHYAKTAKKMD	Condensin complex subunit 2 (78;501;613;626;637)	Nucleus
	ITKAQKKDGKKRK	Histone H2B type 1-B (25)	Nucleus
	KKHKKEKKKVKDK	Mediator of RNA polymerase II transcription subunit 1 (445)	Nucleus
		cDNA, FLJ94908, similar to Homo sapiens PPAR binding protein (PPARBP) (1489)	
	KRKRSRKESYSVY	Histone H2B type 1-C/D/E/F/G/H/K/I/M/N/F-S (35)	Nucleus
		HIST1H2BC protein (35)	
	PKKPRGKMSSYAF	Putative high mobility group protein B1-like 1 (12)	Nucleus
		High-mobility group box 1 variant (14)	
	PVGGGQKLLTRKA	Nucleolar and coiled-body phosphoprotein 1(NOLC1) (134;415)	Nucleus
	RKARAKKNKAMKS	Chromodomain-helicase-DNA-binding protein 1 (1338)	Nucleus
	TEKKAKKAKIKVK	Nucleolar protein 58 (461)	Nucleus
Sirt2	ENYRRNKSYSFIA	Calcium homeostasis endoplasmic reticulum protein (CHERP), (366;386;912)	Cytoplasm
	FAKNVQKRLNRAQ	Amphiphysin (AMPH) (15)	Cytoplasmic vesicle
	GMIFYRKGVKSVD	Serine hydroxymethyltransferase (133;271)	Cytoplasm
	IKQRAAKYANSNP	Histone acetyltransferase p300 (912)	Cytoplasm
	KISAYMKSSRFLP	GSTM1 glutathione S-transferase mu 1 (130;199;218), mu 2 (199;160), mu 4 (199)	Cytoplasm
	KWTNYIKGYQRRW	Oxysterol-binding protein 1 (103)	Cytoplasm
	NMKAVLKTSSPSV	FH1/FH2 domain-containing protein 3 (FHOD3) (516;531;1199;340)	Cytoplasm
	PACRYRKCLQAGM	NR3C1 nuclear receptor subfamily 3, group C, member 1(480;481;83)	Cytoplasm
	RKARAKKNKAMKS	SMAD2 SMAD family member 2 (20)	Cytoplasm
	RNALYIKSSKISR	Usp14 ubiquitin specific peptidase 14 (313)	Cytoplasm
		Ubiquitin carboxyl-terminal hydrolase (267;278;287;302;313)	
	RTVPQYKYAAGVR	PABPC1 poly(A) binding protein, cytoplasmic 1 (21;59;45;165;467;512)	Cytoplasm
		cDNA, highly similar to PABPC1 (467;512;1480)	
	SYVASTKYQKVYG	CTNNA2 catenin (cadherin-associated protein), alpha 2 (623;889)	Cytoplasm
		Catenin alpha 2 isoform CTNNA2b (473)	
Sirt3	AFKDKYKQLFLGG	SLC25A4 solute carrier family 25 member 4 (96;129)	Mitochondrion inner membrane
	ALNFAFKDKYKQI	SLC25A6 solute carrier family 25 member 6 (92)	Mitochondrion inner membrane
		ADP/ATP translocase (44)	
	ANVVHVKSLPGYM	Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial (146)	Mitochondrion
	ARDEGGKAFFKGA	ADP/ATP translocase 2 (268)	Mitochondrion inner membrane
		SLC25A5 protein (293); cDNA FLJ96310, similar to Homo sapiens SLC25A5 (268)	
	FRDEGGKAFFKGA	ADP/ATP translocase 3 (268)	Mitochondrion inner membrane
		ADP,ATP carrier protein, liver isoform T2 variant (293)	
	GAKAFFKGAWSNV	ADP/ATP translocase 1 (272)	Mitochondrion inner membrane
	HYDLLEKNINIVR	ACO2, aconitase 2, mitochondrial (50)	Mitochondrion
	RLLGWKKSAGGSG	cDNA FLJ51705, similar to Aconitate hydratase, mitochondrial (EC 4.2.1.3) (50)	
	IENAYKKTFLPEM	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa (8;14;243)	Mitochondrion matrix
		NDA1 internal alternative NAD(P)H-ubiquinone oxidoreductase A1 (242)	
	KLAQQIKQEVRQE	Methylenetetrahydrofolate dehydrogenase 2, methenyltetrahydrofolate cyclohydrolase (50)	Mitochondrion
	KNPGYIKLRKIRA	Prohibitin-2 (250); PNAS-141 (PHB2 prohibitin 2), partial (20)	Mitochondrion inner membrane
	KQKEITKAIKRAQ	28S ribosomal protein S18c, mitochondrial (109;81;80)	Mitochondrion
	QTSPNPKYRGFFH	Tricarboxylate transport protein, mitochondrial (160)	Mitochondrion inner membrane
Sirt4	AAWEAGKFGNEVI	AACAT1 acetyl-CoA acetyltransferase 1 (230)	Mitochondrion
	APVLFNKEMIESM	NAD(P) transhydrogenase, mitochondrial (331;200)	Mitochondrion inner membrane
	DDPEVQKDIKNVP	Stress-70 protein, mitochondrial (135;66)	Mitochondrion
		HSPA9, partial [synthetic construct] (137)	
	EKHFFHKVSERLS	Mitofusin-2 (243)	Mitochondrion outer membrane
		cDNA FLJ57997, similar to Transmembrane GTPase MFN2 (EC 3.6.5.-) (107)	
	ELNTKVKEKFQQL	Vesicle transport protein SEC20 (48)	Mitochondrion
	GCRHFSKTNELLQ	Uracil-DNA glycosylase (295;286); cDNA FLJ54140, highly similar to UNG (252)	Mitochondrion
	IHDTETKMEEFKD	Stress-70 protein, mitochondrial (595);HSPA9 protein (121;321;597)	Mitochondrion
	QAEMDLKRLRDPL	Calcium uniporter protein, mitochondrial (332)	Mitochondrion inner membrane
	RSKHMPKSTIETA	Translational activator of mitochondrially encoded cytochrome c oxidase I (119)	Mitochondrion
	RSTMNFKIGGVTE	Fumarate hydratase, mitochondrial (80)	Mitochondrion
	TYQHPPKDSSGQH	Aconitate hydratase mitochondrial (ACO2) precursor (549); ACO2 (574)	Mitochondrion
	YSNNITKLLKAIS	NAD(P) transhydrogenase, mitochondrial (394;263)	Mitochondrion inner membrane
Sirt5	GCRHFSKTNELLQ	Uracil-DNA glycosylase (295;286)	Mitochondrion
	KQSGFGKDLGEEA	ALDH1L2 aldehyde dehydrogenase 1 family, member L2 (903)	Mitochondrion
	LAYGLDKSEDKVI	Stress-70 protein, mitochondrial (234), HSPA9 protein (236)	Mitochondrion
		Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) (102)	
	NDVGIQKDGAPKA	ETFDH electron-transferring-flavoprotein dehydrogenase (58;176;223)	Mitochondrion inner membrane
		ACADM acyl-CoA dehydrogenase, C-4 to C-12 straight chain (223)	
	PANEDQKIGIEII	HSPD1 heat shock 60kDa protein 1 (chaperonin) (462);	Mitochondrion matrix
	PGSPLDKLVSSTS	HMGCS2 3-hydroxy-3-methylglutaryl-CoA synthase 2 (84;437)	Mitochondrion
	EKHFFHKVSERLS	cDNA, similar to HMGCS2 3-hydroxy-3-methylglutaryl-CoA synthase 2 (203; 213)	
	QEGVDPKKLDSLT	HADHA, alpha subunit (55;569)	Mitochondrion
		Epididymis tissue sperm binding protein Li 14m (569)	
	RAIQSLKKIVNSA	ATP5L ATP synthase, H+ transporting, mitochondrial Fo complex, subunit G (54)	Mitochondrion
	RSDPDPKAPANKA	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial (23;176;212)	Mitochondrion matrix
		ACADM acyl-CoA dehydrogenase, C-4 to C-12 straight chain (245)	
	TIRADGKISEQSD	ATP synthase, H+ transporting, mitochondrial F1 complex, a-subunit 1 (7;51;509)	Mitochondrion inner membrane
	TYCDLGKAAKDVF	Voltage-dependent anion-selective channel protein 3 (12)	Mitochondrion outer membrane
Sirt6	AATAAEKYKHRGE	MECP2 methyl CpG binding protein 2 (33;449)	Nucleus
	AKVFIDKQTNLSK	CUGBP (CUG triplet repeat, RNA binding protein), Elav-like family member 1 (436)	Nucleus
		CELF2 CUGBP, Elav-like family member 2 (353;448;458;471)	

	GPSFPLKGITEQQ	X-ray repair cross-complementing protein 5 precursor (195)	Nucleus
		Putative uncharacterized protein XRCC5 (31)	
	IIIHSLKKCDISL	X-ray repair cross-complementing protein 5 (155)	Nucleus
	KLIQILKGQSLLQ	Flap endonuclease GEN homolog 1 (230)	Nucleus
	LKALQEKVEIKQL	X-ray repair cross-complementing protein 5 (665)	Nucleus
	MKEEYDKIQIADL	Pyrin and HIN domain-containing protein 1 (45)	Nucleus
	QKNSNSKNDRRNR	Chromatin modification-related protein MEAF6 (74;52)	Nucleus
	RLEHKLKEEEESL	Nesprin-1 (75;550;573;791;1340;3342;3499)	Nucleus outer membrane
		SYNE1 spectrin repeat containing, nuclear envelope 1 (243;933;1918;2603)	
		Similar to spectrin-like protein of the nuclear envelope and Golgi; Syne-1 (232)	
	RRGRRRKYNPTRN	Methyl-CpG-binding domain protein 6 (956;572)	Nucleus
	SAEGAAKEEPKRR	HMGN1 high mobility group nucleosome binding domain 1 (14;30)	Nucleus
	YVAVMLKVADHSG	COMMD6 COMM domain containing 6 (48;63)	Nucleus
Sirt7	APPSTRKDYPAAK	Myc proto-oncogene protein (317;331)	Nucleus
	EWAYGKKGQPDAK	Peptidyl-prolyl cis-trans isomerase FKBP3(201)	Nucleus
	IVKMLEKYLKGED	AKAP8 A kinase (PRKA) anchor protein 8 (538)	Nucleus matrix
	KRKRSRKESYSVY	Histone H2B type 1-C/D/E/F/G/H/I/K/L/M/N (35), Histone H2B type 2-F/S (35)	Nucleus
	LGMNSRKPDLRVV	MEF2A myocyte enhancer factor 2A (270;200;268;189)	Nucleus
	MMENGIKPVYVFD	FEN1 flap structure-specific endonuclease 1 (80;44)	Nucleus
	MPKPRLKATVTPS	NUCKS1 nuclear casein kinase and cyclin-dependent kinase substrate 1 (175)	Nucleus
	MSSRHFKPEIRVT	PWP2 periodic tryptophan protein homolog (700)	Nucleus
	NKNMQAKSPPPMN	MADS box transcription enhancer factor 2, polypeptide C (191;239;237;257)	Nucleus
	ZMPDPAKSAPAPK	HIST1H2BH histone cluster 1, H2bh (6), HIST1H2BO histone cluster 1, H2bo (6)	Nucleus
		HIST2H2BF histone cluster 2, H2bf (6)	
	ZMPEPAKSAPAPK	Histone H2B type 1-C/E/F/G/I (6), Histone H2B type F-S-like (6)	Nucleus
		HIST1H2BJ histone cluster 1, H2bj (6), HIST1H2BK histone cluster 1, H2bk (6)	
		HIST2H2BE histone cluster 2, H2be (6), HIST1H2BC protein (6)	
	ZMPEPVKSAPVPK	HIST1H2BM histone cluster 1, H2bm (6)	Nucleus

Interestingly, several sirtuins have common targets that might constitute a sign of functional cooperation. Moreover, sirtuins sharing the same subcellular localization have specific, even isoform-specific, targets. Considering the number and diversity of proteins targeted by sirtuins, it is not surprising that these enzymes are involved in multiple physiological and pathophysiological processes.

1.6.5 Sirtuin modulators

Numerous studies in the field of sirtuins use pharmacological modulators of sirtuin activity, with the great majority of them directed at SIRT1. Therefore, before addressing the role of sirtuins in physiology and pathophysiology, we will first discuss the discovery and development of sirtuin activators and inhibitors.

Because of a link made between sirtuins, lifespan and age-related diseases (detailed in **sections 1.6.6-1.6.8**), there was a huge interest in developing sirtuin modulators (168, 169). That field of R&D was strongly boosted by the hypothesis that calorie restriction (CR), the only non-genetic condition known to improve lifespan, increases lifespan through sirtuins (96, 109, 170, 171).

The best known molecule able to activate SIRT1 is the polyphenol resveratrol (172-174). A number of other SIRT1 activators (also called STACs for SIRT1-activating compounds) structurally non-related to resveratrol have been described (175). Although the real impact of sirtuins on lifespan is source of controversy (see **section 1.6.6**) and the specificity of resveratrol for SIRT1 is questionable, STACs have been shown to mimic some of the effects of CR and to improve age related pathologies. Many studies are currently assessing the

therapeutical potential of STACs in pathological conditions. Resveratrol is currently under clinical trial for conditions such as aging, memory, type 2 diabetes, Huntington disease, chronic obstructive disease, and coronary artery disease in patients with metabolic disease (https://clinicaltrials.gov).

Sirtris Pharmaceuticals Inc. is a biotechnology company founded in 2004 by David Sinclair from Harvard University (Cambridge, MA). David Sinclair, at that time in the laboratory of Leonard Guarente, published seminal discoveries suggesting that the yeast *Sir2* gene increases lifespan (168). Sirtris was created with the aim to develop STACs for treating type 2 diabetes, cancer, and other diseases. Sirtris has been purchased in 2008 by GlaxoSmithKline that pursues the development of STACs, with lead candidates SRT2104, SRT1720 and SRT2379. SRT1720 and SRT2104 were recently reported to extend the lifespan of mice (176). Interestingly, the group of van der Poll (AMC, Amsterdam) recently reported that oral administration of SIRT2104 reduced LPS-induced blood levels of IL-6, IL-8 and C-reactive protein, and attenuated coagulation activation in healthy volunteers. TNF production and activation of neutrophils, vascular endothelium and fibrinolytic system, as well as total leukocyte counts and leukocyte transcriptome (177) were not affected by SIRT2104.

Table 5. Selection of commercially available sirtuin inhibitors.

Inhibitor	IC_{50} (μM) or inhibition (%)				
	SIRT1	SIRT2	SIRT3	SIRT5	SIRT6
AGK2	>30	3.5	>50		
AK-7	>50	15.05.2024	>50		
Cambinol	56	59		42% at 300 μM	0% up to 250 μM
CHIC-35	0.06-0.12	2.8	0%		
EX-527	0.098-1	20-33	49	0%	56% at 200 μM
Ginkgolic acids	119-126	80-141			
Inauhzin	0.7-2	0%	0%		
Nicotinamide	50-100	1.2-100	30-43	1600	2200
Salermide	76	25-45	>50		
Sirtinol	37.6-131	38-58	>50		0%
Splitomicin HR73	5	0%	0%		
Suramin	0.297	1.15	0%	22	0%
Tenovin-1	70-90% at 25 μM	10			
Tenovin 6	21	10	67		
Urushiols	52-118	55-143			

Since sirtuins have a detrimental role in several pathologies (see **sections 1.6.7-1.6.9**), sirtuin inhibitors have been developed as promising drugs for cancer and neurodegenerative diseases (178). The most well described inhibitors are listed in **Table 5**. They mainly target SIRT1 and SIRT2, although it should be noted that their specificity towards the entire panel of sirtuins is rarely tested. Since we have been working with

sirtuin inhibitors (see **sections 3.4-3.5**), the chemical structure of the most-relevant ones for our studies is displayed in **Figure 6**. These include cambinol and sirtinol that are SIRT1/SIRT2 inhibitors with IC_{50} of around 50-60 μM and 30-130 μM, respectively. EX-527 and CHIC-35 are SIRT1 inhibitors at concentration lower than 2 μM, while AGK2 and AK-7 are SIRT2 inhibitors with IC_{50} of 3.5 μM and 15-24 μM, respectively.

Cambinol is a chemically stable and cell permeable β-naphtol related to splitomicin. It works through substrate competition, it has no activity on classical Zn-dependent HDACs and it displays low cell toxicity (179). Sirtinol was identified through a high-throughput yeast cell-based phenotypic screening. Similarly to cambinol, sirtinol is a cell permeable molecule and it works through substrate competition (180). EX-527 is a cell permeable and stable indole. It was identified through high-throughput screening using human SIRT1 produced in bacteria. It binds SIRT1 after the release of nicotinamide and prevents the release of deacetylated products (181, 182). CHIC-35 is an EX-527 analog. CHIC-35 binds the catalytic cleft of SIRT1, displacing NAD^+ and modifying SIRT1 into a conformational state that sterically prevents the binding of substrates. (183) AGK2 was identified thanks to screening through an *in vitro* fluorimetric assay of structural analogs of known SIRT2 inhibitors. AGK2 acts by binding to the NAD^+ pocket of SIRT2 (184). Finally, AK-7 was the first identified brain-permeable SIRT2 inhibitor. It was discovered by *in silico* screening of compound libraries and deacetylation assays (185).

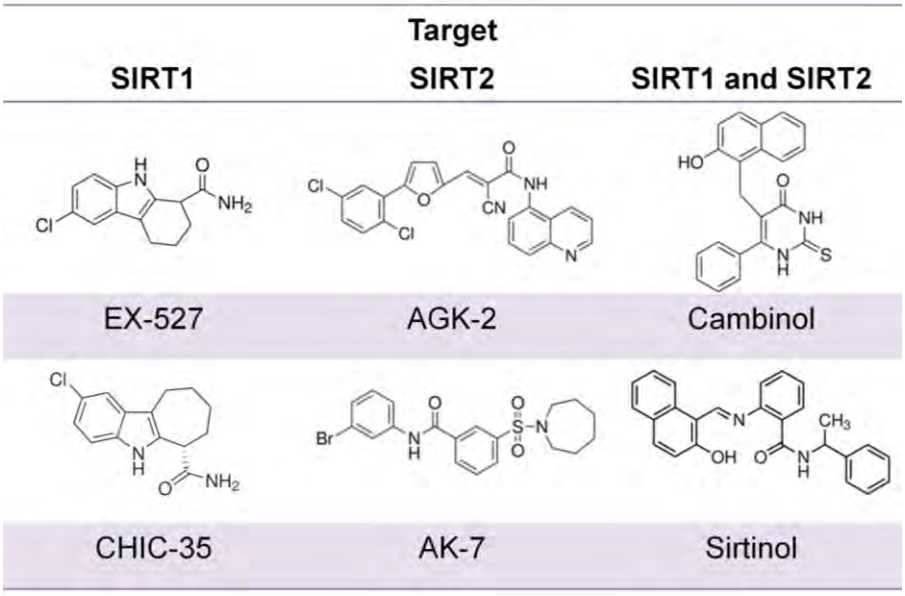

Figure 6. Chemical structure of selected SIRT1 and SIRT2 inhibitors.

1.6.6 Sirtuins and lifespan

The origin of the interest in sirtuins lies in the hypothesis that they might control lifespan. In 1999, the group of Guarente reported that the introduction of an extra copy of the yeast *Sir2* gene in the genome of *Saccharomyces cerevisiae* (*S. cerevisiae*) increases lifespan, whereas an opposite phenotype was observed upon deletion of the *Sir2* gene (168).

In yeast, lifespan is generally defined based on the maximal number of cells that can bud from a parental cell before its death (186). Indeed, cell division happens in an unequal mode, and one of the factors limiting replicative lifespan is the accumulation of ribosomal DNA (rDNA) circles in budding cells (187). This accumulation results from both asymmetric cell division and from the ability of rDNA to self replicate (187). Since deletion of *Sir2* increases rDNA recombination and extrachromosomal rDNA circles and reduces lifespan (168, 188, 189), one of the mechanisms underlying the increased lifespan in yeast with a duplicate *Sir2* gene was proposed to be a more efficient suppression of the formation of the rDNA circles in the genome. Subsequently, an extra copy of *Sir2* was shown to improve lifespan in worms and in fruit flies (189, 190). Therefore, the accumulation of rDNA could not be an exhaustive explanation of the lifespan extension.

Calorie restriction (CR) has been long known to improve lifespan in several species, including yeast, worms, flies, fish and rodents. The first published study, "The Effect of Retarded Growth upon the Length of Life Span and upon the Ultimate Body Size", is from 1935 by McCay et al. and reports that CR extends mean and maximal lifespan of male rats when compared with *ad libitum* fed animals (191) (**Figure 7**).

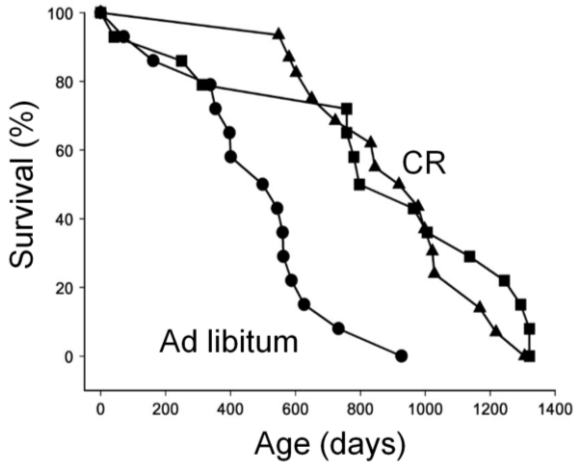

Figure 7. Impact of calorie restriction (CR) on rat lifespan. Survival of male rats fed ad libitum (circles) or under CR without malnutrition starting at weaning (squares) or 2-weeks post-weaning (triangles). The graph is from McDonald and Ramsey (in "*Honoring Clive McCay and 75 years of calorie restriction research*"; (192)) that reanalyzed the original data from McCay, Crowell and Maynard (191). Of note, the ability of CR to extend lifespan was not observed using female rats.

The mechanism by which CR mediates its beneficial effects remains unclear, but seems linked to reduced oxidative stress and metabolism. Indeed, reducing glucose concentration in yeast growth media increases replicative lifespan (193). Upon *Sir2* gene deletion, CR fails to increase lifespan (193, 194). Following these observations, SIR2 was believed to mediate the increased longevity conferred by CR.

Two models on how CR increases SIR2 activity in yeast have been proposed. In the first model, CR induces a metabolic shift from fermentation to respiration provoking an increase of NAD^+ levels required for sirtuin activity (194). In the second model, CR up-regulates the expression of *PNC1*, which encodes for a nicotinamidase, resulting in a decreased concentration of nicotinamide (NAM) that, as a product of deacetylase reaction, acts as a sirtuin inhibitor (195). Wether the effect of CR on longevity in yeast is mediated by sirtuins is still debated (196-198). A more recent study failed to reproduce the beneficial effects of Sir2 overexpression on the lifespan in *Caenorhabditis elegans* (*C. elegans*) and *Drosophila melanogaster* (*D. melanogaster*) (199), and additional debate arises from two contradicting studies assessing the impact of CR on the lifespan of rhesus monkeys (200, 201). The reasons of these discrepant observations are not clear, but may at least in part result from differences in genetic background and housing conditions of the model organisms.

The findings that sirtuins might promote longevity generated a considerable interest in the development of sirtuin modulators, leading to the identification by high-throughput screening of the SIRT1 activator resveratrol, a plant polyphenol (202). Resveratrol was known to exert anticancer, cardioprotective, anti-inflammatory, antidiabetic and neuroprotective effects (172). Yet, the connection between the pharmacological effects of resveratrol and SIRT1 activation is unclear. Whether resveratrol works exclusively or even predominantly through regulation of sirtuin activity remains questionable. Additional screenings identified small molecule activators of SIRT1 (STACs) that improved insulin sensitivity, lowered plasma glucose and increased mitochondrial biogenesis in genetically and diet-induced obese rodents (203). Some of these molecules are currently being evaluated in clinical trials in patients with metabolic diseases.

In mammals the contribution of the sirtuins to lifespan is unclear (204). Most studies concentrate on SIRT1 and to a lesser extent on SIRT3, SIRT5 and SIRT6. Mice overexpressing SIRT1 have lower DNA damage and improved general health status, with reduced incidence of spontaneous carcinomas and sarcoma (205). SIRT1 transgenic mice also display a CR-like phenotype characterized by reduced body weight, fat mass and blood cholesterol levels (206). Conversely, SIRT1 knockdown causes perinatal lethality (207-209). In

inbred 129/Sv mice, embryo development is delayed, newborns are smaller and the majority dies during the early postnatal period (209). In an outbred background, SIRT1 knockdown mice survive to adulthood, but are sterile and have heart and eye abnormalities (208, 209). There is no evidence for a contribution of SIRT3 in mouse lifespan. Although genetic studies identified single nucleotide polymorphisms (SNPs) in *SIRT3* associated with increased survival, this has not been reproduced in other studies (210-214). Male, but not female, transgenic mice overexpressing SIRT6 have a modest but significantly longer lifespan than wild-type mice (215). Reversely, germline deletion of *SIRT6* in inbred 129/Sv mice provokes progressive hypoglycemia, genomic instability and degenerative phenotype from the third week of life, all of which contributing to an early death (136). Finally, associations between lifespan and *SIRT6* and *SIRT5* SNPs were reported in a cohort of elderlies from Iowa (214).

Although the concept that sirtuins are sufficient and necessary regulators of longevity continues to generate controversy (204), it is commonly accepted that sirtuins play a role in pathways critical in aging (216). Indeed, sirtuins have been linked to several age related diseases among which neurodegenerative (Alzheimer, Huntington's and Parkison's diseases), metabolic, autoimmune and oncologic diseases (217).

1.6.7 Sirtuins and neurodegenerative diseases

Considering that sirtuins are connected to lifespan and that neurodegenerative diseases primarily affect the elderly population with massively increasing health costs, the role and potential target value of sirtuins has sparked lots of interest (218).

Alzheimer's disease

SIRT1 is expressed in different areas of the central nervous system in humans and mice. SIRT1 expression decreases along with disease progression in hippocampal samples of Alzheimer's patients (219). Overexpression and knockdown of SIRT1 and usage of drugs directed at SIRT1 support a protective role for SIRT1 from Alzheimer's disease by reducing two classical hallmarks: the amyloid plaques and the neurofibrillary tangles of TAU protein (220-229).

The direct delivery of a SIRT2 inhibitor (AK-1) to the hippocampus of Alzheimer's disease mice (rTg4510 mice which express a mutant form of the TAU protein) reduces neuronal loss (230). Whereas independent cohort studies using patient of different ethnicities led to inconsistent associations of *SIRT2* SNPs and risk of

Alzheimer's disease (231-233), a recent meta-analysis revealed an association between a *SIRT2* SNP (rs10410544) and disease development (171).

Alteration of SIRT3 expression in the cerebral cortex has been observed in two mouse models of Alzheimer's disease, although with discordant outcome (234, 235). Additionally, one study reported that SIRT3 is up-regulated in the temporal neocortex of Alzheimer's patients (235) while another one reported that SIRT3 expression in the gray matter (GM) inversely correlates with disease progression (219). Finally, SIRT5 expression was reported to increase during Alzheimer's disease (219).

Huntington's disease

In two mouse models of Huntington's disease, SIRT1 overexpression promotes the production of neurotrophic factors, protects from neurotoxicity, limits brain atrophy, reduces metabolic abnormalities, and improves motor functions and survival (236, 237). Conversely, RNA silencing of SIRT1 exacerbates neural toxicity (236) and brain-specific SIRT1 knockdown aggravates pathology (237).

In sharp contrast with the above studies, inhibition of SIRT1 with selisistat (6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide), a novel SIRT1 inhibitor, improves Huntington's disease pathology caused by expression of the mutant form of human huntingtin in *D. melanogaster*, mammalian cells and mice (238). In primary neurons and in *D. melanogaster* and *C. elegans* models of Huntington's disease, genetic ablation of SIRT2 and pharmacological inhibition of SIRT2 with AGK2 and AK-1 reduce sterol biosynthesis and confer neuroprotection (239). In line with these results, in acute and chronic mouse models of Huntington's disease, treatment with the SIRT2 inhibitor AK-7 reduces aggregation of mutant huntingtin and brain atrophy, improves motor function and extends survival (240). Finally, immortalized striatal precursor cells expressing a mutated form of protein Huntingtin display reduced expression of SIRT3 (241).

Parkinson's disease

SIRT1 activation by resveratrol has been suggested to protect from Parkinson's disease (242). Mechanistically, SIRT1 deacetylates heat shock factor 1 (HSF-1), that increases the transcription of molecular chaperones and peroxisome proliferator-activated receptor (PPAR)γ coactivator-1α (PGC-1α) that protect dopaminergic neurons from cell degeneration (243). A genetic study, analyzing the promoter region of the *SIRT1* gene in 97 sporadic Parkinson's disease patients and 127 matched controls, identified three heterozygous variants (g.69644133C>G, g.69644213G>A and g.69644351G>A) in Parkinson's disease patients. It was proposed that these variants alter SIRT1 expression and impact on disease development

(244). No follow-up or confirmation study has been published. SIRT2 inhibition provides neuroprotection *in vitro* and *in vivo* in mouse and *D. melanogaster* models of Parkinson's disease (184, 245, 246). Finally, SIRT5 knockdown exacerbates motor deficits in a Parkinson's disease mouse model (247).

1.6.8 Sirtuins and cancer

Expression of sirtuins is altered in many types of cancer suggesting that these enzymes could play a role in tumorigenesis (248). Yet, a complex picture has arisen from studies in the field showing that SIRT2 functions as a tumor suppressor, SIRT5 and SIRT7 as tumor promoters, and SIRT1, SIRT3, SIRT4 and SIRT6 as either tumor suppressors or oncogenic factors depending on the cellular context and study conditions (249). We will here briefly review some of the main observations linking sirtuins with cancer.

SIRT1

As extensively reviewed by Chalkiadaki and Guarente (249), SIRT1 displays both tumor suppressor and oncogenic activities. SIRT1 deacetylates and downregulates the activity of the tumor suppressors p53 and E2F1. SIRT1 expression is increased in mouse lymphoma, sarcoma and lung and prostate cancers and in human leukemia and liver, colon and thyroid cancers. Tumor suppressors down-regulate SIRT1 expression and activity while oncogenes increase SIRT1 expression. In contradiction with these findings, SIRT1 expression is down-regulated in a number of human cancers (colon, prostate, bladder, breast, oral and brain cancer). SIRT1 overexpression protects from spontaneous tumor development (liver cancer, carcinoma and sarcoma) in mice. Treatment of mice with the SIRT1 activator SRT1720 inhibits growth and promotes death of transplanted human MCF-7 breast cancer cells. These conflicting observations may, at least partially, result from the different cancer types studied and depend on the experimental approaches used (inhibitors/activators *vs* transgenic/knockout cells and mice), and could also highlight different behaviors of SIRT1 depending on the overall context (stress, metabolic and inflammatory status).

SIRT2

SIRT2 acts as a tumor suppressor gene and has an essential role in maintaining the integrity of mitosis through multiple mechanisms (249-251). First, during mitosis SIRT2 shuttles from the cytoplasm to the nucleus where it directly deacetylates H4K16 (252) and indirectly promotes the monomethylation of H4K20 (253) contributing to chromatin condensation and reorganization. Second, SIRT2 regulates the activity of anaphase-promoting complex/cyclosome (necessary for mitotic exit) (254). Third, SIRT2 promotes genomic

fidelity by stabilizing the multidomain protein kinase BUBR1 of the spindle assembly checkpoint and by preventing premature separation of sister chromatids during the G2-M transition (255). Accordingly, SIRT2 levels are decreased in human breast, prostate and renal cancer, and liver and glioma tumors (248, 254). Moreover, SIRT2 knockout mice display genomic instability (253) and develop tumors in the mammary glands, liver, lung, pancreas, stomach, duodenum and prostate (254). Finally, in a model of chemically induced skin cancer, SIRT2 knockout mice develop more and larger aggressive tumors responsible for increased mortality rate (254).

SIRT3-7

SIRT3 knockout cells and mice are more prone to tumorigenesis (254) and human tumors are characterized by deletion or reduction of SIRT3 expression (254, 256). Nevertheless, there is also evidence for an oncologic role of SIRT3 in brain and oral tumors (257-259), and pharmacological inhibition of SIRT3 has anti-tumorigenic properties *in vitro* (260). SIRT4 knockout mice develop spontaneously lung tumors and lymphoma (261, 262). In line with that, SIRT4 expression is decreased in human lung, stomach and bladder tumors and in leukemia (249). Nonetheless, it has recently been suggested that SIRT4 promotes tumorigenesis *in vitro* (263). Little is known about the impact of SIRT5 on cancer although there is evidence for an oncogene function for SIRT5 (264). SIRT6 expression is reduced or absent in 20% of all cancers, but high expression is also associated with resistance to therapy and increased angiogenesis and metastasis (249). SIRT7 deacetylates H3K18 provoking cellular growth and loss of contact inhibition and shRNA-mediated SIRT7 depletion reverses cancer cell phenotypes and inhibits tumor cell growth *in vivo* (265). Moreover, SIRT7 is overexpressed in many human cancer types (249).

1.6.9 Sirtuins and immune functions

The impact of sirtuins on immune function is fragmentary. Most of the studies in the field concern SIRT1 and SIRT6 with recent evidence about SIRT2. As for data obtained in the fields of neurodegenerative disease and cancer (see **sections 1.6.7.-1.6.8**), contradictory findings have been reported, with both proinflammatory and anti-inflammatory activities ascribed to SIRT1, SIRT6 and SIRT2. The reasons why proinflammatory and anti-inflammatory properties have been observed are not clear. Whereas different sirtuins may have opposing properties, additional differences in cell types, human versus murine system, stimuli, usage of inhibitory drugs or si/shRNA and knockouts can partially explain controversial results. Moreover, sirtuins modulate circadian clock and are subjected themselves to circadian oscillation (266). Therefore, also

experimental settings, including *in vivo*, might determine the variability of results. As we will discuss later (see **section 1.6.10**), sirtuins are linked to the energetic status of the cell and, as a consequence, it is possible that qualitative and quantitative differences in caloric input might lead to different observations. Finally, it is now clearly established that the microbiota generates numerous metabolites that impact directly on immune responses (see also **section 3.2**), and therefore differences in microbiota composition might affect outcome in *in vivo* models. Despite all these incertitude, I will summarize in the next sections the principal observations linking sirtuins and immune responses.

SIRT1

Evidences for anti-inflammatory activities of SIRT1. Numerous studies have shown that SIRT1 down-regulates the activitiy of NF-κB and AP-1 that are central transcription factors regulating immune gene expression and inflammation (Galli, Van Gool et al. 2011, Kong, McBurney et al. 2012). Knockdown of SIRT1 in mouse macrophages increases I-κB degradation, NF-κB activation and TNF production in response to LPS stimulation (267). Accordingly, bone marrow-derived macrophages (BMDMs) from myeloid-specific SIRT1 knockout mice display increased NF-κB acetylation and NF-κB-mediated proinflammatory gene expression (268). Of note, myeloid deletion of SIRT1 increases proinflammatory gene expression in M1, but not in M2 macrophages (269). In line with these results, SIRT1 knockdown is associated with severe renal morphological and functional damages linked to an increased infiltration of neutrophils and production of proinflammatory cytokines in a model of endotoxemia (270). Moreover, in a mouse model of sepsis following cecal ligation and puncture, SIRT1 knockdown increases Iκ-B phosphorylation and degradation, NF-κB activation, inflammation and injury in lungs (271). SIRT1 expression and activity in the lung are decreased after exposure to cigarette smoke in rats and in patients with chronic obstructive pulmonary disease (COPD) (272, 273). Those are associated with increased acetylation and activity of NF-κB and release of proinflammatory mediators. Moreover, exposure of SIRT1 deficient mice to cigarette smoke induces higher oxidative stress and reduces antioxidant enzymes, whereas oxidative stress is attenuated in SIRT1 transgenic mice (274). SIRT1 has also been investigated in the context of self-tolerance and autoimmunity. Deletion of SIRT1 in DCs increases their ability to induce Th1 and to down-regulate $Foxp3^+$ Treg cell responses (275). SIRT1 deacetylates and promotes the function of the transcription regulator Aire that allows medullary thymic epithelial cells to express self-antigens during negative selection in the thymus (276). Moreover, SIRT1 favors the peripheral anergy of autoreactive T cells that escape negative thymic selection (276). Thus, SIRT1 could promote self-tolerance and prevent autoimmunity. In fact, SIRT1 myeloid deletion

exacerbates arthritis symptoms, at least partly because of the hyperacetylation and activation of NF-κB in macrophages (170). Furthermore, SIRT1 knockout mice are more susceptible to EAE (277).

Evidences for proinflammatory activities of SIRT1. In sharp contrast with the above-mentioned studies, SIRT1 has been associated with proinflammatory conditions. SIRT1 has been linked to the pathogenesis of lupus (278), allograft rejection (deletion of SIRT1 enhances Treg suppressive activity) (279), EAE (SIRT1 deacetylates RAR-related orphan receptor gamma/RORγ and promotes Th17 differentiation and SIRT1 knockout mice are protected from EAE) (280), arthritis (SIRT1 is highly expressed in rheumatoid arthritis synovial tissues where it positively affects the production of proinflammatory mediators) (281), allergic airway inflammation (SIRT1 deletion in DCs increases PPAR-γ activity, decreases DCs migratory activity in the lung and down-regulates Th2 pathogenic responses) (282) and colitis (SIRT1 knockout mice have reduced proinflammatory gene expression and develop milder colitis) (283). SIRT1 is also important for antiviral immune response (284). Finally, SIRT1 promotes HIF-2α activation during hypoxia (285) and authophagy (286).

Overall, on the one hand, SIRT1 has been shown to regulate innate immune responses and to shape self-tolerance and adaptive immunity. On the other hand, SIRT1 was reported to promote inflammatory and autoimmune conditions.

SIRT6

Evidences for anti-inflammatory activities of SIRT6. SIRT6 shapes the development of DCs. SIRT6 knockout bone marrow-derived DCs (BMDCs) produce increased TNF levels upon TLR stimulation when compared with wild-type BMDCs (293). In line with this finding, overexpression of SIRT6 suppresses the expression of NF-κB dependent genes, reduces inflammation and protects mice from chondrocyte degeneration in models of osteoarthritis (294) and arthritis (295). Similarly, SIRT6-overexpressing cardiomyocytes display reduced NF-κB activation and ROS production and increased survival during hypoxia (296), while SIRT6 knockout mice are more sensitive to ischemia/reperfusion injury (297). SIRT6 down-regulation favors the formation of atherosclerotic plaques in diabetic patients (298). Finally, SIRT6 promotes autophagy in human bronchial epithelial cells and probably protects from COPD (299).

Evidences for proinflammatory activities of SIRT6. SIRT6 has an important role in mounting proinflammatory responses. In a seminal paper, Van Gool et al. reported that nicotinamide posphoribosiltransferase (NAMPT) inhibitors impair TNF synthesis by macrophages and DCs and identified SIRT6 as the sirtuin that controls

TNF production by acting at a post-transcriptional level (287). Moreover, SIRT6 regulates TNF secretion through its deacetylation that probably occurs in the endoplasmic reticulum during the secretion process (138). Additionally, SIRT6 increases nuclear levels of NFAT (288), and, reducing the bioavailability of NAD^+ through inhibition or deletion of the NAMPT, impairs the production of TNF and IFNγ by phytohemagglutinin-stimulated peripheral blood lymphocytes, and of TNF in mice challenged with LPS (289, 290). Overexpression of SIRT6 sensitizes neurons to oxidative stress-induced mortality (291) and promotes insulin sensitivity during high caloric diet (292).

SIRT2

Evidences for anti-inflammatory activities of SIRT2. A first evidence of the involvement of SIRT2 in immune responses date back to 2010, when Rothgiesser et al. reported that SIRT2 decreases NF-κB activity in mouse embryonic fibroblasts (MEFs). Mechanistically, SIRT2 binds and deacetylates NF-κB p65 subunit at lysine 310 (K310). Upon TNF stimulation, p65/SIRT2 complex formation is enhanced, preventing NF-κB from promoting the transcription of a subset of target genes (300). SIRT2 was then shown to have anti-inflammatory properties and to be protective in models of:

- Collagen-induced arthritis (CIA). SIRT2 knockout mice develop more severe swelling, erythema, articular destruction, joint rigidity and displacement in the hind paws ankles and have higher levels of proinflammatory cytokines in both serum and joint tissue due to higher levels of acetylated p65 (301).
- Chemically induced colitis. SIRT2 knockout mice have more severe intestinal inflammation and weight loss (302).
- LPS-induced renal injury. SIRT2 knockdown increases the acetylation of MAPK phosphatase-1 (MKP-1) suppressing MAPK activation and inhibits p65 binding to the promoters of chemokine receptors CXCL2 (targeting neutrophils) and CCL2 (targeting monocytes and macrophages). As a consequence, SIRT2 knockout mice express reduced levels of CXCL2 and CCL2 in mouse proximal tubular epithelia and display less neutrophil and macrophage infiltration, and decreased TNF, IL1β and IL-6 expression in the kidney (303).
- LPS-induced inflammation in the central nervous system. SIRT2 is expressed by murine microglia cells and is downregulated upon intracortical injection of LPS. In SIRT2 knockout mice, cortical microglia cells express increased mRNA levels of proinflammatory cytokines (304).

- Brain injury. In a model of traumatic brain injury, administration of AK-7 increases p65 K310 acetylation, NF-κB nuclear translocation, microglial activation and production of proinflammatory mediators and worsens edema (305).

Evidences for proinflammatory activities or weak/no immunomodulatory activity, of SIRT2. SIRT2 knockout BMDMs stimulated with LPS display lower NF-κB nuclear translocation, express less iNOS and produce lower levels of NO and ROS than wild-type BMDMs (306). Much interestingly, SIRT2 was reported to favor the pathogenesis of *L. monocytogenes* infection. *L. monocytogenes* exploits host SIRT2 to dampen the immune response of infected cells. The listerial virulence factor InlB induces the nuclear translocation of SIRT2 that deacetylates H3K18, thus repressing the expression of DNA binding proteins, transcription factors and immune response elements (307). Finally, myeloid conditional SIRT2 knockout mice challenged with *Mycobacterium tuberculosis* (*M. tuberculosis*), delivered through aerosol, have slightly higher bacterial counts at the early stage of the disease, but no alteration of immune cell proportion and cytokine production in lungs. The initial mycobacterial burden in lungs is lost at later stages of the disease suggesting that SIRT2 ablation does not really affect *M. tuberculosis* infection process (308).

1.6.10 Sirtuins, metabolism and immunometabolism

Sirtuin activity is intrinsically linked to the metabolic status of the cell through the dependency on NAD^+. NAD exists in two forms, oxidized NAD^+ and a reduced NADH. NAD^+ is an oxidizing agent that accepts electrons from other molecules to generate NADH which can in turn be used as a reducing agent to donate electrons. Briefly, NADH is formed during glycolysis, fatty acid oxidation, citric cycle and tricarboxylic acid (TCA or Krebs) cycle. It is converted to NAD^+ by the electron transport chain (ETC). Once used by sirtuins, NAD^+ is recycled inside the cell thanks to the combined action of NAMPT (that converts nicotinamide into nicotinamide mononucleotide) and nicotinamide mononucleotide adenylyltrasferase (NMNAT1, that converts nicotinamide mononucleotide to NAD^+) (309, 310).

Sirtuins modulate the activity of transcriptional regulators and enzymes directly involved in the control of metabolic pathways. To give just a few examples, SIRT1 sustains gluconeogenesis, inhibits glycolysis and increases fatty acid catabolism. SIRT2 inhibits adipogenesis and promotes lipolysis. SIRT3 supports TCA cycle and oxidative burst. SIRT4 inhibits glutamine metabolism and promotes fatty acid synthesis. SIRT5 reduces glucose metabolism. SIRT6 inhibits glycolysis and promotes fatty acid oxidation. SIRT7 promotes

oxidative phosphorylation and triglyceride synthesis (311-313). A summary of our current knowledge is presented in **Table 6** which describes, for each sirtuin, the metabolic pathway analyzed, the effect of SIRT1-7 (↑ = increased, ↓ = decreased activity) and the target through which sirtuins mediate their effects.

A growing body of evidence indicates the existence of a tight relationship between metabolism and immune functions. Metabolic reprogramming routinely happens in cells to adapt to energy conditions, and in immune cells sensing pathogens through PRRs or cytokines through cytokine receptors. Indeed, important metabolic changes occur during immune cell activation and the switch between metabolic pathways determines immunological phenotypes. For instance, while resting cells usually rely on oxidative phosphorylation as a source of energy, activated immune cells such as macrophages, DCs and Th17 cells switch towards glycolysis (359-361).

In agreement, classically activated M1 macrophages and alternatively activated M2 macrophages have distinct functions that are amazingly mirrored by metabolic polarization (**Figure 8**). M1 macrophages switch from oxidative phosphorylation to glycolysis and pentose phosphate pathway during activation, favoring ROS production, glucose uptake and conversion of pyruvate into lactate. In M1 macrophages, iNOS is upregulated promoting the catabolism of arginine to citrulline and production of NO. The TCA cycle is broken leading to the accumulation of succinate and citrate. Citrate is exported from the mitochondria to the cytosol where it sustains ROS and NO production and promotes phospholipid synthesis, leading to arachidonic acid and prostaglandin production (359, 362). Succinate activates HIF-1α, which reaches HIF-1α binding site of the *IL1b* promoter and promotes IL-1β mRNA expression (363). All these metabolic changes are necessary to rapidly produce energy to support secretory and bactericidal functions (359, 364).

Table 6. Effect of mammalian sirtuins on metabolic pathways, with intermediate targets.

Sirtuin	Metabolic pathway	Effect	Target	Ref.
SIRT1	Fatty acid oxidation	↑	PGC1α, PPARα	(314-318)
	Lipid synthesis	↓	SREBP-1c	(314, 319, 320)
	Gluconeogenesis	↑	PGC1α	(321, 322)
	Gluconeogenesis(late fasting)	↓	CRTC2, G6Pase and PEPCK	(323)
	Glycolysis	↓	PGAM1, GK	(321, 324)
	Reverse cholesterol transport	↑	LXRs	(325)
	Biosynthesis of bile /cholesterol catabolism	↑	FXR	(326)
SIRT2	Adipogenesis	↓	PPARγ, FOXO1, ACLY	(327, 328)
	Fatty acid oxidation	↑	PGC1α	(329)
	Gluconeogenesis	↑	PEPCK1	(330)
	Insulin sensitivity	↑	Akt, TUG	(331, 332)
		↓	Akt, GSK3β	(333)
SIRT3	Fatty acid oxidation	↑	LCAD	(334, 335)
	Urea cycle	↑	OTC	(335)
	Ketogenesis	↑	HMGCS2	(336)
	Oxidative metabolism	↑	PHD	(337)
	TCA cycle	↑	IDH2	(338, 339)
SIRT4	Fatty acid oxidation	↓	PPARα	(262)
	Lipid synthesis	↑	MCD	(340)
	Glutamine catabolism	↑	GDH	(341)
	Oxidative metabolism	↓	PDH	(342)
SIRT5	TCA cycle	↓	SDH	(343)
	Oxidative metabolism	↓	PDC	(344)
	Oxidative phosphorylation	↑	IDH1	(345)
	Urea cycle	↑	CPS1	(346, 347)
	Glutamine catabolism	↓	GLS	(348)
	Ketogenesis	↓	HMGCS2	(349)
SIRT6	Glycolysis	↓	HIF-1α, H3K9, glycogenic genes	(350, 351)
	Triglyceride synthesis	↓	H3K9, lipogenic genes	(351)
	Gluconeogenesis	↓	PGC-1α	(352)
	Cholesterol uptake	↓/↑	SREBP-2, FOXO3, H3K9, H3K56	(353)
	Cholesterol uptake	↓	PCSK9, SREBP1/SREBP2	(354, 355)
	Fatty acid oxidation	↑	MiR-122	(356)
SIRT7	Oxidative phosphorylation	↑	Complex I, II, IV, V	(357)
	Triglyceride synthesis	↑	NR2C2	(358)

ACLY: ATP citrate lyase; AKT: Protein kinase B; CPS1: Carbamoyl phosphate synthetase; CRTC2: CREB regulated transcription coactivator 2; FOXO: Forkhead box protein O; FXR: Farnesoid X receptor; GLS: Glutaminase; G6Pase: Glucose-6-phosphatase; GDC: Glycine Decarboxylase Complex; GK: Glucokinase; GSK3β: Glycogen synthase kinase 3 beta; HIF-1α: Hypoxia-inducible factor 1-alpha; HMGCS2: 3-hydroxy-3-methylglutaryl CoA synthase 2; IDH1: Isocitrate dehydrogenase 1; IDH2: Isocitrate dehydrogenase 2; LCAD: Long-chain-specific acyl coenzyme A dehydrogenase; LXRs: Liver X receptor (LXR) proteins; MCD: Malonyl CoA decarboxylase; NR2C2: nuclear receptor subfamily 2 group C member 2; OTC: Ornithine transcarbamoylase; PCSK9: Proprotein convertase subtilisin/kexin type 9; PDC: Pyruvate Dehydrogenase Complex; PDH: pyruvate dehydrogenase complex; PEPCK: Phosphoenolpyruvate carboxykinase; PGAM1: Phosphoglycerate mutase-1; PGC1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PHD: Pyruvate dehydrogenase; PPAR: Peroxisome proliferator-activated receptorSDH: Succinate dehydrogenase; SREBP: Sterol-regulatory element binding protein.

M2 macrophages keep an oxidative metabolism sustained by normal levels of fatty acid oxidation that provides energy for a long period as requested for a healing process (364, 365) (**Figure 8**). To support fatty acid oxidation, M2 macrophages take up triglycerides through scavenger receptors (such as CD36) and hydrolyze them with lysosomal acid lipases (366). M2 macrophages also up-regulate arginase-1 (Arg1) to produce urea, polyamines and ornithine that are important metabolites involved in healing. Interestingly, inhibition of oxidative metabolism in M2 macrophages triggers a M1-like phenotype. Conversely, forcing oxidative metabolism in M1 macrophages provokes a shift to a M2-like phenotype.(364, 365, 367)

The metabolic switches operated by the host in response to infections lead to the production of metabolites that directly target pathogens, for example ROS and NO as mentioned above. Moreover, the accumulation of citrate in M1 macrophages results in the synthesis of itaconic acid that negatively impacts on *S. thyphimurium* and *M. turberculosis* metabolism and limits their viability (368). Another example is given by type I IFN signals which inhibit lipid synthesis and increase lipid uptake by host cells. This reduces lipid bioavailability for microorganisms (369).

An additional level of complexity is given by the observation that pathogens can hijack host metabolic pathways. *Trypanasoma* for example produces indolepyruvate that inhibits the metabolic switch towards glycolysis and HIF-1α and IL-1β production, hence favoring immune evasion. Overall, the balance between host and pathogens will impact on the outcome of the infection (*Metabolic reprogramming in innate immunity: electron flow in mitochondria as an arbiter of cytokine production*; by Luke O'Neill from Trinity College, Dublin; talk given at TOLL 2015 – Targeting innate immunity, Marbella, Spain, 3 Oct 2015). Obviously there is more and more interest in identifying metabolic dysfunctions in sepsis as this may represent new opportunities to identify therapeutic targets (52, 53).

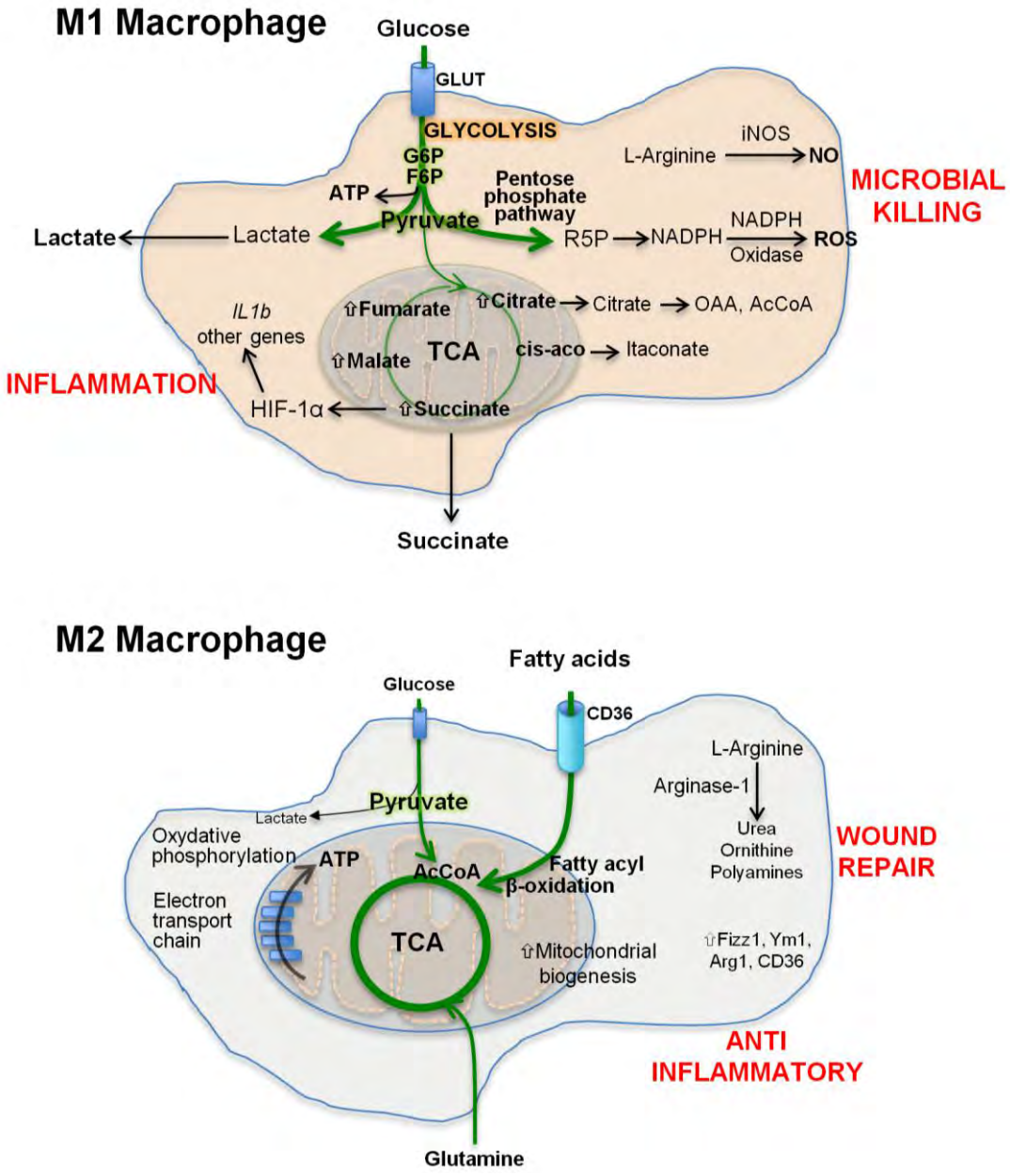

Figure 8. Distinct metabolic profiles of M1 and M2 macrophages. M1 macrophages are characterized by high glucose uptake and active glycolysis and pentose phosphate pathway activity, while M2 macrophages are characterized by high triglyceride uptake and active fatty acid oxidation and oxidative phosphorylation. AcCoA: acetyl-coenzyme A; Arg1: arginase -1; F6P: fructose-6-phosphate; G6P: glucose-6-phosphate; OAA: oxaloacetic acid; R5P: ribose-5-phosphate; TCA: tricarboxylic acid.

2 AIM AND OBJECTIVES

The overall aim of this work was to contribute to decipher the impact of HDACs and inhibitors of HDACs on innate immune responses to microbial infection. The first part of the result section (**section 5.1**) is presented in the form of a review that summarizes the knowledge, available at the start of this work, about HDACi and innate immune responses, with a focus on sepsis. Four specific objectives were then defined according to past results from the lab and current developments in the field:

1. Considering that a) diet determines microbiome composition, b) dysbiosis is associated with several immunopathologies, and c) commensal bacteria produce short chain fatty acids (SCFAs) that may have immunomodulatory properties, the **first objective** was to study the impact of propionate, one of the most abundantly expressed SCFA, on innate immune responses *in vitro* and *in vivo* (**section 3.2**).
2. The **second objective** was to analyze the expression pattern of SIRT1-7 in immune cells (particularly macrophages) and main organs, as well as the impact of MAMPs, DAMPs and cytokines on SIRT1-7 expression levels (**section 3.3**).
3. Considering that a) classical HDACs and SIRT1 and SIRT2 share several targets, among which the master transcription factor NF-κB, and b) HDACi have strong immunosuppressive properties, the **third objective** was to characterize the innate immunomodulatory properties of cambinol, a newly developed SIRT1/SIRT2 inhibitor with anti-cancer activity (**section 3.4 and 3.5**).
4. The **fourth objective** was to investigate *in vitro* and *in vivo* the impact of SIRT2 on immune system development and host defense mechanisms by making use of SIRT2 knockout mice (**section 3.6**).

3 RESULTS

This part is divided into the six following sections:

3.1 Epigenetics in sepsis: targeting histone deacetylases

3.2 Characterization of the impact of propionate, a short chain fatty acid, on antimicrobial host defenses *in vitro* and *in vivo*

3.3 Expression pattern of sirtuins in immune cells and organs

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

3.5 Screening the impact of sirtuin inhibitors on inflammatory and innate immune responses of macrophages and in a mouse model of endotoxic shock

3.6 Sirtuin 2 deficiency increases bacterial phagocytosis by macrophages and protects from chronic staphylococcal infection

3.1 Epigenetics in sepsis: targeting histone deacetylases

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Summary

This review summarizes our knowledge about the biological effects of inhibitors of HDACs (HDACi) on innate immune responses specifically in the context of infection and sepsis. HDACi were identified in the late 70's and rapidly shown to have potent antitumor activity, motivating the development of HDACi as anti-cancer drugs. This field has been very successful with several molecules entering into clinical use in the 2000's (romidepsin/FK228 in 2004, SAHA/vorinostat in 2006, and panobinostat/LBH-589 in 2015). Already in 2002 Leoni et al. (*Proc Natl Acad Sci USA* 99:2995) reported the anti-inflammatory activity of SAHA, suggesting that HDACi could be used for treating inflammatory or autoimmune diseases, but also that they could interfere with innate immune responses. Here, we summarize the results of studies showing how HDACi negatively impact on innate immune responses *in vitro* and *in vivo*, how HDACi might be used to improve lethal sepsis, and how HDACi might sensitize to non-severe infections. Importantly, we highlight the need for a close survey of the immunological and infection status of patients treated with HDACi, especially oncologic immuno-deficient patients.

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Epigenetics in sepsis: targeting histone deacetylases

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ABSTRACT

Severe sepsis and septic shock are lethal complications of infection, characterised by dysregulated inflammatory and immune responses. Our understanding of the pathogenesis of sepsis has improved markedly in recent years, but unfortunately has not been translated into efficient treatment strategies. Epigenetic mechanisms such as covalent modification of histones by acetylation are master regulators of gene expression under physiological and pathological conditions, and strongly impact on inflammatory and host defence responses. Histone acetylation is controlled by histone acetyltransferases and histone deacetylases (HDACs), which affect gene expression also by targeting non-histone transcriptional regulators. Numerous HDAC inhibitors (HDACi) are being tested in clinical trials, primarily for the treatment of cancer. We performed the first comprehensive study of the impact of HDACi on innate immune responses in vitro and in vivo. We showed that HDACi act essentially as negative regulators of the expression of critical immune receptors and antimicrobial pathways in innate immune cells. In agreement, HDACi impaired phagocytosis and killing of bacteria by macrophages, and increased susceptibility to non-severe bacterial and fungal infections. Strikingly, proof-of-principle studies demonstrated that HDACi protect from lethal toxic shock and septic shock. Overall, our observations argue for a close monitoring of the immunological and infection status of patients treated with HDACi, especially immunocompromised cancer patients. They also support the concept of pharmacological inhibitors of HDACs as promising drugs to treat inflammatory diseases, including sepsis.

1. Innate immunity

Host integrity is preserved by the innate immune system, which provides the first line of defence against microbial infections. Sensing of pathogens by innate immune cells is mediated by pattern recognition receptors (PRRs) or molecules specialised in the recognition of conserved structures of micro-organisms. Families of PRRs include the Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs) and AIM2-like receptors (ALRs). Recognition of invasive pathogens by immune cells results from their capacity to detect microbial-associated molecular patterns (MAMPs). MAMPs include components of the microbial cell wall [lipopolysaccharide (LPS), lipoproteins, peptidoglycan, glucans, mannans, etc.] or flagellum and nucleic acids [1]. The innate immune system is also triggered by the recognition of damage (or danger)-associated molecular patterns (DAMPs), also known as alarmins, which are endogenous signals commonly released by injured cells, such as RNA, DNA, histones, uric acid crystals, ATP, cytochrome c, S100 molecules and HMGB1. DAMPs are sensed through PRRs, primarily through the NLRP3 inflammasome that controls the secretion of interleukin-1beta (IL-1β) and IL-18 [2].

Recognition of MAMPs or DAMPs by PRRs engages multiple adaptors and transducers that trigger rapid activation of the nuclear factor kappaB (NF-κB), mitogen-activated protein kinase (MAPK) and interferon (IFN) response factor signalling pathways. These pathways stimulate the production of soluble mediators as well as adhesion, major histocompatibility complex (MHC) and co-stimulatory molecules. Among the soluble mediators, cytokines, chemokines and IFNs play a crucial role in the initiation and regulation of the inflammatory response and in the co-ordination of cellular and humoral responses aimed at eradicating or containing invasive pathogens [1,2].

2. Sepsis

Innate immune responses have to be tightly regulated, and failure to mount an appropriate defence response to microbial invasion may have dramatic consequences for the host. Severe sepsis and septic shock are life-threatening complications of infection.

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Despite recent advances in antimicrobial therapy and supportive care, the prognosis of patients with severe infections remains grim, with mortality rates ranging from 20% to 80%. Severe sepsis and septic shock may be caused by highly virulent pathogens or by genetic predisposition impairing the mounting of appropriate inflammatory responses. Either by default or by excess, unbalanced inflammatory and immune responses can result in uncontrolled microbial growth or devastating inflammatory responses with tissue injury, vascular collapse and organ dysfunction [3–5]. Indeed, experimental studies have shown that blocking or deficiency in PRRs and cytokines or their receptors increase susceptibility to infections, and human association studies have linked polymorphisms in innate immune genes with an augmented propensity to develop infections [6,7].

3. The inflammatory response in sepsis

Our understanding of the pathogenesis of sepsis has progressed considerably over previous decades. The earlier view of sepsis resulting merely from an exuberant inflammatory response has been recently reconsidered. The postulate of an initial overwhelming inflammatory response [the systemic inflammatory response syndrome (SIRS)] followed by a state of immunosuppression [the compensatory anti-inflammatory response syndrome (CARS)] does not reflect the complexity of events occurring in severely ill patients, and there is no clear temporal separation between the pro- and anti-inflammatory responses occurring in sepsis [3–5]. Nevertheless, an excessive inflammatory response mediates tissue damage and organ failure, whereas sepsis-associated immune suppression accounts for the development of secondary infections.

The earlier reductionist views of sepsis pathophysiology had the advantage of driving clinical development. Indeed, the association of high levels of pro-inflammatory mediators with poor outcome in septic patients has been the basis of the concept that blocking pro-inflammatory mediators released by the host or blocking pro-inflammatory microbial components may improve survival. Despite promising results in pre-clinical investigations, anti-cytokine therapies had a modest impact on the survival of septic patients. Thus, blocking one single mediator may not be sufficient to interfere with the inflammatory cascade and its dramatic consequences, as could be expected from the complex dynamics of sepsis. Other factors may account for the limited efficacy of anti-cytokine therapies, including the heterogeneity of patients for genetic background, underlying diseases, sepsis severity, infectious agent and site of infection. Recently, recombinant human activated protein C (Xigris®; Eli Lilly) has been withdrawn from the sepsis market following negative results from the PROWESS-SHOCK study. Moreover, study of the efficacy of the TLR4 antagonist Eritoran (Eisai) failed to meet the primary endpoint in the ACCESS phase III trial. It remains more than ever imperative to better characterise sepsis pathogenesis in order to conceive original therapeutic strategies.

4. Epigenetic control of gene expression

The term epigenetics was introduced to characterise heritable mechanisms affecting gene expression without affecting the DNA sequence itself. This definition has been extended in past years to those changes not entirely heritable but functionally linked to the genome [8]. Epigenetic information includes DNA methylation, non-coding RNAs and post-translational modification of histone proteins. In the following sections, we will focus on histone modifications by acetylation as an important mechanism affecting innate immune responses, as well as on recent advances in inhibitors of histone deacetylases (HDACs).

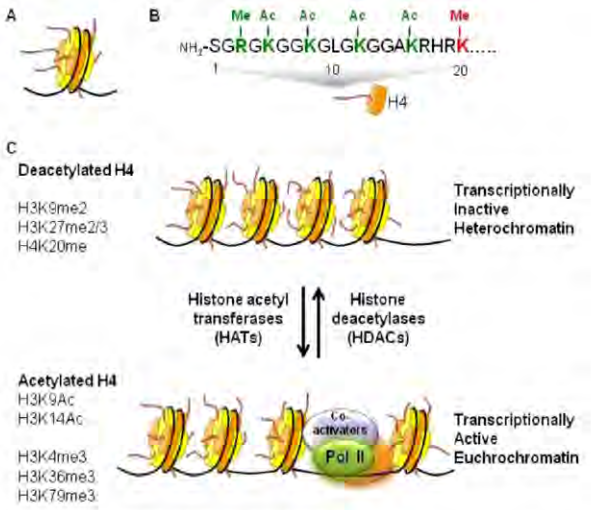

Fig. 1. Histone modifications by acetylation and methylation. (A) Octamers of histones compose the core of the nucleosome onto which DNA is wrapped. (B) Protruding histone tails are covalently modified by methylation (Me) and acetylation (Ac) at arginine (R) and lysine (K) residues. The figure shows the N-terminal sequence of histone 4 (H4) with modifications associated with active (green) and repressed (red) transcription. (C) Main histone modifications associated with transcriptionally inactive heterochromatin and transcriptionally active euchromatin. The open structure of euchromatin allows the recruitments of co-activators and RNA polymerase II (Pol II). me2, dimethylation; me3, trimethylation.

4.1. Histone structure

The basic unit of chromatin is the nucleosome, an octamer composed of two copies of the core histones H2A, H2B, H3 and H4, around which 147 DNA base pairs are wrapped (Fig. 1 A). The amino-terminal tails of core histones protrude from the nucleosome and are subjected to covalent modifications by methylation, acetylation, phosphorylation, ubiquitination, SUMOylation and ADP-ribosylation (Fig. 1B). These modifications affect gene expression in two main ways: (i) they modify the structure of chromatin thus promoting or preventing interaction between DNA and nucleosomes and among nucleosomes; and (ii) they create specific landscapes influencing the recruitment of non-histone proteins, acting as readers of histone marks, which engage additional factors promoting or repressing gene transcription [8]. During cell replication, nucleosomes are disassembled and histones are recycled on-site, conferring histone modification a certain level of heritability [9]. Methylation and acetylation are by far the most studied histone modifications and will be the focus of the following sections.

4.2. Histone methylation

Methyltransferases transfer methyl (me) groups from S-adenosyl methionine to lysine (K) or arginine (R) of histone tails (Fig. 1B). Dimethylation (me2) and trimethylation (me3) may occur simultaneously at different sites. H3K9me2, H3K27me2, H3K27me3 and H4K20me are enriched in transcriptionally inactive compacted heterochromatin, whereas H3K4me3, H3K36me3 and H3K79me3 are enriched in transcriptionally active open euchromatin (Fig. 1C). Methyl groups are removed by demethylases [10]. Histone methylation impacts on various biological processes, and altered histone methylation has been related to several diseases, primarily cancer [11].

Table 1
Impact of histone deacetylase inhibitors (HDACi) on host defences and sepsis.

HDACi[a]	Model	Effect of HDACi	Reference(s)
ITF2357, NaB, LAQ824, SAHA, TSA, VPA	PBMCs, monocytes, macrophages and DCs stimulated with microbial products and pro-inflammatory cytokines	Decreased expression of cytokines (TNF, IL-1β, IL-6, IL-12, IFNγ), chemokines, nitric oxide and co-stimulatory molecules	[14–17,30–32,35]
LAQ824	Transcriptome of LPS-stimulated human macrophages	Decreased production of molecules involved in Th1 effector cell activation and migration	[34]
TSA	Transcriptome of resting and LPS- and Pam_3CSK_4-stimulated BMDMs	Decreased expression of PRRs, cytokines, growth factors, complement, and adhesion and co-stimulatory molecules	[32]
TSA, VPA	BMDMs, resting and exposed to *Escherichia coli* or *Staphylococcus aureus*	Decreased expression of phagocytic receptors, and inhibition of oxidative and nitrosative burst. Decreased phagocytosis and killing of bacteria	[39]
SAHA, TSA, tubastatin	In vivo and in vitro models (mouse), human PBMCs	Increased generation and function of $Foxp3^+$ T-regulatory cells	[18,19,33]
SAHA, TSA	LPS+IFNγ-stimulated moDCs, LPS-treated mice, differentiation of IL-17-producing T-cells in vitro	Decreased production of Th17-polarising cytokines and generation of Th17 cells	[19,35]
TSA, SAHA, VPA	Monocytic cell lines, BMDMs, TSA-treated mice	Decreased MIF expression	[36,37]
ITF2357	Endotoxaemia	Decreased serum cytokines	[14]
VPA	Non-severe pneumonia (*Klebsiella pneumoniae*)	Increased bacterial burden. Increased mortality	[32]
VPA	Non-severe candidiasis	Increased mortality	[32]
SAHA, VPA	Lethal endotoxaemia and Pam_3CSK_4-induced fulminant toxic shock	Decreased serum cytokines. Decreased lung inflammation. Increased survival	[32,40]
NaB, TSA, VPA	Lethal CLP	Increased survival	[32,41]

NaB, sodium butyrate; SAHA, suberoylanilide hydroxamic acid; TSA, trichostatin A; VPA, valproic acid; PBMCs, peripheral blood mononuclear cells; DCs, dendritic cells; TNF, tumour necrosis factor; IL, interleukin; IFN, interferon; LPS, lipopolysaccharide; BMDMs, bone-marrow-derived macrophages; PRRs, pattern recognition receptors; moDCs, myeloid-derived DCs (human); MIF, macrophage migration inhibitory factor; CLP, caecal ligation and puncture.
[a] Other names of HDACi: ITF2357, givinostat; LAQ824, dacinostat; MS-275, entinostat; SAHA, vorinostat.

4.3. Histone acetylation

The acetylation status of chromatin is controlled by the antagonistic activities of histone acetyltransferases (HATs) and HDACs. HATs catalyse the transfer of acetyl groups from acetyl-coenzyme A to the ε-amino group of lysines in the amino-terminal region of histones. Histone acetylation favours active transcription through two mechanisms (Fig. 1C). First, acetylation neutralises positive charges of lysines, thus releasing the chromatin structure. Second, newly acetylated lysines function as molecular tags for transcriptional activators. In contrast to HATs, HDACs are linked to compacted chromatin and transcriptional repression. In the following sections, we will describe HDACs and the impact of HDAC inhibitors (HDACi) on antimicrobial defence mechanisms.

5. Histone deacetylases

HDACs were named according to their first identified substrates, i.e. histones. As the list of non-histone targets of HDACs is constantly increasing [12], some have proposed to rename HDACs as lysine deacetylases (KDACs). The 18 mammalian HDACs are classified in classes I–IV based on their homology with yeast HDACs, subcellular localisation and enzymatic activity. Classes I, II and IV comprise the 'classical' Zn^{2+}-dependent HDAC1–11, whilst class III encompasses the NAD^+-dependent sirtuins (SIRT1–7). Here we will discuss 'classical' HDACs. HDACs lack an intrinsic DNA-binding activity and are recruited to chromatin through multiprotein complexes. The function of HDACs is being deciphered mainly using HDACi and, more recently, using *Hdac* knock-out mice. Deficiency in HDACs results in early embryonic lethality (HDAC1, 3), perinatal lethality (HDAC2, 4, 8), and viability with discrete developmental abnormalities (HDAC5, 6, 9). These and other observations suggest unique and essential roles exerted by HDACs. Moreover, it was shown that HDACs are involved in the development of age-related diseases including cancer, diabetes, and cardiovascular, neurodegenerative and autoimmune diseases [13].

6. Histone deacetylase inhibitors and their impact on inflammatory diseases

In the late 1970s, butyrate, a short fatty acid, was reported to have anti-cancer activities and to suppress histone deacetylation. Ten years later, trichostatin A (TSA) was identified as the first specific HDACi. These pioneer studies stimulated the development of new HDACi as anti-cancer drugs. Research in the field has been very successful. Suberoylanilide hydroxamic acid (SAHA) (vorinostat, Zolinza™) and romidepsin (FK228, Istodax®, also known as depsipeptide FR901228) have recently been approved by the US Food and Drug Administration (FDA) for the treatment of advanced and refractory cutaneous T-cell lymphoma. Many other HDACi are currently being tested in clinical trials alone or combined with other anti-cancer agents.

In 2002, Leoni et al. reported that SAHA, at concentrations 20-fold lower than those used to inhibit tumour cell proliferation, significantly reduces the production of tumour necrosis factor (TNF), IL-1β, IL-12 and IFNγ by human peripheral blood mononuclear cells (PBMCs) and reduces circulating levels of cytokines in LPS-treated mice [14] (Table 1). Quickly after, HDACi have been reported to downregulate pro-inflammatory parameters and to improve outcome in experimental models of systemic lupus erythematosus, arthritis, multiple sclerosis, graft-versus-host disease, asthma and colitis (Table 2). In line with these findings, dendritic cells (DCs) and regulatory T-cells (Treg), which play a key role in establishing self-tolerance and preventing autoimmune diseases, are key targets of HDACi (Table 1) [30–33]. Several clinical trials are underway to test the efficacy of HDACi as alternative therapy for autoimmune diseases [13].

7. Histone deacetylase inhibitors impair host defences: the bad and the good sides

Following the initial description of the anti-inflammatory activity of HDACi, several studies reported that HDACi impact on innate

Table 2
Pre-clinical models of inflammatory and immune diseases treated with histone deacetylase inhibitors (HDACi).

Disease	HDACi[a]	Reference(s)
Systemic lupus erythematosus	SAHA, TSA	[20,21]
Rheumatoid arthritis	Depsipeptide FR901228, NaB, TSA	[22,23]
Multiple sclerosis	NaB, TSA	[24,25]
Graft-versus-host disease	ITF2357, SAHA	[15,26]
Asthma	TSA	[27]
Colitis	ITF2357, TSA, SAHA, VPA	[18,28,29]
Septic shock	NaB, SAHA, TSA, VPA	[32,40,41]

SAHA, suberoylanilide hydroxamic acid; TSA, trichostatin A; NaB, sodium butyrate; VPA, valproic acid.
[a] Other names of HDACi: depsipeptide, romidepsin; ITF2357, givinostat; LAQ824, dacinostat; MS-275, entinostat; SAHA, vorinostat.

immune responses at many levels. HDACi inhibit cytokine and nitric oxide (NO) release and co-stimulatory molecule expression by PBMCs, monocytes, macrophages and DCs stimulated with microbial products and pro-inflammatory cytokines (Table 1). Moreover, HDACi block the production of Th1- and Th17-polarising cytokines, which drive the Th1 and Th17 protective responses against intracellular and extracellular pathogens [34,35].

In a highly comprehensive study, we described the effects of TSA, valproate and SAHA on innate immune responses in vitro and in vivo. Genome-wide expression studies revealed that HDACi act essentially as negative regulators of critical immune receptors and antimicrobial products by mouse and human immune cells (macrophages, DCs and whole blood) at rest or exposed to a broad range of bacterial and fungal products [32]. HDACi also inhibit the expression of macrophage migration inhibitory factor (MIF) [36,37], a key regulator of innate and inflammatory responses [38]. HDACi markedly impair the uptake and killing of Gram-positive and Gram-negative bacteria by macrophages, these effects being associated with reduced expression of phagocytic receptors and inhibition of reactive oxygen and nitrogen species production [39]. Going well along with these results, and as expected from any agent interfering with innate immune responses, valproate increases microbial burden and mortality of mice subjected to non-severe *Klebsiella pneumoniae* pneumonia and candidiasis [32].

An important question arising from our observations is whether HDACi impair immunity in patients. Although clinical trials have been performed, no definitive answer can be drawn for the following reasons. First, clinical trials are typically performed in cancer patients with confounding underlying components and reduced life expectancy. Second, few patients have been treated for long time periods with HDACi. Third, whether patients were treated prophylactically with antimicrobial agents and whether episodes of infection occurred may have not been always accurately reported. Yet it is worthwhile to mention that patients treated with HDACi commonly experience thrombocytopenia, leukopenia and neutropenia, which are risk factors for developing infections. Moreover, episodes of severe infection have been reported in patients treated with HDACi, even without neutropenia. We thus propose a close monitoring of the immunological and infection status of patients treated with HDACi, especially immunocompromised cancer patients.

Anti-inflammatory agents may confer protection to septic shock by blocking the activity of pro-inflammatory mediators. We thus tested whether HDACi could improve the outcome of septic shock. These studies revealed that valproate reduces the cytokine storm and confers protection against lethal toxic shock induced by Pam_3CSK_4 lipopeptide (mimicking Gram-positive bacteria lipopeptides) and caecal ligation and puncture (CLP) [32]. Other groups have reported a protective effect of sodium butyrate, TSA and SAHA in models of lethal endotoxaemia and CLP [40,41]. These data support the contention that HDACi could represent adjunctive therapy for inflammatory diseases, including sepsis (Table 1).

8. Perspectives

HDACi represent a class of promising drugs not only in the field of cancer but also in the field of immune diseases. HDACi may offer advantages compared with cytokine blocking agents, including transient inhibition of cytokine production and inducing 'global' gene remodelling. HDACi may also offer a certain level of selectivity, as different HDACi influence specific signalling cascades. This advantage will be improved with the synthesis of HDAC-isotype-specific inhibitors. Interestingly, the doses of HDACi mediating anti-inflammatory effects are significantly lower than those needed for cancer therapy, and several HDACi can be administrated per os. Yet we are missing data regarding the long-term toxicity of HDACi. Similarly, whether HDACi affect natural host defences of patients should be addressed carefully. Nevertheless, converging in vitro and in vivo data support the concept of HDACi as attractive therapeutics for treating inflammatory and immune-related diseases, among which sepsis may have its place.

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3.2 Characterization of the impact of propionate, a short chain fatty acid, on antimicrobial host defenses *in vitro* and *in vivo*

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Characterization of the impact of propionate, a short chain fatty acid, on antimicrobial host defenses *in vitro* and *in vivo*

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Running title: Impact of propionate on antimicrobial host defenses

Abstract

Short chain fatty acids (SCFAs) produced by intestinal microbes mediate antioxidative, anticarcinogenic and anti-inflammatory effects through direct inhibition of histone deacetylases or signaling via metabolite sensing G-protein coupled receptors. Propionate is one of the most abundantly produced SCFAs. Yet, whether propionate impacts on host defense responses remains largely unknown. Here we show that propionate dampened the response of innate immune cells to microbial stimulation, inhibiting cytokine and NO production by mouse or human monocytes/macrophages, splenocytes, whole blood and, less efficiently, by dendritic cells (DCs). Propionate given per os or intraperitoneally neither sensitized nor conferred protection in models of endotoxemia, infection and lethal sepsis due to gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*), gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*) and *Candida albicans*, even when mice were beforehand depleted of gut microbiota. Yet, propionate reduced anti-Klebsiella and anti-Streptococcus IgG titers in mice surviving pneumoniae, indicating that propionate interfered to some extend with anti-microbial host response. Altogether, these data suggest that, despite evident anti-inflammatory properties, propionate treatment has no significant impact on host susceptibility to primary infection and support the development of therapies using propionate or directed at the diet or the microbiota for treating non-infectious inflammation-related disorders.

Keywords (3-10):

Propionate, Short chain fatty acid, Histone deacetylase inhibitor, Epigenetics, Innate immunity, Cytokine, Macrophage, Endotoxemia, Sepsis, Microbiota.

Introduction

Host defenses against infection rely on innate immune cells that sense microbial derived products through pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), c-type lectins, NOD-like receptors, RIG-I-like receptors and intra-cytosolic DNA sensors. The interaction of microbial ligands with PRRs activates immune cells to produce immunomodulatory molecules like cytokines and co-stimulatory molecules [1-3]. Pro-inflammatory cytokines play an essential role in coordinating the development of the innate and adaptive immune responses aimed at the eradication or containment of invading pathogens. Yet, inflammation has to be timely and tightly regulated since it may become life-threatening both by default of by excess [3-6].

Short chain fatty acids (SCFAs) are end products of the fermentation of resistant starches and dietary fiber by intestinal bacteria, with the most abundant metabolites produced being acetate, propionate and butyrate [7]. SCFAs reach elevated concentrations in the gut lumen (50-100 mM) and are absorbed into the portal circulation, acting as the primary source of SCFAs in the bloodstream (0.1-1 mM) [8-11]. SCFAs, primarily butyrate, not only serve as a source of energy, but also stimulate neural and hormonal signals regulating energy homeostasis [12]. Beside their trophic effects, SCFAs possess antioxidative, anticarcinogenic and anti-inflammatory properties and play an essential role in maintaining gastrointestinal and immune homeostasis [7, 10, 11]. Butyrate has been reported to affect activation, proliferation, apoptosis, migration of, and cytokine production by immune cells [13].

Both extracellular and intracellular SCFAs exert immunosuppressive effects. Extracellular SCFAs act through metabolite sensing G-protein coupled receptors (GPCRs) such as GPR41, GPR43 and GPR109A [7, 14]. Although the expression patterns and the signaling effects of GPCRs are poorly defined, GPCRs were recently shown to mediate the anti-inflammatory effects of SCFAs and protect form colitis, rheumatoid arthritis and airway hyper-responsiveness [15-17]. GPCRs propagate anti-inflammatory effects at least through a β2-arrestin-dependent stabilization of IκBα and inhibition of NF-κB-dependent transcription, and by promoting the generation of T regulatory (Treg) cells [7, 10].

Intracellular SCFAs inhibit zinc-dependent histone deacetylases (HDACs) [18]. Mammalian HDACs are classified into four subfamilies based on their sequence homology to yeast HDACs and domain organization: class I (HDAC1-3, 8), class IIa/IIb (HDAC4, 5, 7, 9/HDAC6, 10) and class IV (HDAC11) zinc-dependent HDACs and class III NAD-dependent sirtuins (SIRT1-7) [19]. HDACs are major epigenetic erasers catalyzing the deacetylation of histones, which leads to chromatin compaction and transcriptional repression [20]. HDACs also target numerous signaling molecules and transcription factors. Inhibitors of HDAC (HDIs) impair directly or indirectly NF-κB activity and mediate powerful anticancer, anti-neurodegenerative and anti-inflammatory activities. Considering the broad therapeutic potential of HDIs, numerous compounds are tested in clinical trials [20-22]. Valproate, a SCFA used since decades as a mood stabilizer and anti-epileptic, suberanilohydroxamic acid (SAHA, vorinostat) and romidepsin (FK228/FR901228) have been approved for the treatment of patients with cutaneous T-cell lymphoma and panobinostat (LBH-589) for the treatment of patients with multiple myeloma who experienced two prior therapies [22]. In agreement with their anti-inflammatory properties, HDIs interfere with the development of innate immune responses, protect against lethal sepsis, and increase susceptibility to infection [23-28].

The impact of propionate on innate immune responses is poorly characterized. To fill in that gap, we analyzed the response of macrophages, dendritic cells (DCs), splenocytes and whole blood to bacterial and fungal compounds and used a panel of preclinical mouse models of endotoxemia, gram-positive and gram-negative bacterial and fungal infection of diverse severity. Our results show that propionate inhibits innate immune responses *in vitro* but neither increases susceptibility to infection nor protects from lethal sepsis *in vivo*.

Results

Impact of propionate on the response of immune cells to microbial stimulation.

To address the effects of propionate on the response of immune cells to microbial stimulation, bone marrow-derived macrophages (BMDMs) were exposed for 8 h to LPS (a TLR4 agonist), Pam_3CSK_4 (a lipopeptide triggering cells through TLR1/TLR2) and *E. coli* and *S. aureus*, used as representative gram-negative and gram-positive bacteria. The levels of TNF, IL-6 and IL-12p40 produced by BMDMs were quantified by ELISA (**Figure 1A**). Propionate (0.5-4 mM) dose-dependently inhibited TNF production induced by Pam_3CSK_4 and *S. aureus*, and IL-6 and IL-12p40 production induced by LPS, Pam_3CSK_4, *E. coli* and *S. aureus*. Similar to other HDIs [27, 29, 30], propionate did not inhibit TNF production induced by LPS and *E. coli*, and in fact amplified TNF response to *E. coli*. In agreement with these results, propionate powerfully inhibited LPS and Pam_3CSK_4-induced *Il6* and *Il12b* mRNA, to a lesser extent Pam_3CSK_4-induced *Tnf* mRNA, but not LPS-induced *Tnf* mRNA expression (**Figure 1B**).

The anti-inflammatory activity of propionate was compared to that of butyrate and valproate by defining the IC_{50} of each of the SCFAs for LPS-induced IL-6 and IL-12p40 production. Similar IC_{50s} were obtained for IL-6 and IL-12p40: 0.01-0.05 mM for butyrate, 0.2-0.4 mM for valproate and 0.2-0.3 mM for propionate. Thus, propionate is as potent as valproate at inhibiting IL-6 and IL-12p40 production by BMDMs, but 8-20 folds less efficient than butyrate. The concentrations of G-CSF, IL-10, IL-18, IP10/CXCL10, MCP1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4 and RANTES/CCL5 released by BMDMs exposed to LPS, *E. coli*, Pam_3CSK_4 and *S. aureus* were measured by Luminex (**Figure 1C**). Whereas LPS and *E. coli* induced the secretion of all mediators, Pam_3CSK_4 and *S. aureus* did not induce appreciable levels of G-CSF, IL-10 and IL-18. Propionate inhibited G-CSF, IL-10 and IL-18 induced by LPS and *E. coli*, and CCL5 and CXCL10 induced by LPS. Propionate also inhibited CCL3, CCL4, CCL5 and CXCL10 induced by Pam_3CSK_4 and CCL4 and CXCL10 induced by *S. aureus*. Overall, propionate impaired more powerfully cytokine/chemokine secretion induced by Pam_3CSK_4 than LPS, and more efficiently secretion induced by pure microbial ligands than whole bacteria triggering similar PRRs (*i.e.* LPS versus *E. coli*, and Pam_3CSK_4 versus *S. aureus*). Propionate also inhibited the production of nitric oxide (NO) induced by *E. coli* or IFNγ/LPS in BMDMs (50% inhibition using 0.6 mM and 4 mM propionate, respectively (**Figure 1D**)).

Bone marrow-derived dendritic cells (BMDCs) were less sensitive than BMDMs to the anti-inflammatory effects of propionate. Indeed, when looking at TNF, IL-6 and IL-12p40 production induced by LPS, Pam_3CSK_4, *E. coli* and *S. aureus*, propionate significantly inhibited Pam_3CSK_4-induced TNF and LPS, Pam_3CSK_4 and *S. aureus*-induced IL-12p40 only (**Figure 2A**). Of note, propionate slightly increased *E. coli*-

induced IL-6 and IL-12p40 production by BMDCs. The viability of BMDMs and BMDCs incubated for 18 h with up to 8 mM propionate was greater than 98%, suggesting that propionate's effects were not related to cytotoxicity. Along with a good tolerability of immune cells to propionate, propionate barely effected the proliferation of splenocytes exposed to *E. coli* whereas it efficiently inhibited IFNγ production (**Figure 2B**).

We then tested the impact of propionate on human cells (**Figure 3**). Propionate dose-dependently inhibited TNF and IL-6 production by whole blood exposed to LPS, albeit less efficiently than butyrate (TNF: 61±6% *vs* 96±4% and IL-6: 41±7% *vs* 70±10% inhibition using propionate *vs* butyrate at 2 mM, n = 3 donors; $P < 0.05$; **Figure 3A**). A Luminex analysis extended to IL-1β, IL-10, IL-12p40, CCL2 and CXCL10 the spectrum of cytokines and chemokines whose expression was significantly inhibited by at least 50% in the majority (*i.e.* 2 or 3 out of 3) of the donors (**Figure 3B**). In parallel experiments, butyrate inhibited more powerfully than propionate the secretion of IL-10 (in 3/3 vs 2/3 donors), CCL2 (3/3 vs 2/3) and CCL4 (2/3 vs 1/3). Butyrate also impaired the release of IL-1RA (3/3 donors) and CXCL8 (1/3). Flow cytometry analyses of intracellular cytokine expression in human monocytes exposed to LPS and Pam_3CSK_4 revealed that propionate reduced the percentage (11-24% reduction) and more strikingly the mean fluorescent intensity (1.7-3.4 fold reduction) of TNF and IL-6 positive cells (**Figure 3C and D**).

Overall, propionate inhibited in a cell-specific manner the response of mouse and human immune cells to microbial stimulation *in vitro*; opening the possibility that propionate might impact on innate immune responses *in vivo*.

Propionate does not confer protection to lethal endotoxemia and severe lethal sepsis.

In agreement with the concept that overwhelming inflammatory responses are deleterious for the host, inhibition of the release of pro-inflammatory mediators confers protection in preclinical models of sepsis [3-5]. Moreover, HDIs were shown to protect from toxic shock [31]. Thus, we tested propionate in a mouse model of acute endotoxemia. Propionate at 200 mM was administrated through the drinking water, a treatment commonly used to study the impact of SCFAs *in vivo* (370-375). One month of propionate treatment had no impact on animal weight (**Figure 4A**). In mice challenged with LPS, severity scores and survival rates were similar whether or not animals were treated with propionate ($P > 0.5$ and $P = 0.3$; **Figure 4B and C**).

The class of innate immune responses challenged by propionate was extended using models of severe lethal sepsis induced by gram-negative (*Klebsiella pneumoniae*), gram-positive (*S. aureus*) and fungal (*Candida albicans*) pathogens administrated either intranasally (i.n., *K. pneumoniae*) or intravenously (i.v., *S. aureus* and *C. albicans*). In mice challenged i.n. with 200 CFU *K. pneumoniae*, bacterial loads in lungs ($P = 0.4$) and mortality (70% vs 90% in control vs propionate groups; $P = 0.8$) were not significantly affected by propionate treatment (**Figure 5A and B**). Using a 10-fold lower inoculum (20 CFU) of *K. pneumoniae*, mortality (50% vs 60% in control vs propionate group; $P = 0.7$; **Figure 5C**) was similarly reduced in control and propionate-treated mice. In the severe model of systemic infection with *S. aureus*, severity scores, weight lost, bacterial counts in blood ($P = 0.9$) and mortality (100% vs 93% in control vs propionate groups; $P = 0.6$) were comparable with or without propionate treatment (**Figure 5D and E**). Finally, in the acute model of candidiasis, all mice died within 4 days, irrespective of the treatment applied ($P = 0.1$; **Figure 5F**).

Propionate does not sensitize to infection with *Escherichia coli*, *Streptococcus pneumoniae* and *Candida albicans*.

Compromising innate immune responses may increase susceptibility to infection. To analyze the impact of propionate on infection, propionate was given either *per os* or intraperitoneally (p.o.: 200 mM in water; i.p.: 1 g/kg i.p. every other day) to mice subsequently challenged with *E. coli* titrated to cause a mild infection. Bacterial counts (P = 0.9; **Figure 6A**) and survival rates (77% vs 70 % and 60% vs 70% in control vs propionate groups upon p.o. and i.p. treatments; P = 0.7 and P = 0.6; **Figure 6B and C**) were similar with or without oral or intraperitoneal propionate administration. Confirming that propionate does not sensitize mice to infection, 90% (9/10) of control mice and 100% (9/9) of propionate-treated mice infected i.n. with 10^4 CFU *Streptococcus pneumoniae* (*S. pneumoniae*) survived infection.

The inoculum of *C. albicans* was adjusted to produce a mild form of candidiasis during which mortality occurs 5 to 10 days after infection. Weight lost, monitored during the first 5 days, and survival (14.3% and 12.5%) were comparable in untreated and propionate-treated mice (P > 0.1 and P = 0.8; **Figure 6D and E**). Taking all together, these data suggested that propionate did not increase susceptibility to *E. coli* peritonitis and *S. pneumoniae* pneumonia or accelerate candidiasis.

In order to test whether propionate treatment had any impact *in vivo*, we measured anti-*K. pneumoniae* and anti-*S. pneumoniae* IgGs in the blood of mice surviving infection with 20 CFU *K. pneumoniae* (4 controls and 5 propionate-treated mice) and 10^4 CFU *S. pneumoniae* (9 controls and 10 propionate-treated mice). Anti-bacteria IgG titers were reduced in mice treated with propionate (**Figure 7A and B**). Results reached statistical significance for anti-*S. pneumoniae* IgGs (P = 0.01), but not for anti-*K. pneumoniae* IgGs (P = 0.1) because of the low number of experimental points. Therefore, although propionate did not interfere with morbidity and mortality in models of infection, it impacted to some extent on anti-microbial host responses.

Propionate does not protect mice depleted of gut microbiota from candidiasis.

Although propionate was shown to impact on immune parameters of mice with a normal microbiota [16, 17, 30, 32, 35], propionate produced by the gut microbiota may attenuate the impact of propionate supplementation in the models of infection described above. To address that issue, mice were treated with a combination of ciprofloxacin and metronidazole (CM) in order to deplete the gut flora and decrease the levels of endogenous SCFAs [32, 36]. CM-treated mice lost 17 % weight during the first week of treatment and recovered their initial weight after 3 weeks. CM-treated mice were more sensitive to candidiasis than untreated mice run in parallel (median survival time: 9.5 days for CM *vs* 11.5 days for controls; n = 10 mice/group; P = 0.05). Co-treatment with CM plus propionate slightly increased weight loss and impaired weight rebound of uninfected mice (**Figure 8A**). CM-treated mice died in between days 6 and 15 after *Candida* challenge, and propionate supplementation did not protect CM-treated mice from candidiasis (P = 0.4; **Figure 8B**).

Discussion

The gut microbiota and its metabolites deeply impact on human health. Among bacterial metabolites, SCFAs have attracted much attention since the demonstration of their beneficial impact onto the development of inflammation-related pathologies, and because their production can be influenced by the diet [7, 10, 37, 38]. Here we show that propionate has powerful, yet selective, anti-inflammatory activity *in vitro*, but neither sensitizes to infection nor protects from lethal sepsis. This observation is particularly relevant in light of the development of diet or microbiota targeting strategies as promising approaches for several diseases.

Propionate impaired cytokine production by innate immune cells, albeit differentially according to the cell type, the microbial trigger and the cytokine analyzed. Similar disparities have been observed with other SCFAs [27, 30, 39, 40]. BMDCs were more resistant to propionate than BMDMs, human monocytes and whole blood. In both human monocyte-derived DCs (moDCs) and BMDCs exposed to LPS, propionate affected modestly IL-6 but efficiently IL-12p40 production (**Figure 2** and [40]). Additionally, propionate did not inhibit MHC-II and CD86 expression but impaired CD83 expression by moDCs [40]. Disparate cell responses to propionate may reflect, at least in part, differential expression of GPCRs. Although GPCR expression has been occasionally reported in immune cells (monocytes/macrophages, DCs, mast cells, neutrophils, Treg cells), in depth analyses of the pattern and the expression levels of cell-surface GPCRs is still missing. How redundant behave GPCRs *in vitro* and *in vivo* is another unresolved important issue, as well as their specificity for one or another SCFAs. For example, mice deficient in either GPR43 or GPR109A were susceptible to gut inflammation and developed exacerbated colitis, and mice deficient in either GPR41 or GPR43 were similarly susceptible to allergic airway inflammation [16, 17, 35, 41].

Besides proceeding through GPCRs, SCFAs act as inhibitors of class I and II HDACs (HDIs). HDIs impair innate and adaptive immune responses at multiple levels, among others TLR and IFN signaling and cytokine production, bacterial phagocytosis and killing, leukocyte adhesion and migration, antigen presentation by DCs, cell proliferation and apoptosis, and Treg development and function [20, 31, 42]. In T cells, acetate, propionate and butyrate suppressed HDAC activity independent of GPR41 and GPR43 [33]. Whether SCFAs mediate HDAC inhibition also through GPCRs is questionable, but it is worth mention that GPCR signaling modulates kinase, redox and acetylation pathways that impact on the cellular distribution and the activity of histone acetyl transferases and HDACs [43].

Butyrate is a more potent HDI than propionate, and acetate the least potent. This ranking parallels the effectiveness of the anti-inflammatory activity of SCFAs. Butyrate and propionate like trichostatin A (TSA), SAHA and other HDIs unrelated to SCFAs, but unlike acetate, potentiated the generation of peripheral Treg cells, a phenomenon associated with increased acetylation of the FOXP3 transcription factor [32, 44]. Acetate could however promote Treg cells in the colon, suggesting discrete and site specific effects of SCFAs (374). Of note, SCFAs were recently reported to promote the generation of Th1 and Th17 cells during *Citrobacter rodentium* infection, implying a complex, context-dependent impact of SCFAs on immune responses [33].

Propionate was less efficient than butyrate at inhibiting cytokine production by immune cells, whereas acetate showed poor anti-inflammatory activity [32, 45, 46]. Additionally, propionate failed to inhibit TNF but not IL-6 and IL-12p40 induced by LPS in BMDMs, which mirrored previous observations obtained

with TSA and SAHA [27, 29, 47-49]. It is therefore tempting to speculate that, rather that acting through GPRs, butyrate and propionate diffuse into BMDMs to inhibit HDACs and impact on cell response. Supporting this assumption, mRNA expression levels of *free fatty acid receptor 2* (*Ffar2*) and *Ffar3* encoding for GPR43 and GPR41 were very low, or below the detection level, in resting BMDMs and BMDMs stimulated with LPS or Pam_3CSK_4 (Ciarlo et al. unpublished gene array and real-time PCR analyses). Similarly, *Ffra2* and *Ffra3* were not detected in immature and LPS-matured BMDCs [33].

Acetate, propionate and butyrate are found at molar ratios of 60/20/20 in the intestinal tract and 90-55/35-5/10-4 in blood depending on portal, hepatic and peripheral origins, where they altogether reach concentrations of around 50-150 mM and 0.1-1 mM, respectively [8, 35]. The high plasma concentrations of propionate compared to butyrate may counterbalance its weaker anti-inflammatory activity. Further work will be required to analyze the effects of combinational treatments with SCFAs on innate immune cells.

Propionate is produced primarily by Bacteroidetes, via the succinate pathway, and some Firmicutes, through the lactate and succinate pathways, acetate by many enteric bacteria and butyrate by Firmicutes [10]. SCFAs themselves modify the composition of the gut microbiota. Propionate stimulates the growth of *Bifidobacterium* [50]. Furthermore, Bacteroidaceae and Bifidobacteriaceae proportions increased in the gut of mice fed with a high fiber diet, elevating acetate and propionate but decreasing butyrate concentrations in cecal content and blood [35]. Therefore, changing microbiome composition affects SCFA levels locally and systemically. In the perspective of targeting the diet or the microbiota for treating inflammatory conditions [7, 10, 37, 38], it was of primary interest to analyze the impact of propionate in preclinical models of infection.

The main observation of this study is that propionate had no obvious impact on the outcome to infections in terms of morbidity and mortality. In rapidly lethal models of endotoxemia and sepsis in which acute inflammation mediated mortality, propionate treatment had no clinical benefit, unlike butyrate and valproate that conferred protection in models of toxic shock and lethal sepsis [25, 27, 51, 52]. This suggested that, albeit anti-inflammatory *per se*, propionate was not efficient enough to revert the dramatic outcome of these severe conditions, which contrasts with the effectiveness of SCFAs at ameliorating the clinical outcome in chronic inflammatory diseases like rheumatoid arthritis, colitis and airway allergy [16, 17, 30, 32, 35, 53]. Propionate regimen was unlikely responsible of the failure to protect septic animals since identical or even shorter treatments were demonstrated to have an immune impact [32, 35]. Moreover, propionate-treated animals had lower anti-*Streptoccocus* and anti-*Klebsiella* IgG titers, indicating that propionate impacted on immune responses during the course of infections. In agreement with this finding, propionate was recently reported to impair the production of house dust mite-specific IgGs in a mouse model of allergic airway disease, supporting the contention that SCFAs impair innate immune defenses of the airways through an intestinal-bone marrow-lung axis [35]. At least in the endotoxemia model, a likely explanation is that propionate was poorly efficient at inhibiting the swift production of TNF, a critical and central mediator of endotoxemia.

Albeit surprising at first glance, propionate did not increase the mortality of mice subjected to mild infection with *E. coli*, *K. pneumoniae*, *S. pneumoniae* and *C. albicans*, even when mice were beforehand depleted of gut microbiota. Indeed, one of the possible collateral damages of administrating immunomodulatory compounds is the increased risk of infections. A well-known example is anti-TNF therapies associated with reactivation of latent tuberculosis and viral infections and increased opportunistic

infections [54]. Moreover, episodes of severe infection have been reported in patients treated with HDIs [55-59]. In the present study, we tested models of both systemic and local (peritonitis and pneumoniae) infections, which may warranty the safety of propionate supplementation. Future studies should extent to other anatomical compartments the spectrum of preclinical models of infections and address to which extend propionate-mediated reduction of the humoral response to bacteria affect host defenses to re-infection.

The production of propionate by intestinal bacteria has been proposed to represent a mechanism through which host response to commensals is kept under control by bacteria-derived SCFAs to avoid local inflammation and tissue damage. Using several preclinical mouse models, we report that administration of propionate neither protects from lethal sepsis nor increases susceptibility to mild infections. These results are encouraging in the perspective of developing therapies using propionate or directed at the diet or the microbiota for treating inflammation-related disorders such as obesity, atherosclerosis, inflammatory bowel diseases, allergy and cancer.

Materials and methods

Ethics statement. Animal experimentations were approved by the Office Vétérinaire du Canton de Vaud (authorizations n° 876.7, 876.8, 877.7 and 877.8) and performed according to our institutional and ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines).

Mice, cells and reagents. Female BALB/cByJ mice (8-10 week-old; Charles River Laboratories, Saint-Germain-sur-l'Arbresle, France) were housed under specific pathogen-free (SPF) conditions. Bone marrow cells were collected from femurs and tibias and cultured for 7 days 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) [60]. Splenocytes were cultured in RPMI 1640 medium containing 2 mM glutamine and 50 μM 2-ME [27, 61]. Culture media (Invitrogen, San Diego, CA) were supplemented with 10% heat-inactivated FCS (Sigma-Aldrich St. Louis, MO), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen).

Propionate and butyrate were purchased from Sigma-Adldrich, valproate from Desitin (Hamburg, Germany), *Salmonella minnesota* ultra pure lipopolysaccharide (LPS) from List Biologicals Laboratories (Campbell, CA) and Pam_3CSK_4 from EMC microcollections (Tübingen, Germany). Bacterial and fungal strains were isolated from septic patients: *Escherichia coli* O18:K1:H7 (*E. coli*), *Klebsiella pneumonia* caroli (*K. pneumonia*), *Staphylococcus aureus* AW7 (*S. aureus*), *Streptococcus pneumonia* 6303 (*S. pneumoniae*), and *Candida albicans* (*C. albicans*). *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* were grown in brain heart infusion broth, *C. albicans* in yeast extract-peptone-dextrose (BD Biosciences, Erembodegem, Belgium). For *in vitro* experiments, microorganisms were heat-inactivated for 2 h at 56°C before usage.

Cell viability assay. Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation and Viability Assay and a Synergy H1 microplate reader (BioTek, Winooski, VT) [62]. On each 96-well cell culture plate, serial quantities of cells (0.3×10^4 - 5×10^5) were seeded to establish a standard curve.

Whole blood assay. Heparinized whole blood (50 µl) collected from healthy subjects was diluted 5-fold in RPMI 1640 medium and incubated with or without propionate and microbial products in 96-wells plates. Reaction mixtures were incubated for 24 h at 37°C in the presence of 5% CO_2. Cell-free supernatants were stored at -80°C until cytokine measurement.

Cytokine and NO measurements. Cell culture supernatants and plasma were used to quantify the concentrations of TNF, IL-6, IL-12p40 and IFN-γ by DuoSet ELISA kits (R&D Systems, Abingdon, UK), cytokines/chemokines using mouse (G-CSF, IL-1β, IL-10, IL-12p70, IL-18, CXCL10/IP10, CCL2/MCP1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES) and human (TNF, IL-1β, IL-1ra, IL-10, IL-12p40, CCL2, CCL3, CCL4, CXCL8/IL-8, CXCL10) Luminex assays (Affimetrix eBioscience, Vienna, Austria) [63], and NO using the Griess reagent [64]. Intracellular cytokine staining was performed essentially as described previously [65]. Briefly, 0.5 x 10^6 peripheral blood mononuclear cells (PBMCs) were incubated for 1 h with propionate and then for 4 h with 1 µg/ml brefeldin A (BioLegend, San Diego, CA) with or without LPS (100 ng/ml) or Pam_3CSK_4 (1 µg/ml). PBMCs were stained with the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Molecular Probes, ThermoFisher Scientific, Baarrerstrasse, Switzerland), washed with Cell Stain Medium (CSM: PBS, 0.5% bovine serum albumin, 0.02% NaN_3 and 2 mM EDTA), incubated with Human TruStain FcX™ (BioLegend) to block Fc receptors and stained with anti-CD3-PerCP/Cy5.5 (clone UCHT1) and anti-CD14-Pacific Blue (clone M5E2). Cells were washed with CSM, fixed using 2.4% formaldehyde in PBS, washed with CSM containing 0.3% saponin (Sigma-Aldrich) and stained with anti-IL-6-allophycocyanin and anti-TNF-phycoerythrin/Dazzle™ 594 antibodies (clones MQ2-13A5 and MAB11). Staining steps were performed at 20°C for 20 min. All antibodies were from BioLegend. Acquisition was performed on a LSR II flow cytometer (BD Biosciences). Unstained and single stained samples were used to calculate compensation in FlowJo vX (FlowJo LCC, Ashland, OR).

RNA analyses by real-time PCR. Total RNA was isolated, reverse transcribed and used for real-time PCR analyses using a QuantStudio™ 12K Flex system (Life Technologies, Carlsbad, CA) [66]. Reactions consisted of 1.25 µl cDNA, 1.25 µl H_2O, 0.62 µl primers and 3.12 µl Fast SYBR® Green Master Mix (Life Technologies). Primer pairs for amplifying *Tnf*, *Il6*, *Il12b* and *Hprt* (*hypoxanthine guanine phosphoribosyl transferase*) cDNA were as published [60]. Samples were tested in triplicates. Gene specific expression was normalized to *Hprt* expression and expressed in arbitrary units relative to the expression in untreated cells.

Proliferation assay. The proliferation of splenocytes (1.5 x 10^5 cells) cultured for 48 h in 96-well plates was assessed by measuring ^{3}H-thymidine incorporation over 18 h using a β-counter (Packard Instrument Inc, Meriden, CT) [27].

***In vivo* models.** Mice (n = 8-16/group) treated or not with propionate (200 mM in drinking water or 1 g/kg i.p. every other day) were challenged with LPS (250 µg i.p.), *E. coli* (4 x 10^4 CFU i.p.), *K. pneumoniae* (20 or 200 CFU i.n.), *S. pneumoniae* (10^4 CFU i.n.), *S. aureus* (2 x 10^7 CFU i.v.) or *C. albicans* (2 or 5 x 10^5 conidia i.v.). Propionate treatment was continued after microbial challenge. In selected experiments, mice were

treated with ampicillin (Roche Life Sciences, Basel, Switzerland), kanamycin (Panreac AppliChem, Darmstadt, Germany), metronidazole (Sintetica S.A., Couver, Switzerland) and vancomycin (TEVA, North Wales, PA) (AKMV, all at 1 mg/ml) or ciprofloxacin (0.2 mg/ml; Fresenius, Brézins, France) and metronidazole (1 mg/ml) in drinking water to deplete the gut microbiota [16, 17, 32, 35, 67]. Unfortunately, AKMV plus propionate treatment killed mice within 1 week, precluding further investigations. Blood was collected 24 h post-infection to quantify cytokines and circulating bacteria [60]. Body weight, severity scores and survival were registered at least once daily as described previously [64].

Detection of anti-bacteria IgG by ELISA. Briefly, 96-well plates (Maxisorp, Affimetrix eBioscience) were coated with 5 x 10^6 heat-killed *K. pneumoniae* or *S. pneumoniae* in bicarbonate/carbonate buffer (100 mM, pH 9.6), blocked with PBS containing 3% (w/v) bovine serum albumin (PBS-BSA) and incubated with mouse serum diluted 1/200 in PBS-BSA. Mouse IgGs were detected by incubation with peroxidase-goat anti-mouse IgG (H+L) and then 3,3′,5,5′-tetramethylbenzidine (TMB) Substrate Solution (ThermoFisher Scientific). Reactions were stopped using 0.16 M sulfuric acid and absorbance measured at 450 nm using a VersaMax ELISA microplate reader (Molecular devices, Sunnyvale, CA). All washing steps were performed using PBS containing 0.05% (v/v) Tween-20.

Statistical analyses. Comparisons between the different groups were performed by analysis of variance followed by two-tailed unpaired Student's t-test. The Kaplan-Meier method was used for building survival curves 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.

Authorship

EC, TH, MM and XZ performed *in vitro* experiments, TH performed Luminex assays, JH performed flow cytometry analyses, EC, TH and DLR performed *in vivo* experiments. TR conceived the project, designed the experiments, performed whole blood assays, and wrote the paper. All authors revised the paper.

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Conflict of Interest Disclosure

The authors declare no competing financial interests

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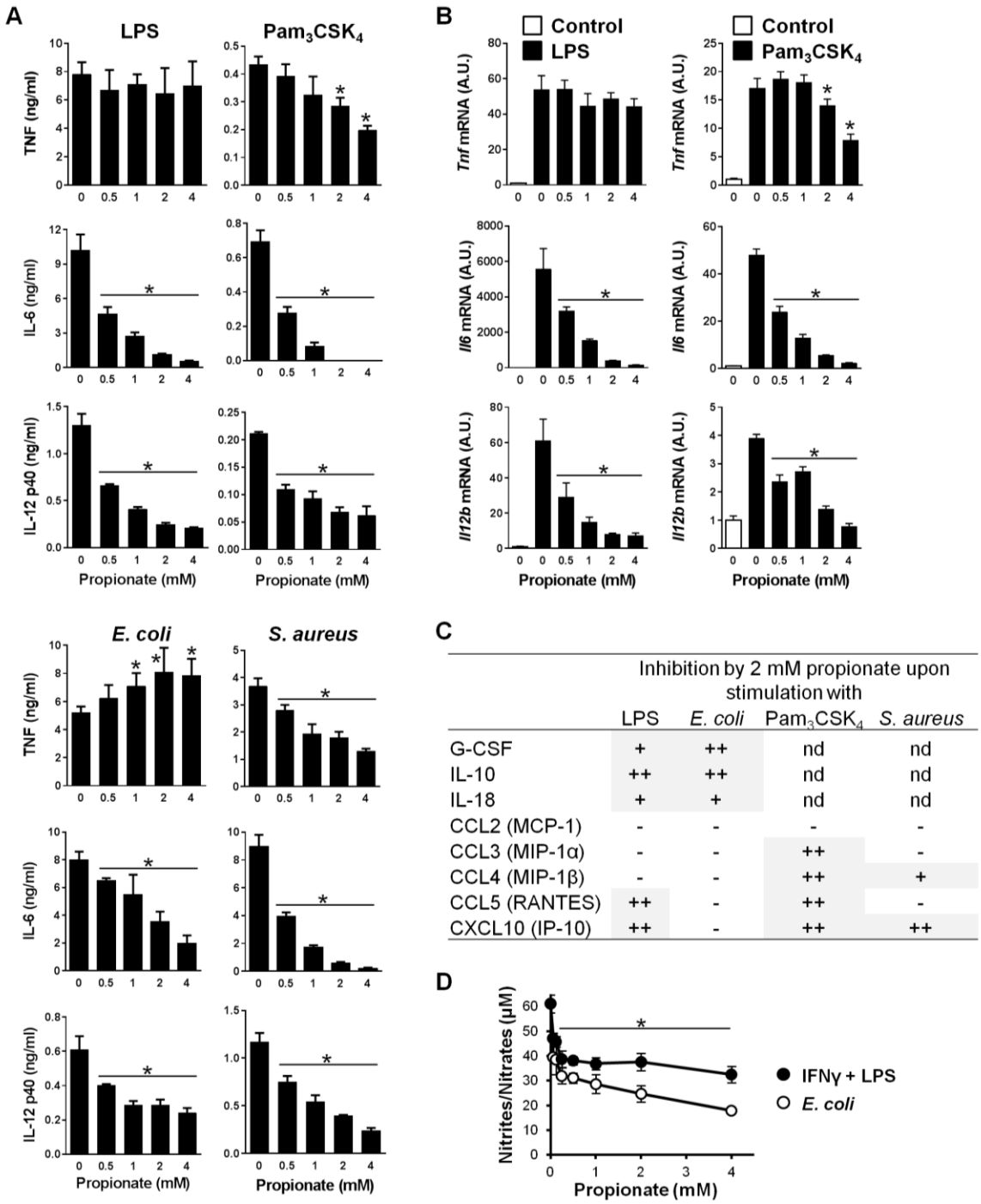


Figure 1. Impact of propionate on the response of macrophages to microbial stimulation. BMDMs were pre-incubated for 1 h with increasing concentrations (0, 0.06, 0.12, 0.25, 0.5, 1, 2 and 4 mM) of propionate before exposure for 4, 8 or 24 h to LPS (10 ng/ml), Pam_3CSK_4 (10 ng/ml), *E. coli* (10^6 CFU/ml), *S. aureus* (10^7 CFU/ml) or a combination of IFN-γ (100 U/ml) plus LPS (10 ng/ml). (A, B) TNF, IL-6 and IL-12p40 concentrations in cell culture supernatants and *Tnf*, *Il6*, *Il12b* mRNA levels were quantified by ELISA (A, t = 8 h) and real time-PCR (B, t = 4 h). No cytokine was detected in the supernatants of unstimulated cells. *Tnf*, *Il6* and *Il12b* mRNA levels were normalized to *Hprt* mRNA levels. Data are means ± SD of triplicate samples from one experiment performed with 4 mice and representative of 2 experiments. *; $P < 0.05$ versus stimulus

without propionate. A.U.: arbitrary units. (C) The production of G-CSF, IL-10, IL-18, CCL2, CCL3, CCL4, CCL5 and CXCL10 was assessed by the Luminex technology (t = 8 h). Data summarize the impact of 2 mM propionate on mediators produced in response to LPS, *E. coli*, Pam_3CSK_4 and *S. aureus*: -, no inhibition; +, 1.5-2-fold inhibition; ++, > 2-fold inhibition. Quantification is from one experiment performed with 4 mice. (D) Nitrites/nitrates were quantified using the Griess reagent (t = 24 h). Data are means ± SD of quadruplicate samples from one experiment performed with 4 mice. $P < 0.05$ when comparing propionate at all concentrations versus no propionate.

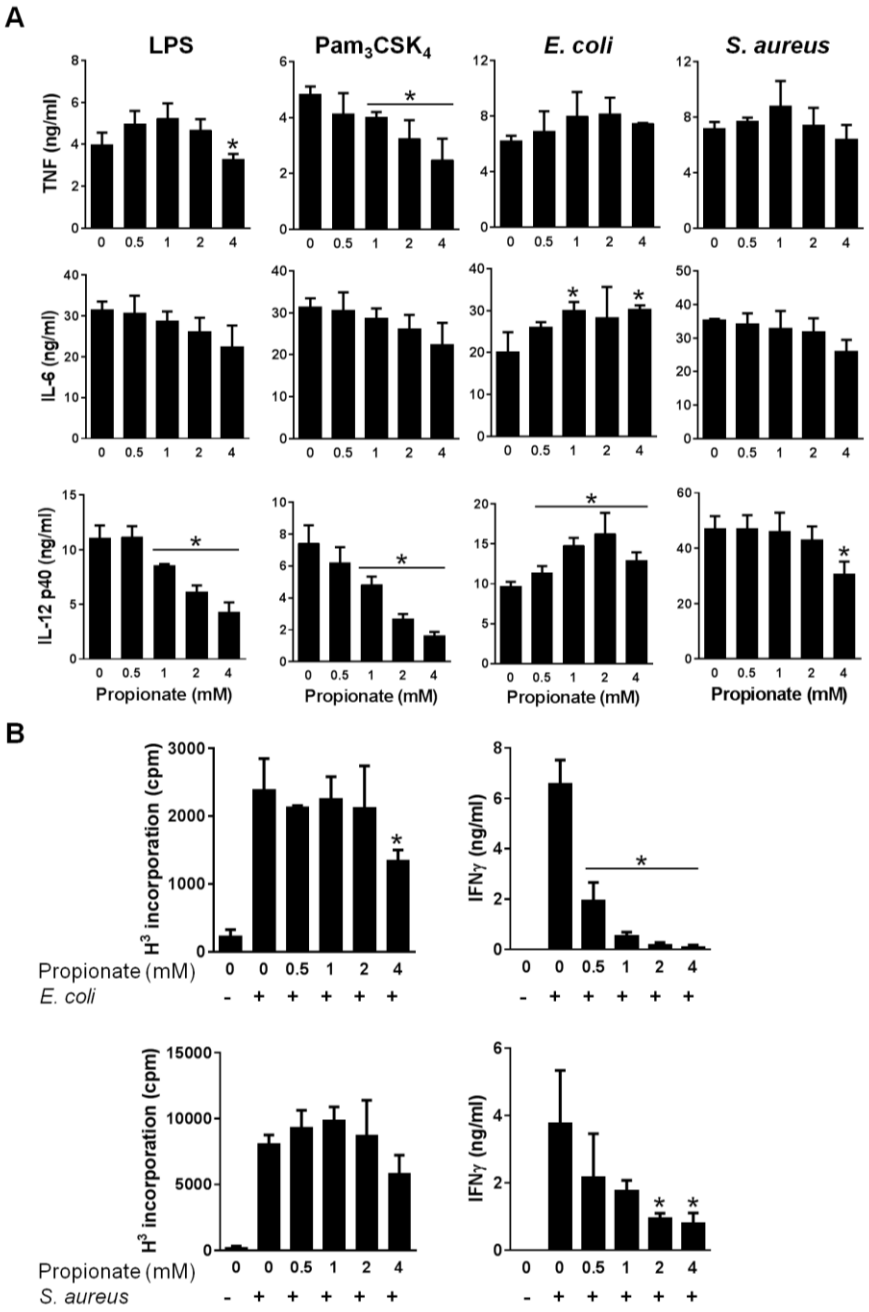
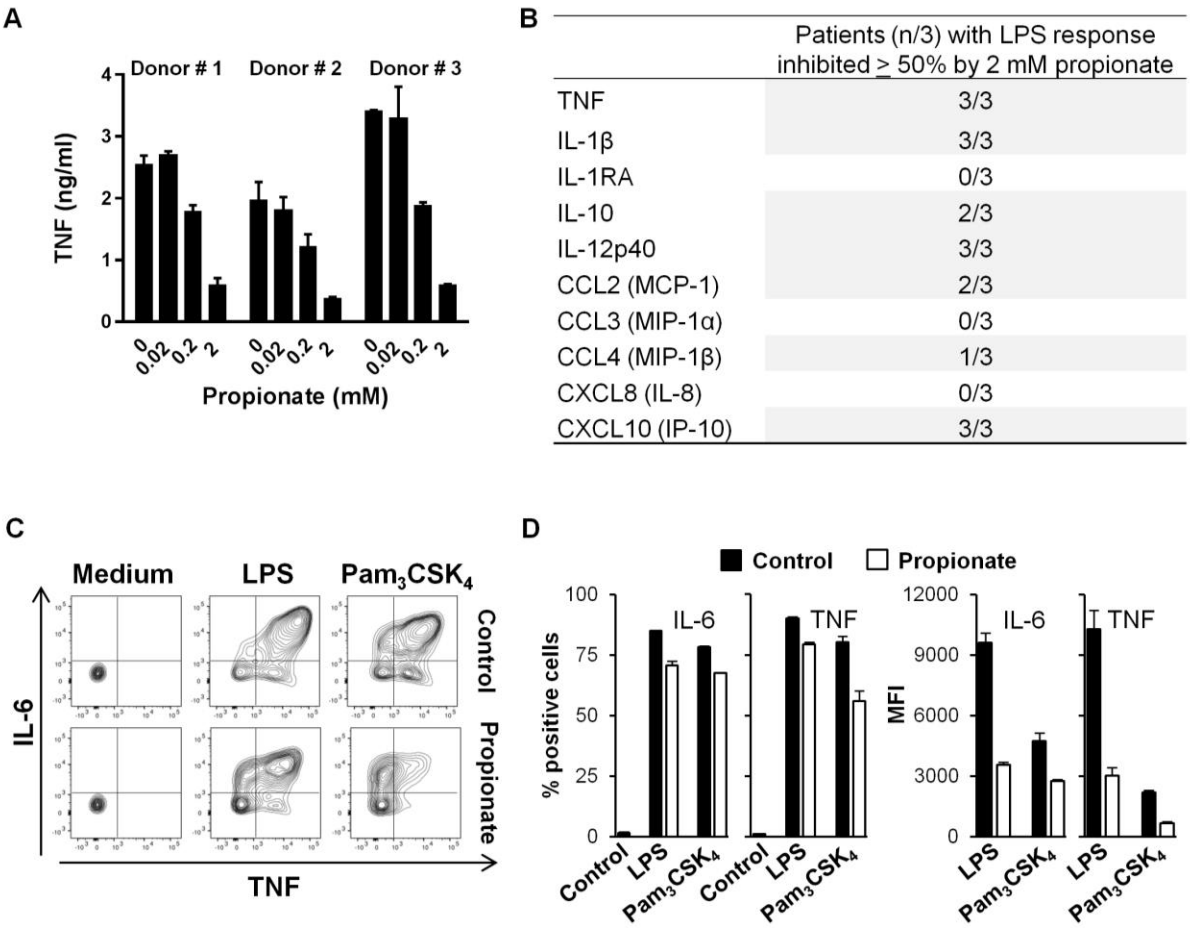


Figure 2. Impact of propionate on the response of dendritic cells and splenocytes. (A) BMDCs were pre-incubated for 1 h with increasing concentrations (0, 0.5, 1, 2 and 4 mM) of propionate before exposure for 8 h to LPS (10 ng/ml), Pam_3CSK_4 (10 ng/ml), *E. coli* (10^6 CFU/ml) and *S. aureus* (10^7 CFU/ml). TNF, IL-6 and IL-12p40 concentrations in cell culture supernatants were quantified by ELISA. Data are means ± SD of triplicate samples from one experiment performed with 4 mice and representative of 2 experiments. No cytokine was detected in the supernatants of unstimulated cells. (B) Mouse splenocytes were incubated for 48 h with or without propionate and *E. coli* (10^6 CFU/ml). Proliferation was measured by 3H-thymidine incorporation. IFN-γ concentrations in cell culture supernatants were quantified by ELISA. Data are means ± SD of triplicate samples from one experiment performed with 4 mice. *; $P < 0.05$ versus stimulus without propionate.



	Patients (n/3) with LPS response inhibited ≥ 50% by 2 mM propionate
TNF	3/3
IL-1β	3/3
IL-1RA	0/3
IL-10	2/3
IL-12p40	3/3
CCL2 (MCP-1)	2/3
CCL3 (MIP-1α)	0/3
CCL4 (MIP-1β)	1/3
CXCL8 (IL-8)	0/3
CXCL10 (IP-10)	3/3

Figure 3. Impact of propionate on the response of human whole blood and monocytes. Whole blood from 3 healthy subjects was incubated for 18 h with propionate and LPS (100 ng/ml), *E. coli* (10^6 CFU/ml) and *S. aureus* (10^7 CFU/ml). (A) TNF concentrations were quantified by ELISA. Data are means ± SD of triplicate determinations. $P < 0.005$ when comparing 0.2 and 2 mM propionate with 0 mM propionate. (B) TNF, IL-1β, IL-1RA, IL-10, IL-12p40, CCL2, CCL3, CCL4, CXCL8 and CXCL10 were quantified by Luminex. Results summarize the number of donors in whom propionate inhibited significantly ($P < 0.05$) and by at least 50% cytokine release. (C, D) PBMCs were incubated for 1 h with 2 mM propionate and stimulated for 4 h with LPS (100 ng/ml) and Pam_3CSK_4 (1 µg/ml). TNF and IL-6 expression in $CD14^+$ monocytes was analyzed by flow cytometry (C) to calculate the percentage of positive cells and mean fluorescence intensity (MFI). Data are means ± SD (D).

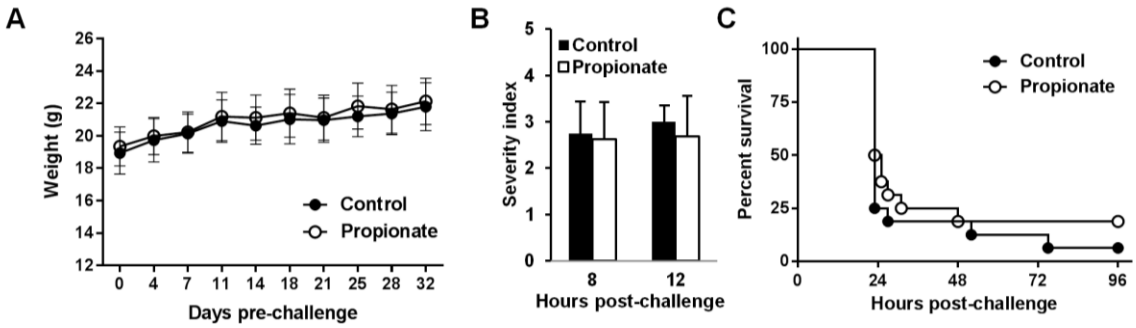


Figure 4. Propionate does not protect from endotoxemia. BALB/c mice (n = 16 per group) were treated with 200 mM propionate in drinking water for 1 month. (A) Weight of animals under propionate treatment. (B, C) Severity scores and survival (*P* = 0.3) of mice challenged with LPS (250 µg i.p.).

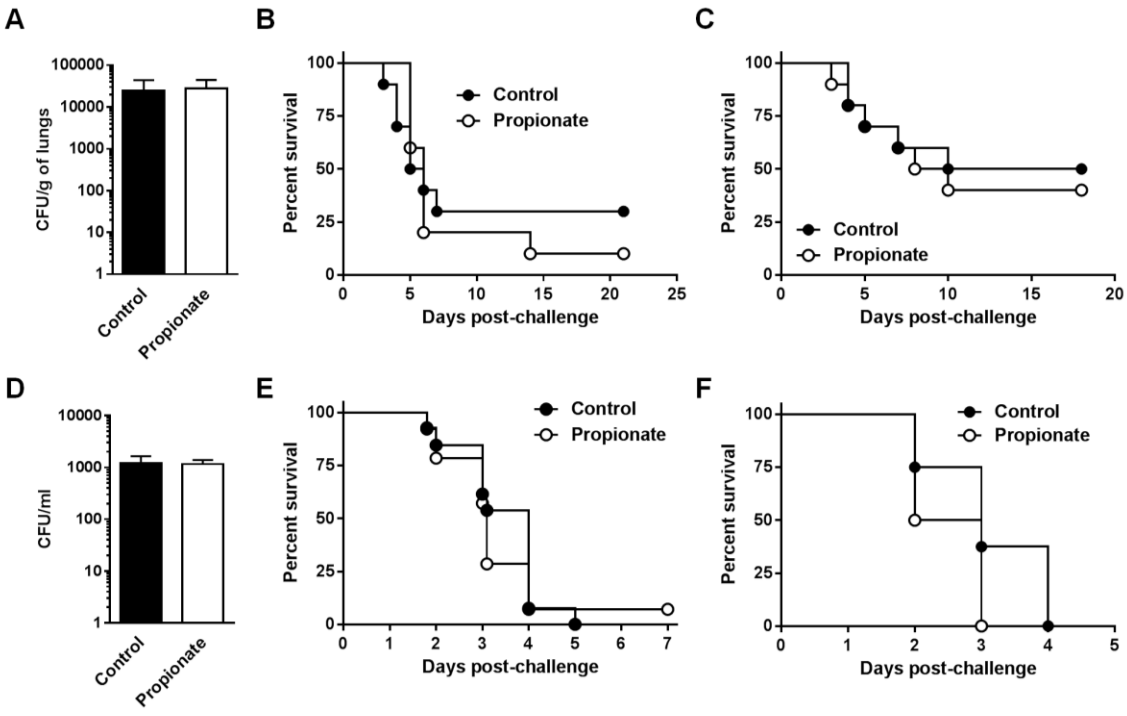


Figure 5. Propionate does not protect from lethal sepsis. BALB/c mice were treated with 200 mM propionate in drinking water for 3 weeks. (A-C) Bacterial counts in lungs 48 h post-infection and survival of mice (n = 10 per group) challenged i.n. with 200 CFU (A, B) or 20 CFU (C) of *K. pneumoniae*. *P* = 0.4, 0.8 and 0.7, respectively. (D, E) Bacterial counts in blood 24 h post-infection and survival of mice (n = 15 per group) challenged with *S. aureus* (2 x 10^7 CFU i.v.). *P* = 0.9 and *P* = 0.6. (F) Survival of mice (n = 8 per group) challenged with *C. albicans* (5 x 10^5 CFU i.v.). *P* = 0.1.

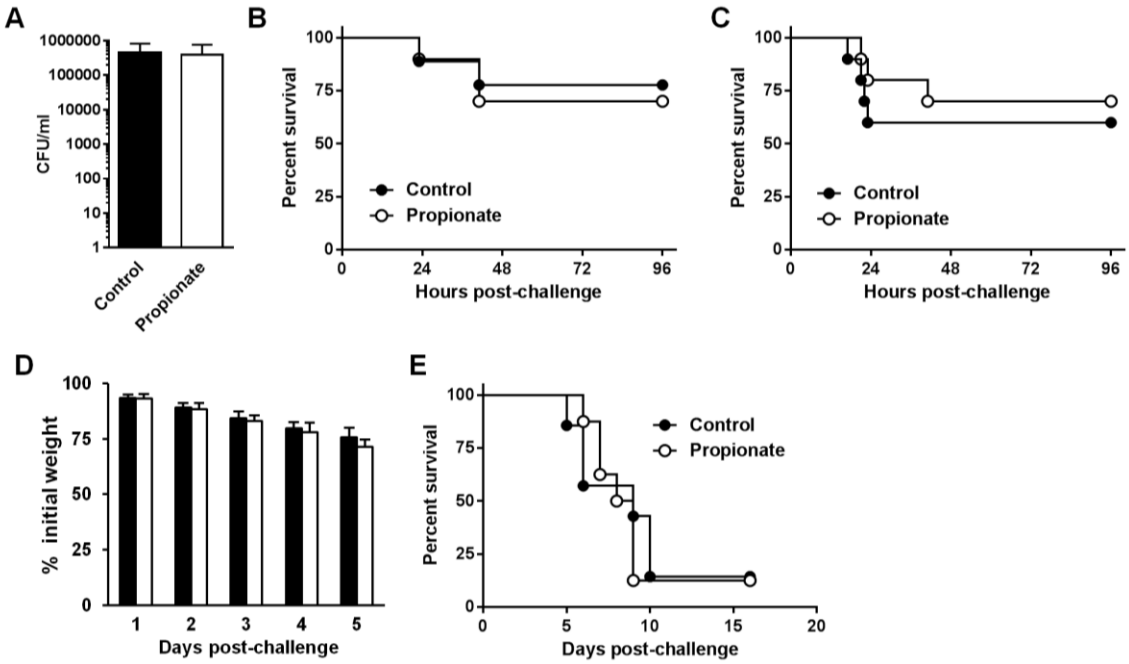


Figure 6. Propionate does not impair host defenses against *E. coli* and *C. albicans* infection. BALB/c mice (n = 10 and 8 per group in A-C and D-E, respectively) were treated with 200 mM propionate in drinking water (A, B, D, E) or 1 g/kg propionate given i.p. every other day (C) for 3 weeks and challenged with *E. coli* (4 x 10^4 CFU i.p.; A-C) or *C. albicans* (2 x 10^5 CFU i.v.; D, E). (A) Bacterial counts in blood 24 h post-infection. *P* = 0.9. (B, C) Survival of mice. *P* = 0.7 and 0.6. (D) Body weight. (E) Survival of mice. *P* = 0.8.

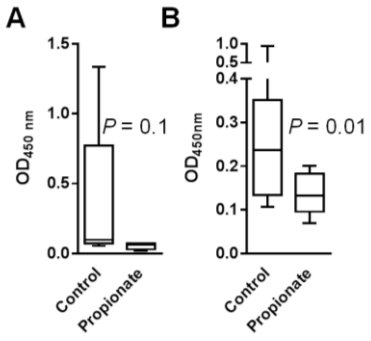


Figure 7. Propionate impairs anti-bacterial IgG response. (A) Blood was collected 17 days post-infection from BALB/c mice surviving i.n. infection with 20 CFU *K. pneumoniae* (Figure 5C; n = 4 control and 5 propionate-treated mice) and (B) from BALB/c mice (n = 9 control and 10 propionate-treated mice) treated with 200 mM propionate in drinking water for 3 weeks and infected i.n. with a sublethal dose of *S. pneumoniae* (10^4 CFU). Box and min-to-max wisker plots represent the $OD_{450\ nm}$ of anti-bacterial IgG titers using plasma diluted 1/200. *P* = 0.1 and = 0.01.

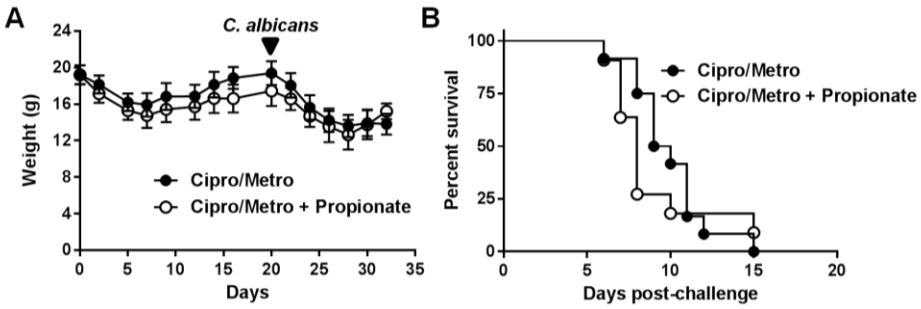


Figure 8. Propionate does not protect from candidiasis mice depleted of gut microbiota. BALB/c mice (n = 10 per group) were treated with ciprofloxacin and metronidazole with or without propionate (200 mM) in drinking water for 3 weeks and challenged with *C. albicans* (2 x 10^5 CFU i.v.). (A) Body weight. (B) Survival of mice. *P* = 0.4.

3.3 Expression pattern of sirtuins in immune cells and organs

No comprehensive study of the expression pattern of sirtuins in immune cells has been reported. Considering our interest in innate immune responses, we analyzed the expression of sirtuins at baseline and after stimulation with MAMPs/DAMPs, whole bacteria and cytokines *in vitro* and *in vivo*, with a particular focus on immune cells and organs.

3.3.1 Sirtuin expression is modulated by microbial stimulation *in vitro*

As described thoroughly in **section 3.6 (Figure 1)**, an extensive analysis of SIRT1-7 expression using data extracted from the BioGPS resource (http://biogps.org) and performed by real time-PCR (RT-PCR) in BMDMs, BMDCs, FMS-like tyrosine kinase 3 ligand (Flt3l)-derived DCs and RAW 264.7 and J774.1 macrophage cell lines (**Figure 9A**) revealed that SIRT2 was the most expressed sirtuin in myeloid-derived immune cell populations.

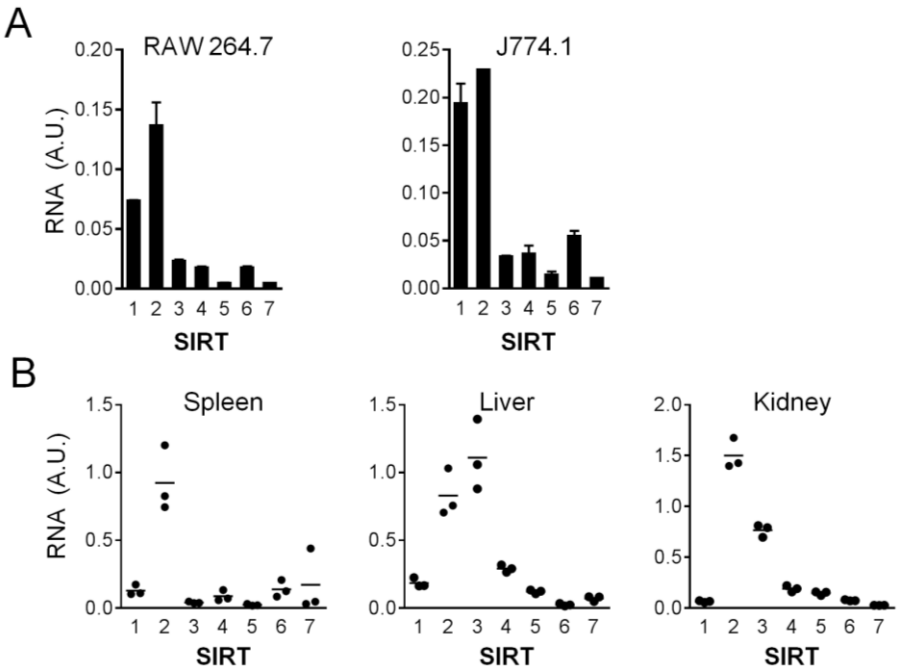


Figure 9. Expression of SIRT1-7 in macrophage cell lines, spleen liver and kidney. RNA was extracted from RAW 264.7 and J774.1 macrophages (**A**) and spleen, liver and kidney of BALB/c mice (B). SIRT1-7 expression was quantified by RT-PCR and normalized to Hprt mRNA levels. Data are means ± SD of triplicate samples from one experiment representative of two experiments. A.U.: arbitrary units.

Western blot analyses confirmed higher SIRT2 than SIRT1 signals in BMDMs (**section 3.4, Figure 7C**), suggesting that differences at the mRNA level translate to the protein expression level. RT-PCR analyses using spleen, liver and kidney showed high SIRT2 mRNA expression levels in these organs and also high SIRT3 mRNA expression in the liver and kidney (**Figure 9B)**.

We then analyzed SIRT1-SIRT7 mRNA abundance in BMDMs exposed for 4 and 18 hours to a broad panel of microbial products proinflammatory and anti-inflammatory cytokines and ATP. Sirtuin expression was overall modestly, either positively or negatively, modulated by these treatments, especially cytokines. SIRT1, SIRT2, SIRT6 and SIRT7 were upregulated upon stimulation with pure microbial products and, to a lesser extent, by microorganisms and ATP (**Table 7**). Conversely, mitochondrial sirtuins, especially SIRT3, showed a trend towards down-regulation upon microbial stimulation.

Table 7. Impact of microbial products, cytokines and ATP on sirtuin mRNA expression in BMDMs.

		SIRT1		SIRT2		SIRT3		SIRT4		SIRT5		SIRT6		SIRT7	
		4h	18h	4h	18h	4h	18h	4h	18h	4h	18h	4h	18h	4h	18h
Microbial products	LPS	++	0	+	0	--	0	0	+	-	0	+++	++	+	0
	Pam_3CSK_4	0	-	+	0	-	0	0	0	0	0	++	0	+	0
	CpG ODN 1826	+	-	++	0	-	-	0	0	0	0	++	0	++	0
	LTA	+	0	+	+	--	0	-	+	--	+	++	++	+	0
	Poly (I:C)	+++	+	+	+	0	0	0	0	0	--	+	++	0	0
	Peptidoglycan	++	0	+	0	-	0	0	0	-	0	++	+	+	0
	Curdlan	+	0	+	+	--	0	-	+	0	+	++	++	0	0
	Zymosan	++	0	++	+	--	0	-	+	0	+	++	++	+	0
Microorganisms	*E. coli* 018	+	0	++	0	--	0	-	+	--	0	++	+	+	0
	S. typhi C5	+	0	+	0	--	0	-	0	--	+	++	+	0	0
	C. albicans 5102 hyphae	0	0	0	+	0	0	0	0	0	0	0	0	0	0
	C. albicans 5102 yeast	0	0	0	+	-	0	0	+	0	+	0	+	0	+
Pro-inflammatory cytokines	TNF	0	0	+	0	0	0	0	0	0	0	+	++	+	0
	IL-1ß	0	0	+	0	0	0	0	0	0	0	0	0	+	0
	IL-2	0	0	0	0	0	0	0	0	0	0	0	0	+	0
	M-CSF	0	+	0	+	0	0	0	0	0	0	0	0	--	0
	GM-CSF	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Anti-inflammatory cytokines	TGF-ß	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	IL-10	0	0	0	0	0	0	0	0	0	0	0	0	+	0
	IL-4	0	0	0	0	0	0	0	0	0	0	0	0	+	0
DAMPs	ATP	++	0	++	+	+	0	+	0	0	0	+	0	++	0

BMDMs were stimulated for 4 or 18 hours with 10 ng/ml LPS and Pam_3CSK_4, 2 µg/ml CpG ODN 1826, 10 µg/ml lipoteichoic acid (LTA), poly (I:C) and zymosan, 20 µg/ml peptidoglycan, 100 µg/ml curdlan, 10^6 CFU/ml *E. coli* O18 and *Salmonella enterica* serovar Typhimurium C5 (*S. typhi* C5), 100 µg/ml *C. albicans* 5102 hyphae and yeast, 10 ng/ml TNF, IL-1ß, IL-2, M-CSF, GM-CSF, TGF-ß, 1 IL-10 and IL-4 and 3 mM ATP. SIRT1-7 mRNA levels were quantified by RT-PCR and normalized to Hprt mRNA levels. Data are means of four independent experiments and results show fold increase compared to unstimulated cells expressed as follows: +++ > 3; 3 ≥ ++ > 2; 2 ≥ + > 1.5; 1.5 ≥ 0 > 0.67; 0.67 ≥ - > 0.5; 05 ≥ -- > 0.33; --- ≤ 0.33.

3.3.2 Sirtuin expression is modulated during endotoxemia

We quantified sirtuin expression in organs of endotoxemic mice. In a first experiment, C57BL/6J mice were injected intraperitoneally (i.p.) with 350 µg of LPS, corresponding to a LD_{90}. Animals were sacrificed 0, 4 and 24 hours after challenge to quantify SIRT1-7 mRNA expression levels by RT-PCR in spleen, liver and kidney (**Table 8**).

Table 8. Sirtuin mRNA expression in spleen, liver and kidney of mice injected with a high dose LPS.

Sirtuin	Spleen (hours LPS)			Liver (hours LPS)			Kidney (hours LPS)		
	0	4	24	0	4	24	0	4	24
SIRT1	0	0	0	0	0	-	0	0	-
SIRT2	0	-	0	0	---	--	0	0	-
SIRT3	0	--	0	0	---	---	0	--	--
SIRT4	0	---	0	0	---	---	0	--	-
SIRT5	0	---	0	0	---	0	0	--	0
SIRT6	0	-	0	0	0	0	0	-	0
SIRT7	0	--	-	0	---	0	0	-	-

Mice were injected intraperitoneally (i.p.) with 350 µg of LPS. SIRT1-7 mRNA levels were quantified by RT-PCR in organs collected 0, 4 and 24 hours after challenge and normalized to Hprt mRNA levels. Data are means of two independent experiments and results show fold increase compared to untreated animals (0 hour) expressed as follows: +++ > 3; 3 ≥ ++ > 2; 2 ≥ + > 1.5; 1.5 ≥ 0 > 0.67; 0.67 ≥ - > 0.5; 05 ≥ -- > 0.33; --- ≤ 0.33.

Four hours after challenge, the expression of sirtuins decreased in all organs, except SIRT1 in spleen, liver and kidney, SIRT2 in kidney and SIRT6 in liver. After 24 hours, SIRT1-6 levels rescued to baseline levels in spleen, SIRT5-7 in liver and SIRT5-6 in kidney. To define whether sirtuin expression could be restored at a later time point or upon less stringent conditions, we performed a second experiment in which mice were injected with 100 µg LPS equivalent to a LD_{10}, and collected liver and kidney after 4, 24 and 48 hours. We confirmed the general down-regulation of sirtuins at 4 and 24 hours in both organs. SIRT1-7 levels returned to baseline levels after 48 hours in the kidney but not in the liver, except for SIRT1 (**Table 9**).

Table 9. Sirtuin mRNA expression in liver and kidney of mice injected with a low dose of LPS.

Sirtuin	Liver (hours LPS)				Kidney (hours LPS)			
	0	4	24	48	0	4	24	48
SIRT1	0	-	0	0	0	--	0	0
SIRT2	0	--	--	--	0	-	--	0
SIRT3	0	---	-	-	0	-	0	0
SIRT4	0	--	-	-	0	--	-	0
SIRT5	0	--	--	--	0	--	--	0
SIRT6	0	0	0	-	0	0	0	0
SIRT7	0	---	--	--	0	--	-	0

Mice were injected i.p. with 100 µg of LPS. SIRT1-7 mRNA levels were quantified by RT-PCR in organs collected 0, 4, 24 and 48 hours after challenge and normalized to Hprt mRNA levels. Data are means of two independent experiments and results show fold increase compared to untreated animals (0 hour) expressed as follows: +++ > 3; 3 ≥ ++ > 2; 2 ≥ + > 1.5; 1.5 ≥ 0 > 0.67; 0.67 ≥ - > 0.5; 05 ≥ -- > 0.33; --- ≤ 0.33.

3.3.3 Conclusions

In this section, we have shown that sirtuins are constitutively expressed in innate immune cells and organs. Observations made at the mRNA level should be validated by protein expression studies, but those are limited by the availability of good anti-sirtuin antibodies and the fact that large quantities of cells are required to trace SIRT3-5 expression using purified mitochondria.

Strikingly, at baseline, SIRT2 was the most expressed sirtuin in all samples analyzed apart from the liver in which SIRT2 closely followed SIRT3 expression levels. This suggests that SIRT2 might have an important physiological role in innate immune cells. As reported in **section 3.6**, we addressed that specific question by using SIRT2 knockout mice.

Sirtuin mRNA expression was modulated by microbial stimulation *in vitro*. In primary macrophages, cytoplasmic and nuclear sirtuins were overall upregulated by microbial stimulation, a trend that contrasted with mitochondrial sirtuins, especially SIRT3, that was downregulated after stimulation. The otherwise quasi-absence of modulation of sirtuin expression by proinflammatory and anti-inflammatory cytokines strongly advocate that microbial stimulation did not modulate sirtuin expression through an indirect autocrine or paracrine action of cytokines.

The constitutive expression of sirtuins in immune cells and organs together with their modulation in macrophages exposed to microbial products suggests an important physiological role of these enzymes in the control of metabolic adaptation and inflammatory and immune responses. Unfortunately, our knowledge

about the impact of sirtuins on innate immune responses is still fragmentary. Whether the modulation of sirtuin expression triggered immune responses or had a feedback regulatory impact on inflammatory responses cannot be concluded from our data and will require further investigations. Importantly, the fact that LPS upregulated most sirtuins in macrophages while basically all sirtuins were down-regulated in organs of mice challenged with LPS warns that expression studies at a population level have to be interpreted with caution in the context of a full organism. Moreover, it would be interesting to test whether TLR ligands other than LPS or microorganisms/infections impact on sirtuin expression *in vivo*.

SIRT6 was the strongest upregulated sirtuin in BMDMs exposed to microbial products and was much less affected than other sirtuins in the organs of endotoxemic mice. SIRT6 and NAD^+ intracellular levels are crucial for TNF production and secretion (138, 287). Further investigation will be needed to define whether overexpression of SIRT6, induced upon microbial stimulation, participates to promote TNF secretion.

In organs of endotoxemic mice, we observed a reduction of sirtuin expression that was rescued in kidney and spleen but not in liver. We can envisage that the inability to restore baseline levels of sirtuins in some organs might contribute to unfavorable outcome. A greater reduction was noticed for the mitochondrial SIRT3 and SIRT4 in the liver of endotoxemic mice. It is known that endotoxemia induces liver injury, and one of the mechanisms underlying liver damage is an excessive production of ROS. This could be linked to SIRT3 downregulation, since SIRT3 decreases cellular ROS through deacetylation and activation of the antioxidant enzyme superoxide dismutase 2 (SOD2) (376). Moreover, we observed a smaller effect on SIRT3 expression in liver of mice injected with a low -less damaging- dose of LPS compared to a high dose of LPS.

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

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Summary

Since SIRT2 is the most expressed sirtuin in macrophages, we hypothesized that it could play a role in innate immune responses. To test our hypothesis, we took advantage of pharmacological inhibitors of sirtuins recently developed for their therapeutic potential in oncologic diseases. We selected cambinol, a cell permeable β-naphthol compound, because it was reported to be selective and non toxic. Cambinol inhibits SIRT1 and SIRT2 with similar IC_{50}.

Cambinol dose-dependently inhibited cytokine secretion by BMDMs, BMDCs, PBMCs and whole blood exposed to LPS, Pam_3CSK_4, CpG, *E. coli* and *S. aureus*, *Nos2* induction and NO production by BMDMs, CD40 upregulation by BMDCs, and proliferation and IFNγ production by splenocytes. At the molecular level, cambinol impaired MAPK signaling and AP-1-mediated transcriptional activity, without affecting NF-κB p65 nuclear translocation. In agreement with these findings, cambinol protected mice from endotoxemia and *K. pneumonia* sepsis.

Unexpectedly, selective inhibitors of SIRT1 (EX-527 and CHIC-35) or SIRT2 (AGK2 and AK-7) used either alone or in combination did not reproduce the anti-inflammatory effects of cambinol on cytokine mRNA and protein expression. It is known that cambinol does not inhibit SIRT3 activity, and we demonstrate that it does not inhibit SIRT6 activity. Altogether, our data indicate that cambinol has powerful anti-inflammatory properties by targeting more than just SIRT1 and SIRT2, and that it may protect from lethal sepsis.

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Review

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]-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).

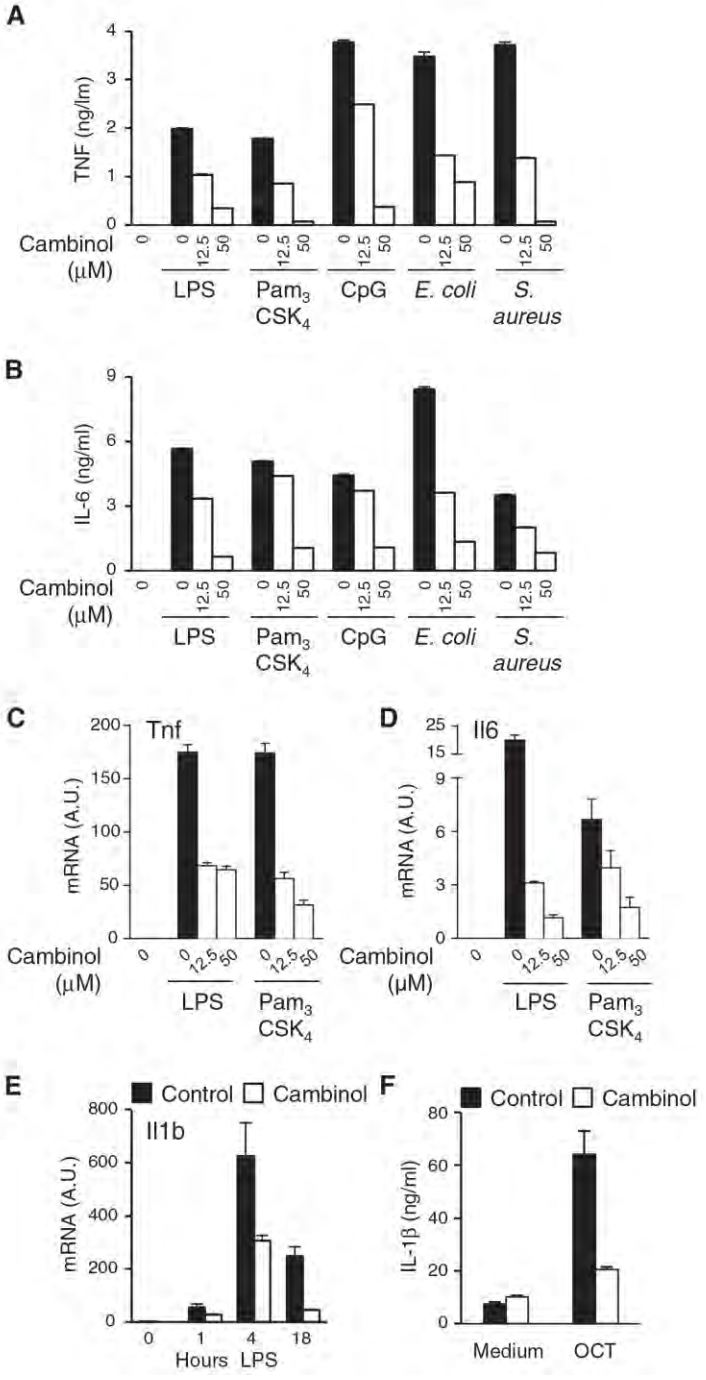
To define whether inhibition of SIRT1 or SIRT2 alone have anti-inflammatory effects, we used the selective SIRT1 inhibitors EX-527 ($IC_{50\ SIRT1} = 0.1$–1 μM; $IC_{50\ SIRT2} = 20$–33 μM) and CHIC-35 ($IC_{50\ SIRT1} = 0.06$–0.12 μM; $IC_{50\ SIRT2} = 2.8$ μM), and the selective SIRT2 inhibitors AGK2 ($IC_{50\ SIRT2} = 3.5$ 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 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 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.



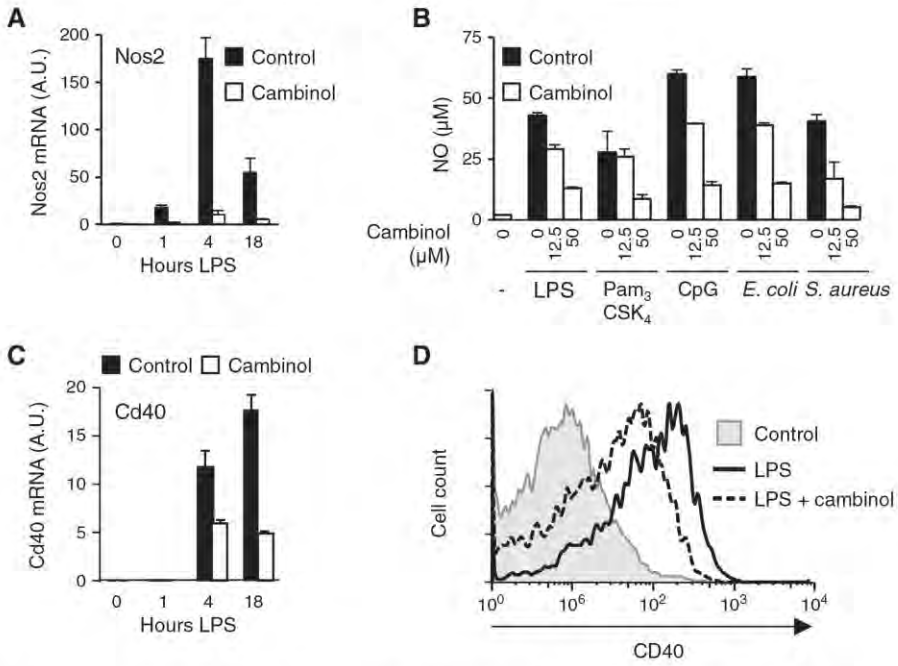


Fig. 2. Cambinol inhibits the response of macrophages to Toll-like receptor stimuli. (A–B) BMDMs were primed 18 h with IFN-γ (100 U/ml), washed, pre-incubated for 1 h with or without cambinol (50 μM in A) and exposed to stimuli as mentioned in Fig. 1. (A) Nos2 mRNA levels were quantified by real time-PCR 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.

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. 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.

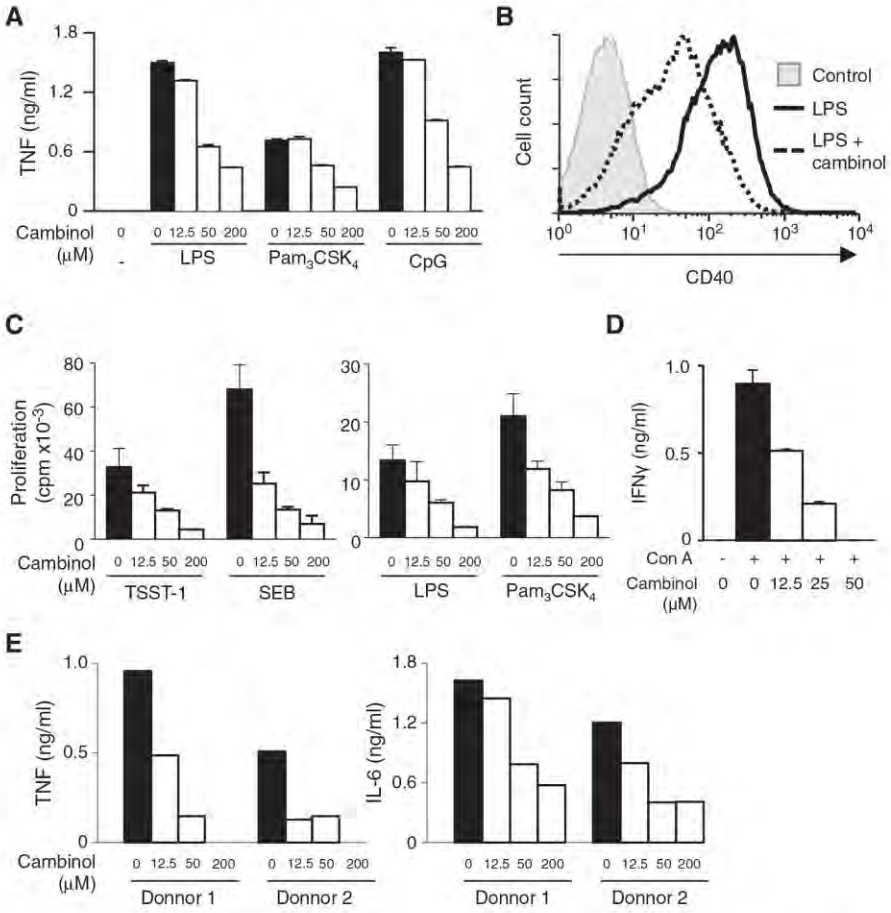


Fig. 3. Cambinol inhibits the response of dendritic cells, splenocytes and whole blood. BMDCs were pre-incubated for 1 h with or without cambinol before exposure for 4 h (A) or 18 h (B) to LPS (10 ng/ml), Pam_3CSK_4 (10 ng/ml) and CpG (2 μg/ml). (A) TNF concentrations in cell culture supernatants were 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.

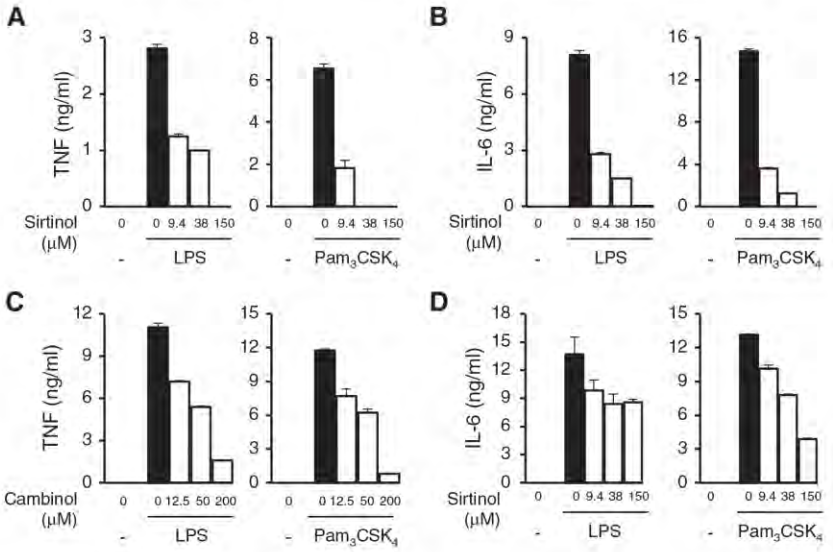


Fig. 4. Sirtinol inhibits cytokine production by macrophages. BMDMs (A–B) and RAW 264.7 macrophages (C–D) were pre-incubated for 1 h with or without sirtinol and cambinol before exposure for 4 h (A, C) or 18 h (B, D) to LPS (10 ng/ml) and Pam_3CSK_4 (10 ng/ml). Concentrations of TNF and IL-6 in cell 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

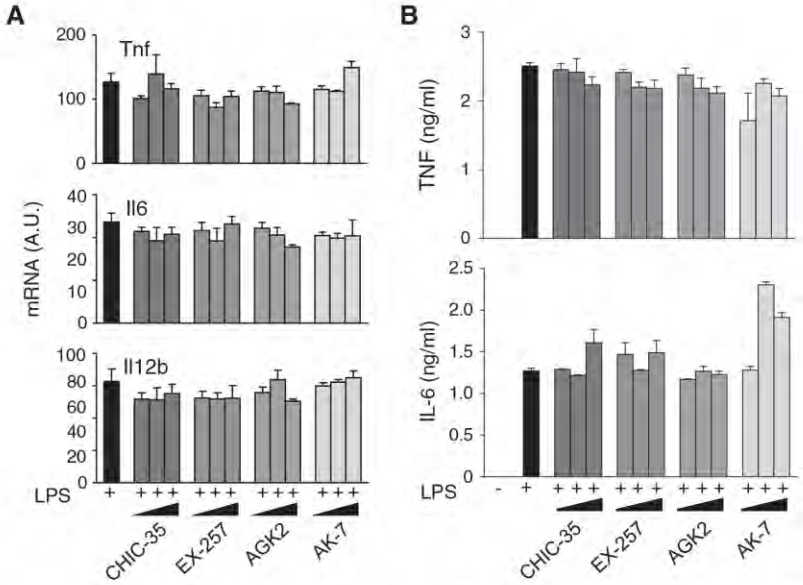


Fig. 5. Selective inhibitors of Sirt1 or Sirt2 used alone or in combination do not inhibit cytokine production by macrophages. BMDMs were pre-incubated for 1 h with or without EX-527 (0.25, 1, and 4 μM), AGK2 (0.8, 3.5, and 14 μM), AK-7 (6, 24, and 96 μM), CHIC-35 (30, 120, and 480 nM) before exposure 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.

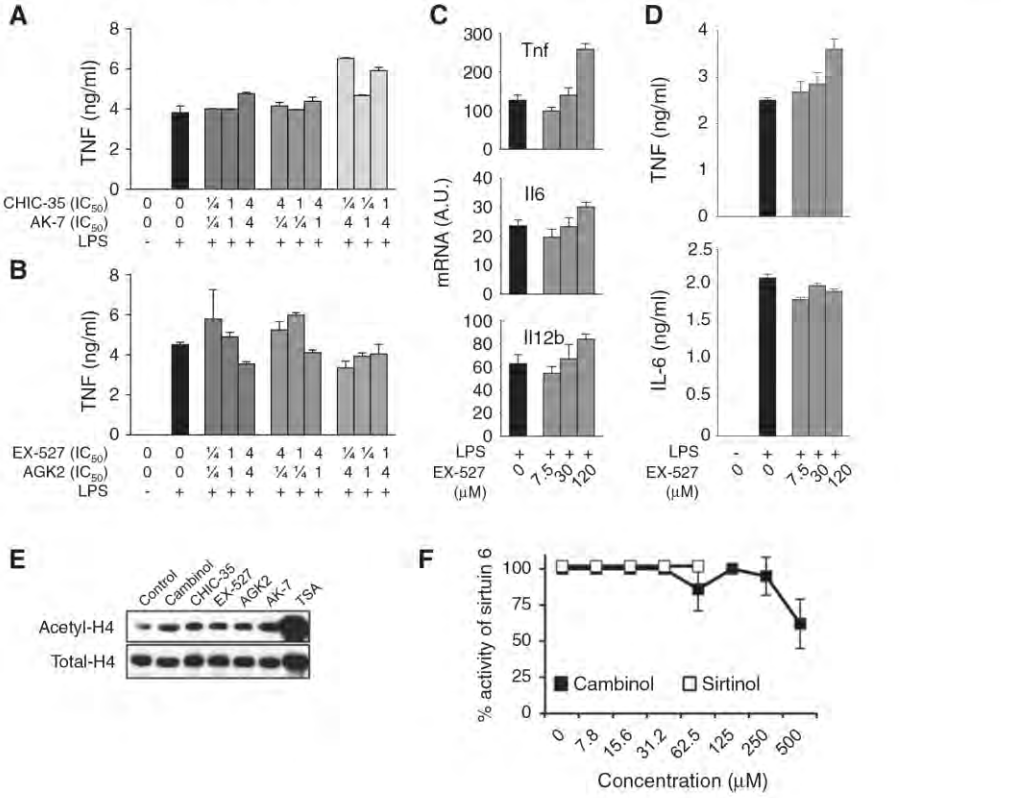


Fig. 6. Dual inhibition of Sirt1 or Sirt2 does not reduce cytokine production by macrophages. BMDMs were pre-incubated for 1 h with combination of CHIC-35 (1/4, 1 and 4: 30, 120 and 480 nM) and AK-7 (1/4, 1 and 4: 6, 24, and 96 μM) (A) or EX-527 (1/4, 1 and 4: 0.25, 1, and 4 μM) and AGK2 (1/4, 1 and 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

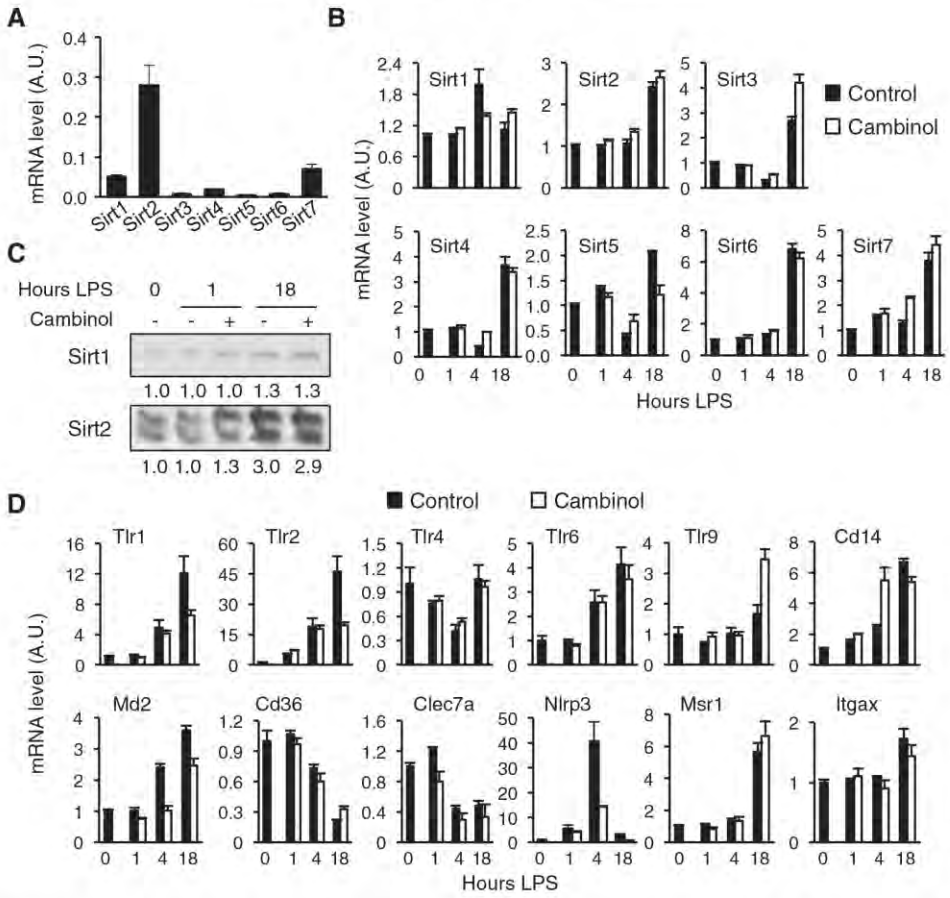


Fig. 7. Effect of cambinol on the expression of sirtuins and pattern recognition receptors. (A) Sirt1-7 mRNA levels in resting BMDMs were quantified by real time-PCR and normalized to GAPDH mRNA levels. Data are means ± SD of triplicate samples from one experiment representative of three experiments. (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

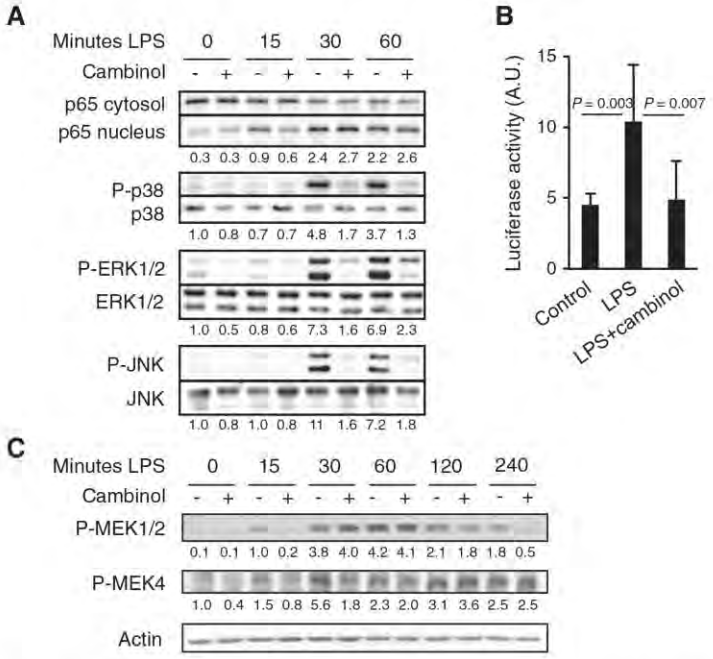


Fig. 8. Cambinol inhibits the MAPK signaling pathway. RAW 264.7 macrophages, transfected with AP-1-reporter luciferase vectors in (B), were pre-incubated for 1 h with (+) or without (−) cambinol (50 μM) and exposed to LPS (10 ng/ml). (A, C) Expression levels of nuclear and cytosolic NF-κB p65, phospho (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.

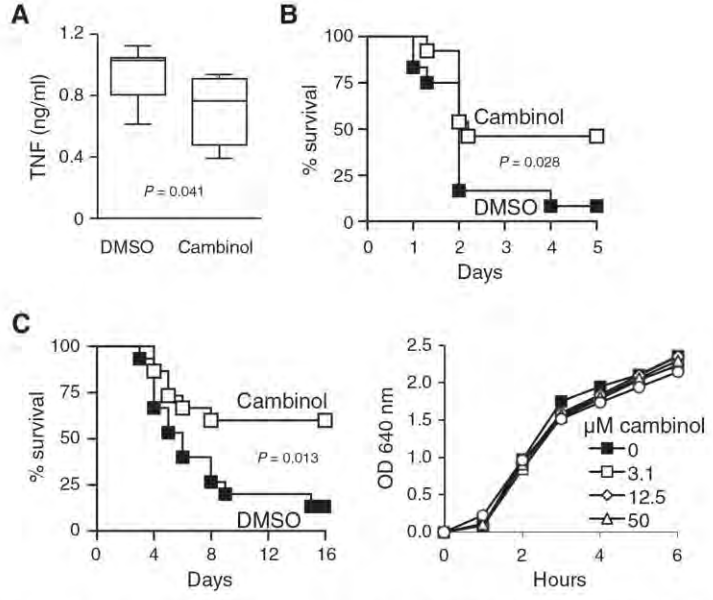


Fig. 9. Cambinol protects from endotoxemia and septic shock. (A–B) BALB/c mice were injected i.p. with LPS (17.5 mg/kg). Cambinol (10 mg/kg) and vehicle (DMSO) were administrated i.p. 1 h before and 24 h after LPS challenge. (A) TNF concentrations in blood collected 1 h after LPS challenge (n = 7–8 per 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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3.5 Screening the impact of sirtuin inhibitors on inflammatory and innate immune responses of macrophages and in a mouse model of endotoxic shock

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This review provides a general and straightforward approach to study the impact of sirtuin inhibitors on innate immune responses *in vitro* and *in vivo*. Procedures are described as laboratory protocols and several *Notes* gives tips and tricks, troubleshooting or possible substitutions.

This manuscript is based on our experience and on our developed and routinely used protocols. It incorporates some representative unpublished data showing that cambinol but not SIRT1 or SIRT2-specific inhibitors reduces cytokine release by RAW 264.7 macrophages and protects from lethal endotoxemia.

Chapter 21

Screening the Impact of Sirtuin Inhibitors on Inflammatory and Innate Immune Responses of Macrophages and in a Mouse Model of Endotoxic Shock

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Abstract

The development and screening of pharmacological modulators of histone deacetylases (HDACs), and particularly sirtuins, is a promising field for the identification of new drugs susceptible to be used for treatment strategies in a large array of welfare-associated, autoimmune and oncologic diseases. Here we describe a comprehensive protocol to evaluate the impact of sirtuin-targeting drugs on inflammatory and innate immune responses in vitro and in a preclinical mouse model of endotoxemia. We first provide an overview on strategies to design in vitro experiments, then focus on the analysis of cytokine production by primary macrophages and RAW 267.7 macrophages at the mRNA and protein levels, and finally describe the setup and follow-up of a mouse model of inflammation-driven endotoxic shock.

Key words Histone deacetylases, Sirtuins, Pharmacological inhibitors, Epigenetics, Innate immunity, Cytokine, Macrophage, Endotoxemia, Sepsis

1 Introduction

The superfamily of histone deacetylases (HDACs) comprises 18 members in mammals, 11 Zn-dependent HDACs (HDAC1-11) and 7 NAD-dependent sirtuins (SIRT1-7). The first gene member of the sirtuin family, silent information regulator-2 (*SIR2*) from *S. cerevisiae*, was described more than 35 years ago as mating-type regulator 1 (*MAR1*) [1]. Yet the role of sirtuins in physiological and pathological processes is far from being fully elucidated.

Mammalian sirtuins differ according to their sub-cellular localization, enzymatic activity and substrate specificity. SIRT1 and SIRT6 are essentially nuclear, SIRT2 cytoplasmic, SIRT3-5 mitochondrial, and SIRT7 nucleolar proteins, although the distribution of some isoforms (e.g., SIRT1, SIRT2, SIRT3, and SIRT5) is dynamic and not fully restricted to a specific subcellular compartment [2]. Sirtuins are characterized by their deacetylase activity. Similarly to other HDACs, most sirtuins remove acetyl groups from lysine residues on histones

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and non-histone proteins thereby modifying their expression or activity. Yet SIRT4 has no appreciable deacetylase activity, and SIRT6 is probably a stronger deacylase than deacetylase [3]. Both SIRT4 and SIRT6 work as ADP-ribosyltransferases [4, 5]. SIRT5 is a weak deacetylase but has efficient demalonylase, desuccinylase, and deglutarylase activities [6, 7]. Sirtuins have numerous targets such as transcription regulators, enzymes, and structural proteins, in line with the fact that more than 6800 acetylation sites have been identified in mammalian proteins [8].

Sirtuins are involved in multiple biological processes. Their dependence on NAD+ naturally links sirtuin activity to the metabolic status, and most sirtuins are connected to metabolic processes such as gluconeogenesis, fatty acid metabolism, oxidative phosphorylation, amino acid metabolism, urea cycle, and mitochondrial biogenesis. Sirtuins also control cell cycle, rDNA transcription, DNA repair, telomere homeostasis, microtubule stability, neuronal development, and circadian functions [2, 9, 10]. It is therefore not surprising that changes in expression or activity of sirtuins are associated with the development of metabolic (type 2 diabetes and obesity), cardiovascular, neurodegenerative (Alzheimer, Parkinson, and Huntington's diseases), oncologic, and other age-associated diseases [11–13]. Sirtuins have also been associated with the development of inflammatory and autoimmune diseases, but here the picture is less clear. Indeed, sirtuins have either a protective or a deletary role in experimental airway diseases, autoimmune encephalomyelitis and arthritis [14–20], possibly due to different settings and strategies used to address the role of sirtuins (inhibitory drugs, si/shRNA, germline and tissue specific knockouts).

In recent years, quite some efforts have been devoted to the discovery and design of sirtuin inhibitors. Several compounds inhibiting primarily SIRT1 and SIRT2 are promising drugs for cancer and neurodegenerative diseases such as Parkinson and Huntington's diseases [21, 22]. Although the panel of sirtuin inhibitors obtained thus far is rather restricted and mainly directed towards SIRT1 and SIRT2, there is little doubt that numerous more selective and more powerful compounds will be available in a near future. Considering that inflammation is central to the development of metabolic, oncologic, neurologic, autoimmune, and infectious diseases, sirtuin inhibitors should be systematically tested for their inflammatory activity, as we recently reported for the SIRT1/SIRT2 inhibitor cambinol [23]. Reinforcing this idea, classical HDAC share with sirtuins several targets among which some crucial regulators of inflammatory and immune responses, and inhibitors of classical HDACs have demonstrated strong anti-inflammatory and immunomodulatory properties [24–27].

Here we describe simple and comprehensive protocols to test the impact of sirtuin inhibitors on immune responses in vitro and in vivo. In vitro testing is performed using macrophages that, as a

main source of cytokines, are central initiators of inflammatory-driven pathologies. The readout is the production of proinflammatory cytokines by both primary macrophages and RAW 264.7 macrophages exposed to microbial ligands such as lipopolysaccharide (LPS, also known as endotoxin), the main proinflammatory component of the outer membrane of gram-negative bacteria [28, 29]. The relevance of in vivo results is then addressed in a mouse model of endotoxemia in which animals die from overwhelming inflammation.

2 Materials

Prepare the solutions sterile, handle material with gloves. Use only ultrapure water and analytical grade reagents. In Subheadings 2.1, 2.3, and 2.4, all products are cell culture certified or appropriately sterilized before usage. Always use polypropylene tubes for macrophage preparations in order to minimize cell adherence to the tube wall. In Subheadings 2.5 and 2.6, use only RNase free plastic disposals and solutions. Cell culture incubators are set at 37 °C with 5% CO_2, and cold means 4 °C.

2.1 Stock Solutions of Stimuli and Sirtuin Inhibitors

Stimuli and inhibitors are generally obtained as powders that are stored at +4 °C unless specified otherwise. The screening is usually performed with LPS. We propose two additional stimuli, Pam_3CSK_4 and CpG ODN, which can be used secondly to validate the results obtained with LPS. Prepare stock solutions, aliquot and store at −20 °C (*see* **Note 1**).

1. LPS (also called endotoxin, *see* **Note 2**): 1 mg/mL in phosphate-buffered saline (PBS).
2. Pam_3CSK4 (*see* **Note 3**): 1 mg/mL in PBS.
3. CpG ODN (*see* **Note 4**): 500 μM in PBS.
4. Sirtuin inhibitors: in an appropriate vehicle—water (H_2O), ethanol (EtOH), methanol (MetOH), dimethyl sulfoxide (DMSO), etc.—taking into account compound solubility and vehicle effect/toxicity (*see* **Note 5**).

2.2 Production and Culture of Bone-Marrow Derived Macrophages (BMDM)

1. Mice (*see* **Note 6**), and a carbon dioxide (CO_2) system to euthanize mice.
2. Laminar flow, water bath, refrigerated centrifuge, full ice tray, cell culture incubator, inverted microscope, material for cell counting.
3. Scissors and forceps, pipetboy, micropipettes.
4. Sterile bacterial petri dishes (100 and 150 mm, *see* **Note 7**), 50 mL conical tubes, 20 mL syringes and 25 G needles, pipettes (5 and 10 mL), cell strainers (nylon, 100 μm), cell lifters (*see* **Note 8**), cryotubes.

5. PBS, 70% EtOH, 1× Versene solution (0.48 mM ethylenediaminetetraacetic acid tetrasodium salt (EDTA, Na_4) in PBS).
6. Red blood cells (RBC) lysis solution: 0.65 M ammonium chloride (NH_4Cl), 10 mM potassium bicarbonate ($KHCO_3$), 0.1 mM EDTA in H_2O. Sterilize by filtration (0.22 µm). Store at 4 °C.
7. Basic IMDM: IMDM (Iscove's Modified Dulbecco's Medium) containing GlutaMAX™ and 4.5 g/L glucose.
8. Complete IMDM: basic IMDM supplemented with 10% heat inactivated (*see* **Note 9**) low endotoxin (*see* **Note 10**) fetal calf serum (FCS), 100 UI/mL penicillin, 100 µg/mL streptomycin, and 50 µM 2-mercaptoethanol.
9. Differentiation IMDM: complete IMDM supplemented with 30% of L929 cell-conditioned medium (*see* **Note 11**).

2.3 Culture of RAW 264.7 Mouse Macrophages

1. Stock ampoule of RAW 264.7 macrophages (ATCC® TIB-71™).
2. Laminar flow, water bath, centrifuge, cell culture incubator, inverted microscope, material for cell counting.
3. Pipettes (5 and 10 mL), pipetboy, 30 mL sterile conical tubes, 100 mm cell culture petri dishes, cell lifter (*see* **Note 8**).
4. Complete RPMI medium: RPMI (Roswell Park Memorial Institute) medium containing GlutaMAX™ and 4.5 g/L glucose supplemented with 10% heat inactivated (*see* **Note 9**) low endotoxin FCS (*see* **Note 10**), 100 UI/mL penicillin, 100 µg/mL streptomycin.

2.4 Testing Inhibitors on Cultures of BMDM and RAW 264.7 Macrophages

1. Laminar flow, water bath, refrigerated centrifuge, full ice tray, vortex mixer, cell culture incubator.
2. Pipettes, single channel and multichannel micropipettes, filter tips, tubes, polystyrene flat bottom 96-well cell culture plates.
3. Complete IMDM or RPMI medium, PBS, stimuli, and drugs described in Subheading 2.1.

2.5 RNA Purification and cDNA Synthesis

1. RNA isolation kit (preferably using a column-based procedure).
2. Pipettes, single channel and multichannel micropipettes, filter tips, vortex mixer, refrigerated microcentrifuge, nucleic acid quantification apparatus, full ice tray, flat bottom 96-well cell culture plates, polypropylene tubes.
3. EtOH (70 and 100%), 3 M sodium acetate (NaOAC) pH 5.2, 5 mg/mL glycogen in H_2O.
4. Reverse transcription kit.

2.6 Cytokine Measurement by RT-PCR

1. Pipettes and filter tips, tubes, micropipettes, vortex mixer, refrigerated microcentrifuge, benchtop cooler, thermoblock or water bath at 42 and 95 °C, 96-well plate spinner, full ice tray.

2. Forward and reverse primers (stock solution at 100 μM).
3. DNase free H_2O, Fast SYBR® Green Master Mix.
4. MicroAmp® Fast Optical 96-Well Reaction Plate and Optical Adhesive Film.
5. Real-time PCR apparatus.

2.7 Cytokine Measurement by ELISA

1. ELISA kits for the detection of TNF, IL-6 or any other cytokine of interest. Here we will use DuoSet® kits consisting of capture antibody, biotinylated detection antibody, and streptavidin–horseradish peroxidase (HRP).
2. Multichannel micropipettes and reservoirs, flat-bottom 96-well plate, plate sealers, PBS.
3. Wash buffer: 0.05% Tween® 20 in PBS, pH 7.2–7.4.
4. Reagent diluent: 1% BSA (bovine serum albumin) in PBS, pH 7.2–7.4, 0.22 μm filtered.
5. TMB substrate (*see* **Note 12**).
6. Stop solution: 2 N sulfuric acid (H_2SO_4).

2.8 Cell Viability Assay Using MTT

1. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) powder.
2. Multichannel micropipettes and reservoirs, balance.
3. Lysis buffer (for one plate): 10 mL 2-propanol, 5 mL 20% SDS (sodium dodecyl sulfate), 80 μL 5 M chlorhydric acid (HCl).
4. Ninety-six well plate and luminescence plate reader.

2.9 Mouse Model of Endotoxemia

1. Eight 12-weeks old female BALB/c mice. The number of animals will depend on experimental design and experiments should be planned based on ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and respecting the 3R principles (Reduce, Refine, and Replace) (*see* Subheading 3.9 and **Note 13**.) [30].
2. 1 mL sterile syringes, 25 G needles.
3. Drug and vehicle at an equivalent concentration (for example 0.8 mg/mL cambinol and 8% DMSO in 0.9% sodium chloride (NaCl)).
4. LPS: 1.4 mg/mL in 0.9% NaCl (*see* **Note 14**).
5. Infrared lamp, scalpels, hemostatic gauze, lithium heparin (for ELISA measurements) or potassium EDTA (for multiplex assay measurement by Luminex) coated Microvette® for capillary blood collection, microcentrifuge, mice digital weighing scale, non-wetting sterile water gel for animal hydration such as HydroGel®.

3 Methods

3.1 Preparation of BMDMs

If possible, use separate laminar flows dedicated to organ collection and cell culture.

1. Day 0: put under the laminar flow an aluminum foil, scissors, and forceps and prepare three petri dishes: A and C containing 15 mL of PBS and B containing 15 mL of 70% EtOH.
2. Sacrifice the mouse with CO_2 (*see* **Note 13**).
3. Collect the hind femurs and tibias. Place the mouse on the aluminum foil and spray it with 70% EtOH. Cut with scissors the skin just above the foot and pull it over the hip. Cut the legs above the hip articulation (*see* **Note 15**). Remove the remaining skin from the feet. Cut ankle tendons and destroy the ankle articulation by gently twisting and pulling the feet. Remove the feet with scissors. Cut knee tendons and loosen the articulation by gently twisting femur and tibias. Remove as much as possible flesh from the bones, with the help of scissors and forceps.
4. Soak briefly the cleaned bones in petri dish A, petri dish B, and petri dish C.
5. Cut bone epiphyses and immediately flush the bone marrow (BM): firmly holding the bone with forceps above a 50 mL tube, introduce into the opening of the bone a 25 G needle adapted to a 20 mL syringe filled with 20 mL of basic IMDM, a volume sufficient to flush both the tibia and the femur from one leg. Flush 10 mL/per bone, moving the needle up and down into the cavity of the bone. The bone becomes progressively white as the BM is expelled.
6. Homogenize the cell suspension by pipetting up and down with a 10 mL pipette.
7. Centrifuge for 7 min at 400 × *g* and 4 °C. Discard supernatant (SN). Resuspend the pellet in 3 mL of cold RBC lysis buffer. Incubate for 5 min on ice. Add 40 mL of cold PBS.
8. Filter the suspension through a 100 μm nylon cell strainer into a new tube.
9. Centrifuge for 7 min at 400 × *g* and 4 °C. Discard SN. Resuspend the pellet in 10 mL of complete IMDM and enumerate BM cells (*see* **Note 16**).
10. Plate 3×10^6 BM cells in a 100 mm bacterial petri dish in a volume of 15 mL of differentiation IMDM (to obtain $6–9 \times 10^6$ BMDM after 7 days of culture, *see* **Notes 17** and **18**).
11. Put the petri dish in a cell culture incubator.

12. Freeze BM cells in excess in complete IMDM containing 10% DMSO (*see* **Note 19**).
13. Day 2 and day 4: examine the culture using an inverted microscope. Add 5 mL of preheated differentiation IMDM in the petri dish (*see* **Note 20**).
14. Day 7: examine the culture using an inverted microscope to check for cell growth (the confluency of adhering macrophages should be around 100%) and contaminants. Under the laminar flow, aspirate the SN, wash the cell layer with 10 mL of cold PBS and add 3 mL of cold Versene (*see* **Note 21**).
15. Using a cell lifter strongly pressed onto the surface of the petri dish, detach the cells by performing a round movement to detach cells on the edges, and then linear one-way movements to lift cells on the remaining surface. If this step is performed properly, maximal cell recovery with minimal cell death is obtained.
16. Homogenize the cell suspension with a 5 mL pipette and transfer to a 50 mL tube.
17. Add cold PBS to 40 mL. Centrifuge for 7 min at 400 × *g* and 4 °C. Discard the SN. Resuspend the pellet in 10 mL of complete IMDM and enumerate BMDM.
18. Dilute cell suspension to a concentration of 2×10^6 cells/mL and proceed according to Subheading 3.3.

3.2 Culture of RAW 264.7 Macrophages

1. Thaw quickly (in a water bath at 37 °C) a cryotube of frozen RAW 264.7 macrophages.
2. Pipet the cell suspension in a conical tube containing 9 mL of complete RPMI.
3. Centrifuge for 7 min at 300 × *g* at room temperature. Discard the SN. Resuspend the pellet in 10 mL of complete RPMI. Enumerate cells. Transfer 1×10^6 living cells in a 100 mm cell culture petri dish. Add complete RPMI to reach 10 mL. Incubate in a cell culture incubator.
4. Detach cells with a cell lifter when reaching 70–80% confluence (*see* **Note 22**).
5. Homogenize the cell suspension by pipetting up and down with a 10 mL pipette. Transfer to a tube. Centrifuge for 7 min at 300 × *g*. Discard the SN. Resuspend the pellet in 10 mL of complete RPMI. Enumerate cells. Transfer 1×10^6 or 2.5×10^6 cells to a 100 mm or 150 mm cell culture petri dish, respectively. Add pre-warmed complete RPMI up to 10 or 20 mL.
6. Split cells as indicated in **steps 4** and **5** every 2–4 days. Increase the number of plates to reach the number of cells required in Subheading 3.3.

3.3 Testing Inhibitors on BMDM and RAW 264.7 Macrophages

Prepare a scheme of your experimental plate as exemplified in Fig. 1a (*see* **Note 23**). Don't forget negative controls (*see* **Note 24**). First screening is performed with cells stimulated with 10 ng/mL LPS. Drug effects on cytokine production can be confirmed using cells stimulated with 10 ng/mL Pam_3CSK_4 or 1 μM CpG ODN. Test each condition at least in triplicate. We prefer to seed and treat cells all at once, and then collect samples sequentially according to the incubation time. A first screening can be performed with RAW 264.7 macrophages, which are easier to obtain than BMDMs, but with the possibility that drugs are more toxic for established cell

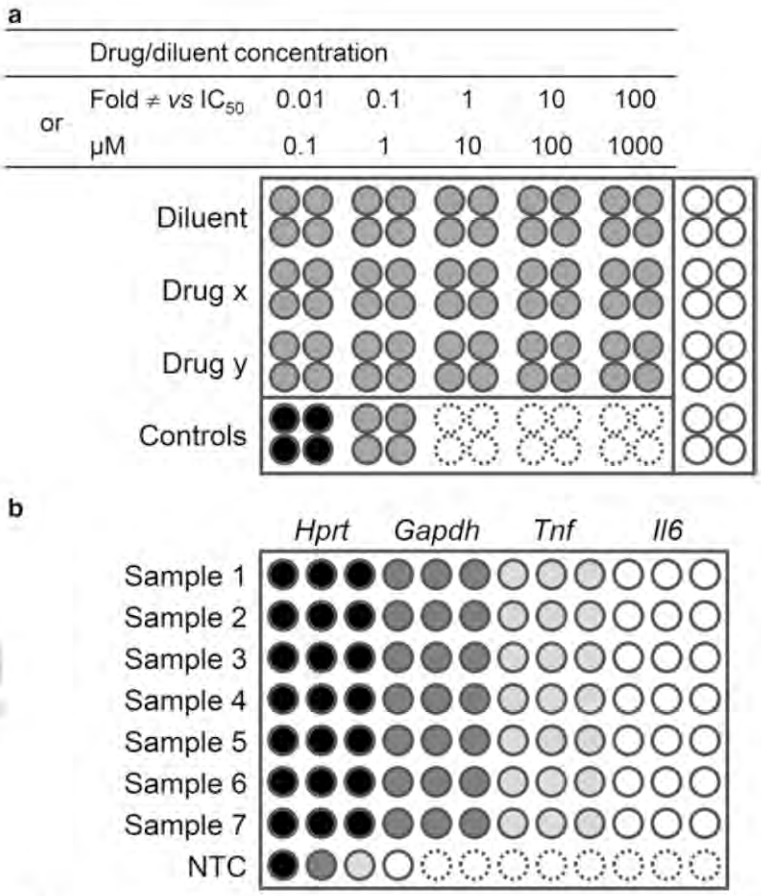

Fig. 1 Example of cell culture and RT-PCR 96-well plate schemes for testing sirtuin inhibitors on cytokine production by macrophages (**a**) Cell culture plate scheme. Cells are seeded in the *grey* or *black* wells. Cells are preincubated with diluent and drug at final concentrations corresponding to 100×, 10×, 1×, 0.1×, and 0.01× of the lowest IC50 or in range from 1 mM to 100 nM as indicated, and then stimulated with (*grey*) or without (*black*) LPS. Each condition is analyzed in quadruplicates. The two right columns are left empty to leave room for standard and blank in ELISA or Luminex assays. (**b**) RT-PCR plate scheme. Samples are distributed horizontally, gene specific mix vertically according to fill color. Each sample is measured in triplicates. NTC: No template control. The *dotted wells* are not used

lines than for primary cells. It is therefore highly recommended to screen the drugs on primary cells, either first-line or second-line, before testing the best-of-class candidate(s) in preclinical models.

1. Prepare suspensions of BMDMs or RAW 264.7 macrophages at 2×10^6 cells/mL in complete IMDM or RPMI medium. Seed 100 µL (2×10^5 cells) per well in 96-well cell culture plates (*see* **Notes 25** and **26**). Incubate in a cell culture incubator while performing **step 2**.
2. Prepare 2× concentrated working solutions of drugs since drugs will be diluted 2× when added to cell cultures. A selection of commercially available sirtuin inhibitors is presented in Table 1. When a drug is tested for the first time, a broad range of final concentrations corresponding to 100×, 10×, 1×, 0.1×, and 0.01× of the lowest IC_{50} for any sirtuin is recommended. If the IC_{50} are unknown, final concentrations should range from 1 mM to 100 nM. Less broad ranging with more frequent concentrations are tested in subsequent experiments to refine results.
3. Add 100 µL of 2× concentrated working solutions of drug to the cells according to your experimental scheme.
4. Incubate for 1 h (*see* **Note 27**).
5. During incubation, prepare 11× concentrated (110 ng/mL) working solution of LPS (*see* **Note 28**).
6. Add 20 µL of LPS working solution to the cells according to your experimental scheme.
7. Incubate cells for 1, 4, and 8 h to measure cytokine mRNA, for 8 and 18 h to measure cytokine secretion (*see* **Notes 29** and **30**), and for 18 h to assess cell viability (*see* **Note 31**).

3.4 RNA Extraction

The RNA extraction protocol is performed using a column-based method. It is adapted to the experimental scheme of Fig. 1a where four replicates are combined. The procedure is run at room temperature. Tubes are kept on ice as soon as RNA is collected.

1. With a 12-channel multipipette, transfer in a new plate 150–200 µL of SN from the plates stimulated for 8 h if you want to quantify cytokines (*see* **Note 30**). Sore at −20 °C for cytokine measurement by ELISA or Luminex. Discard the remaining liquid, and the SN from the plates stimulated for 1 h and 4 h.
2. Add 100 µL of lysis buffer to each well containing cells. Pipet up and down to homogenize. Pool the four replicates in a single pre-labeled 1.5 mL eppendorf tube.
3. Purify RNA following manufacturer's recommendations (*see* **Note 32**). Eluate RNA in 100 µL RNase-free water.
4. Place tubes containing the RNA eluate on ice. The sample can be used for RNA precipitation or stored at −80 °C.

Table 1
Selection of commercially available sirtuin inhibitors with IC_{50}

Inhibitor	IC_{50} (μM)						
	SIRT1	SIRT2	SIRT3	SIRT4	SIRT5	SIRT6	SIRT7
AGK2	>30	3.5	>50	NT	NT	NT	NT
AK-7	>50	15.5–24	>50	NT	NT	NT	NT
Cambinol	56	59	NI	NT	42% inhibition at 300 μM	NI up to 250 μM	NT
CHIC-35	0.06–0.12	2.8	NI	?	?	?	?
EX-527	0.098–1	20–33	49	?	NI	56% inhibition at 200 μM	?
Ginkgolic acids	119–126	80–141	NT	NT	NT	NT	NT
Inauhzin	0.7–2	NI	NI	?	?	?	?
Nicotinamide	50–100	1.2–100	30–43	?	1600	2200	?
Sirtinol	37.6–131	38–58	>50	?	?	No inhibition up to 200 μM	?
Salermide	76	25–45	>50	?	?	?	?
Splitomicin HR73	5	NI	NI	?	?	?	?
Suramin	0.297	1.15	NI	?	22	No inhibition up to 200 μM	?
Tenovin-1	70–90% inhibition in Jurkat and MyLa cells at 25 μM	10	?	?	?	?	?
Tenovin-6	21	10	67	?	?	?	?
Urushiols	52–118	55–143	NT	NT	NT	NT	NT

NI No inhibition observed
NT Not tested
? No report (likely not tested)

3.5 RNA Precipitation and cDNA Synthesis

1. Prepare a mix containing, per sample to precipitate, 10 µL of NaOAC, 2 µL of glycogen, and 275 µL of cold 100% EtOH (*see* **Note 33**). Distribute 287 µL of the mix to each sample. Mix well by inverting the tubes. Incubate overnight at −20 °C (*see* **Note 34**).
2. Centrifuge for 15 min at 1300×*g* and 4 °C.
3. Remove the SN without touching the pellet. Add 0.5 mL of cold 70% EtOH. Centrifuge for 5 min at 1300×*g* and 4 °C.
4. Repeat **step 3**.
5. Remove the SN and let dry the pellet at room temperature (*see* **Note 35**).
6. Add 30 µL of RNase-free water without touching the pellet. Leave on ice for 15 min. Vortex for 2 s to dissolve the pellet and quick spin to collect the liquid at the bottom of the tube.
7. Measure yield and check quality of the RNA preparation (*see* **Note 36**).
8. Pipet 500 ng of RNA from each sample and perform reverse transcription as recommended by the manufacturer (*see* **Notes 37–39**).
9. Dilute the cDNA samples 1:5 in RNase-free water (*see* **Note 40**). Store at −20 °C until use.

3.6 Real-Time PCR (RT-PCR)

The RT-PCR is performed in 96-well plates using the Fast SYBR® Green Master Mix and FAST 7500 apparatus and analyzed using the comparative method (ΔΔCT) (*see* **Note 41**). A triplicate measure of each sample is recommended.

1. Prepare a scheme of your RT-PCR plate (a simple example is given in Fig. 1b). Indicate sample position and gene names. Do not omit "no template controls" (NTC) (*see* **Note 42**).
2. Prepare 10 µM primer working solutions by mixing in a tube 10 µL of each of the forward and reverse primer stock solutions with 80 µL of DNAse free water. A selection of validated primers for housekeeping and cytokine genes is listed in Table 2.
3. Amplification mix (per sample to analyze): 4.5 µL of H_2O, 0.5 µL of primer working solution, and 7.5 µL of Fast SYBR® Green Master Mix (*see* **Notes 33** and **43**). Vortex for 2 s. Short spin. Keep on ice protected from direct light. Repeat for each gene of interest.
4. Pipet 2.5 µL of cDNA into the bottom of the wells (*see* **Note 44**).
5. Add 12.5 µL of the amplification mix on the sidewall. Seal the plate carefully. Spin down.
6. Run the PCR in the FAST 7500 apparatus: initial step of 20 s at 95 °C and 40 amplification cycles with 3 s at 95 °C and 30 s at 60 °C. Analyze data using the FAST 7500 software v2.0.6.

t2.1 **Table 2**
t2.2 **Primers used to amplify cytokine and housekeeping genes by RT-PCR**

t2.3 Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
t2.4 *Gapdh*	CTC ATG ACC ACA GTC CAT GC	CAC ATT GGG GGT AGG AAC AC
t2.5 *Hprt*	GTT GGA TAC AGG CCA GAC TTT GTT G	GAT TCA ACT TGC GCT CAT CTT AGG C
t2.6 *Il6*	CCG GAG AGG AGA CTT CAC AG	CAG AAT TGC CAT TGC ACA AC
t2.7 *Il12b*	GGA AGC ACG GCA GCA GAA TA	AAC TTG AGG GAG AAG TAG GAA TGG
t2.8 *Tnf*	CCA GGC GGT GCC TAT GTC	GGC CAT TTG GGA ACT TCT CAT

3.7 ELISA

Cytokines can be quantified by ELISA or with the Luminex technology using pre-custom or personalized assays like ProcartaPlex™ Multiplex Immunoassays, Bio-Plex Pro™ Mouse Cytokine Assays or Luminex Screening Assays and Luminex Performance Assays. We do not describe Luminex-based methodology because it necessitates specific apparatus. The detection of mouse TNF and IL-6 by ELISA reported here uses DuoSet® ELISA kits but can be achieved with any ELISA kits of choice. Each brand has technical specificities thoroughly detailed in protocol handbooks. Dilutions of reagents are given by the manufacturer. All volumes are given per well.

1. Prepare a scheme of your ELISA plate with the positions of blank, standard, and samples.
2. Coat 96-well MaxiSorp™ plate(s) with 100 µL of capture antibody. Seal the plate and incubate overnight at room temperature.
3. Empty wells. Wash three times with 350 µL of wash buffer (*see* **Note 45**).
4. Add 300 µL of reagent diluent. Incubate at room temperature for 1 h.
5. During the 1 h incubation, dilute samples (*see* **Note 46**) and prepare a standard curve (starting from 1 or 2 ng/mL) made of seven twofold serial dilutions in reagent diluent.
6. Wash the plate three times with 350 µL of wash buffer. Add 100 µL of reagent diluent alone (blank), standard, or sample to the appropriate wells. Cover with an adhesive strip. Incubate for 2 h at room temperature. Wash three times with 350 µL of wash buffer.
7. Add 100 µL of biotinylated detection antibody. Cover with an adhesive strip. Incubate for 2 h at room temperature. Wash three times with 350 µL of wash buffer.
8. Add 100 µL of streptavidin–HRP. Cover with an adhesive strip. Incubate for 20 min at room temperature protected from light. Wash three times with 350 µL of wash buffer.

9. Add 100 μL of TMB substrate solution. Incubate for around 20 min at room temperature avoiding exposure to direct light (*see* **Note 47**).
10. When the color has developed sufficiently, add 50 μL of stop solution. Shake the plate. Measure the ODs using a micro-plate reader set at 450 nm with wavelength correction set at 570 nm.
11. Analyze data using the SoftMax® Pro or equivalent software to generate a four parameter logistic (4-PL) curve-fit, calculate cytokine concentrations and plot data in a graph (an example is given in Fig. 2).

3.8 Cell Viability Assay Using MTT

The MTT assay is used to assess cell viability (*see* **Note 48**). It can be performed with dedicated cell culture plates or with cell culture plates from which the SN has been collected to measure cytokine release (*see* Subheading 3.3 and **Note 31**). To have a more accurate quantification of drug effect on cell viability, a standard curve can be established using values obtained from wells seeded with 0.5, 1,

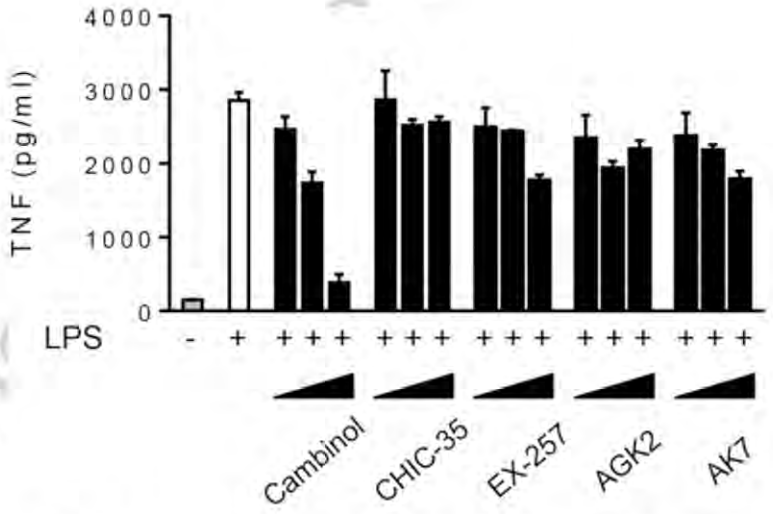

Fig. 2 Impact of sirtuin inhibitors on TNF production by RAW 264.7 macrophages. RAW 264.7 macrophages were preincubated for 1 h with or without cambinol (15, 60, and 240 μM), CHIC-35 (30, 120, and 480 nM), EX-527 (0.25, 1, and 4 μM), AGK2 (0.8, 3.5, and 14 μM), and AK-7 (6, 24, and 96 μM) before exposure for 8 h to LPS (10 ng/mL). Concentrations of TNF in cell culture SN were quantified by ELISA. Data are means ± SD of triplicate samples from one experiment representative of two experiments. The results show that the SIRT1/SIRT2 inhibitor cambinol powerfully inhibits TNF production in a dose-dependent manner whereas SIRT1 and SIRT2 selective inhibitors are much less potent. Note that drug dilutions used here are (0.25×, 1×, and 4× the IC_{50} for SIRT1 or SIRT2, *see* Table 1) were optimized based on pretests performed with a broader panel of dilutions as described in Fig. 1

2, 4, and 8×10^5 cells in complete medium. These cells are used only for the MTT assay (SN is not collected) and are seeded in wells "preserved" for establishing the ELISA standard (*see* **Note 23**). Quantities mentioned below are for one full 96-well plate. Scale up as necessary.

1. Weight 10 mg MTT in a 15 mL tube. Add 5 mL of PBS. Vortex and sonicate until full dissolution. Distribute 50 µL to each well. Incubate the plate for 2 h in a cell culture incubator. During the last 30 min, prepare 15 mL of cell lysis buffer.
2. Empty the plate by inversion in one single movement over a waste container. Add 150 µL of cell lysis buffer.
3. Shake the plate on an orbital shaker until precipitates are dissolved.
4. Measure OD_{570} (quantification) and OD_{690} (correction) of the samples. Report to non-treated controls representing 100% viability, or to a standard curve established with serial quantities of cells. Lower the intensity of the signal, higher is the toxicity.

3.9 Mouse Model of Endotoxemia

The preclinical model of endotoxemia is a well accepted and easy to manage model to test the impact of drugs on inflammation-driven mortality (*see* **Notes 13** and **49**). It has to be setup in a preliminary experiment to define the lethal doses 20 and 90 (LD_{20}, LD_{90}) of LPS as they vary according to the batch of LPS and mouse strain used. If the results obtained in vitro indicate that the drug of interest decreases inflammation, then animals should be challenged with a LD_{90} of LPS to demonstrate drug-mediated protection from endotoxemia. On the contrary, if the drug increases inflammation in vitro, then animals should be challenged with a LD_{20} of LPS to demonstrate drug-mediated increased morbidity/mortality. At least two groups of mice are required to test a drug: a control group injected with vehicle and an experimental group injected with the drug of interest. Power calculation should be performed to define the minimal number of animals to be used to obtain statistically significant results, according to ARRIVE guidelines [30]. Different drug concentrations or treatment schedules may also be considered, respecting the 3R principles. Results from a typical experiment showing the protective effects of cambinol are shown in Fig. 3.

1. Label mice from each cage (*see* **Note 50**). Add food and HydroGel® on the bed layer.
2. Fill 25 G needle-mounted syringes with 250 µL of vehicle or drug (*see* **Note 51**). Inject intraperitoneally into mice. Treatment may be repeated at the time of, or post-LPS challenge.
3. Fill 25 G needle-mounted syringes with 250 µL of LPS. Inject intraperitoneally 1 h after vehicle or drug challenge (*see* **Note 13**).

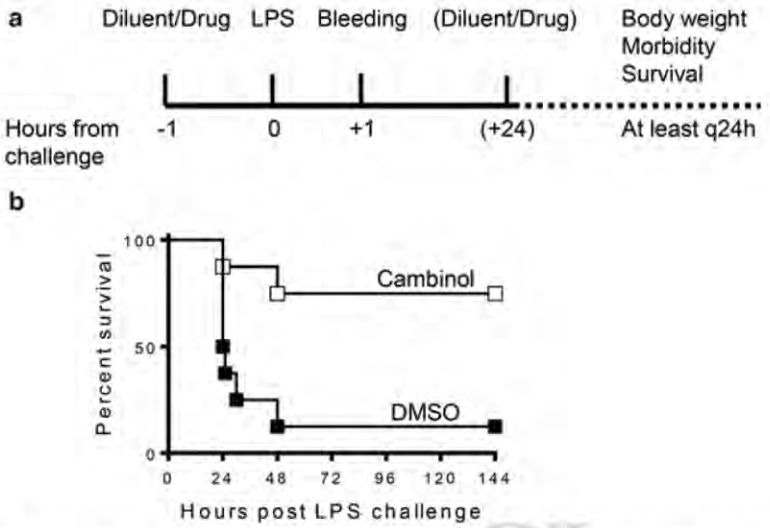

Fig. 3 Cambinol protects mice from endotoxic shock. (**a**) Flowchart of the endotoxemia model. Animals are checked for morbidity and mortality at least twice a day and for body weight once a day up to 6 days. (**b**) BALB/c mice ($n = 8$ per group) were injected i.p. with 17.5 mg/Kg LPS. Cambinol (10 mg/Kg) and an equivalent amount of vehicle (DMSO) were administrated i.p. 1 h before and 24 h after LPS challenge. Survival was recorded regularly to build Kaplan–Meier plots. Differences were analyzed by the log-rank sum test ($P = 0.0085$). The results show that cambinol protects from endotoxemia

4. Bleed the mice exactly 1 h after LPS challenge (*see* **Note 52**). Warm mice a few minutes with an infrared lamp. Make with a scalpel a small incision on the tail vein (*see* **Note 53**). Collect five drops (~100 µL) of blood in a 300 µL microvette. Press the tail with the haemostatic gauze to stop bleeding. Mix the tube by inversions. Keep on ice. Back to the lab, centrifuge for 10 min at 2000 × *g*. Transfer the plasma in a new tube. Store at −20 °C until use.
5. Record weight, severity scores and survival at least once daily, preferably every 8–12 h (*see* **Note 54**).

4 Notes

1. Aliquots of inhibitors and stimuli should not be too small in order to avoid evaporation. This is particularly important if long term storage is considered. Use tubes that close tightly.
2. LPS (lipopolysaccharide, also known as endotoxin) is a major component of the outer membrane of gram-negative bacteria and one of the most powerful inflammatory stimuli [28]. LPS from *Escherichia coli* or *Salmonella minnesota* can be used indifferently. Better use ultra-pure LPS than standard LPS

well-known to contain contaminant bacterial components. Sonication of the LPS solution allows a better dissolution and exposure of the immunostimulatory acyl groups of the molecules.

3. Pam_3CSK_4 (*N*-Palmitoyl-S-[2,3-*bis*(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine) is a synthetic tripalmitoylated lipopeptide that mimics the acylated amino terminus of bacterial lipoproteins.
4. CpG ODN (oligodeoxynucleotides) are short single-stranded synthetic phosphorothioated oligonucleotides that contain unmethylated CpG dinucleotides. CpG ODN mimic microbial DNA. Different classes (A, B, and C) of CpG ODN are commercially available. Class B mouse specific CpG ODN 1826 is a better stimulus of mouse macrophages.
5. Drug stock solutions should be in the mM range (1–100 mM) as far as possible. When drugs are tested in cell culture, vehicles are diluted enough to avoid side effects, especially toxicity. In general, we try to reach a final concentration of diluents that is $\leq 0.1\%$.
6. Any immunocompetent mouse strain (such as BALB/c and C57BL/6) mice can be used. Older (12–20 weeks), male, mice give generally a better yield.
7. The usage of bacterial instead of cell culture petri dishes is mandatory in order to reduce cell adherence and facilitate cell recovery.
8. Prefer a soft polyethylene cell lifter to prevent cell disruption.
9. The activity of the complement system, that affects cell growth and immune responses, is destroyed by incubation for 30 min at 56 °C. Immerse a bottle of room temperature-equilibrated FCS into a water bath pre-warmed at 56 °C. Swirl the bottle every 10 min. The incubation period has to be adapted (around 1 h) to effectively reach 56 °C for 30 min inside the bottle. Immerse the bottle in ice. Aliquot and store the FCS at −20 °C.
10. Using low endotoxin or endotoxin free reagents is mandatory since minute amounts of endotoxin stimulate macrophages and introduce a bias in the results.
11. L929 cells produce macrophage-colony stimulating factor (M-CSF) required for stimulating the differentiation and growth of BMDM. To produce L929 cell-conditioned medium, seed 3×10^6 L929 cells in 150 cm^2 flasks with 100 mL of Dulbecco's Modified Eagle Medium (DMEM) containing GlutaMAX and 4.5 g/L glucose and supplemented with 10% heat inactivated low endotoxin FCS, 100 UI/mL of penicillin and 100 µg/mL of streptomycin. Incubate cells in a cell culture incubator for 1–2 weeks, in any case until cells start to die and the cell culture medium turns yellow. Collect the supernatant,

centrifuge for 10 min at 1800×*g*, filter (0.22 μM), aliquot in 50 mL polypropylene tubes and stock at −20 °C. Once an aliquot is thawed, keep it at +4 °C for a maximum of 7 days. It is recommended to prepare a large stock of conditioned medium by multiplying the number of flasks seeded in parallel with L929 cells as each stock of cell-conditioned medium has to be checked for growth factor properties. If low numbers of BMBM are recovered, the stock has to be discarded and a new batch performed (probably with longer cell incubation period).

12. TMB substrate solution is a 1:1 mixture of hydrogen peroxide (H_2O_2) and 3,3′,5,5′-tetramethylbenzidine. The two reagents are stored separately and are mixed just before usage. Many ready-to-use solutions are commercially available.
13. Only accredited experimenters can perform experimentation on living animals. Animal experiments have to be approved by the relevant office and performed according to institution guidelines. Other methods of euthanasia are possible.
14. The concentration of the working solution of LPS should be adjusted according to the activity of your batch of LPS and the mouse strain used. In our hands, LD_{20} and LD_{90} are around 5 and 17.5 mg/Kg in BALB/c mice, and 10 and 25 mg/Kg in C57BL/6 mice, respectively. *See* also Subheading 3.9.
15. It is important to cut the leg above the hip articulation to avoid rupture of femur.
16. The number of BM cells varies according to the strain, age, and sex of mice. Around 25–100 millions BM cells are obtained from the two tibias and two femurs of one mouse.
17. If a larger number of BMDMs is needed, seed several 100 mm bacterial petri dishes or seed $1–1.4 \times 10^7$ BM cells in 150 mm petri dishes with 30 mL of differentiation IMDM in order to obtain $3–9 \times 10^7$ BMDM at day 7.
18. Daily gentle shaking of petri dishes favors homogeneous distribution of growing cells.
19. Prepare cryotubes containing $1–1.5 \times 10^7$ BM cells. Store in liquid nitrogen. Cells can be thawed to start a new differentiation process.
20. If more practical, differentiation IMDM can be added at day 3 and 5. A better yield is obtained when medium is added three times at day 1, 3, and 5. If cells are seeded in a 150 mm petri dish, 10 mL of differentiation medium are added each feeding.
21. If working with 150 mm petri dishes, use 5 mL of Versene.
22. We suggest splitting cells every 2–4 days according to the time needed to reach 70–80% confluence, which depends on the batch of cells and the quality of FCS. To secure the culture, two plates with different cell numbers can be seeded.

When you have a good idea of cell proliferation rate, splitting can be performed based on dilution factors such 1/4-or 1/10 without counting the cells. Cells are maintained in culture for 5–8 weeks, after what restart from an aliquot of frozen RAW 264.7 macrophages. Restart culture also if cells have overgrown, detached from the plastic surface or changed of shape. Record the number of passages.

23. To facilitate cytokine quantification by ELISA, leave 16 empty wells in your plate. Indeed, the ELISA method described in this chapter requires a standard curve made of eight points (including the blank) performed in duplicate.
24. Perform negative controls: (a) cells without treatment, and (b) cells exposed to vehicle, as vehicle may be cytotoxic. Treatment of cells with inhibitor only may also be tested.
25. The usage of a repetitive pipette is suggested to quicken and standardize the seeding process.
26. To save time the day of cell stimulation, it is possible to recover and plate BMDM in differentiation medium at the end of day 6. Day 7, substitute the medium with 100 µL of fresh, 37 °C pre-warmed complete IMDM.
27. In our experience, 1 h of preincubation with inhibitors prior to cell stimulation is sufficient to reveal an effect on cytokine production [23]. Yet when testing a drug for the first time, different (pre)incubation schedules are recommended (for example −4, −1, 0 h).
28. Stimuli should be 11× concentrated since 20 µL of stimuli are added on the top of 200 µL (100 µL of cells plus 100 µL of drug or vehicle). In the case of Pam_3CSK_4 and CpG ODN, 11× concentrated working solution are 110 ng/mL or 11 µM, respectively.
29. These time points are classically used to analyze cytokine gene and protein expression [26, 31]. Yet it might be worthwhile to perform a more complete pilot kinetic experiment at first test.
30. BMDMs and RAW 264.7 macrophages adhere firmly to cell culture treated plastic. After treatment, some cells may become loosely adherent or detach. Centrifugation of the plate for 7 min at 400×*g* prior to collecting SN is recommended.
31. The same plate incubated for 18 h can be used to collect SN and quantify cytokine release and assess cell viability using MTT. In that case, collect 100 µL SN and proceed with the cell viability assay (*see* Subheading 3.8).
32. If the volume of lysate/mixture is larger than the column capacity, deposit one part on the column, centrifuge the column, discard the flow-through, and refill the column with the remaining mixture.

33. For all solutions, always prepare at least 10% more volume than what needed.
34. Precipitation of RNA can be also performed at −20 °C for 1 h or at −80 °C for 30 min.
35. RNA pellets can be quick dried in a vacuum concentrator, but in this case complete dissolution in water will take more time.
36. The ratio of absorbance 260/280 nm and 260/230 nm should be 1.8–2.0 and 2.0–2.2. If lower values are measured, contaminants (protein, phenol, EDTA, carbohydrates, …) are present in your preparation and the precipitation protocol should be repeated.
37. If the volume of RNA to reach 500 ng is incompatible with kit guidelines, reduce the quantity of RNA up to 100 ng. In this case use undiluted sample for RT-PCR.
38. cDNA synthesis kits usually include a step to destroy DNA contaminants before cDNA synthesis. Some RNA isolation kits also provide material to destroy DNA contaminants.
39. The reverse transcriptase is usually active at 42 °C and inactivated at 94 °C.
40. The 1:5 dilution is optimized for cytokine gene measurement in macrophages, but should be adapted according to the expression level of the gene of interest.
41. SYBR Green is a cyanine dye that binds double-stranded DNA molecules. During the annealing step of each cycle, the SYBR Green emission signal is registered and allows quantification of DNA.
42. The NTC should be prepared for each gene measured in the assay and consist of the mix prepared in **step 3** without DNA. The NTC should not emit any signal. Otherwise, contamination should be considered and the measure repeated with new reagents.
43. The RT-PCR protocol described here uses 96-well plates. It can be convenient to use the 384-well format if large series are to be tested. In this case, the volumes of reagents need to be adjusted. For example, we use 1.25 µL of cDNA to which we add a mix composed of 1.25 µL of H_2O, 0.625 µL of primers, and 3.125 µL of Fast SYBR® Green Master Mix and run reactions using a QuantStudio™ 12 K Flex system. The usage of electronic pipettes is highly recommended for filling 384-well plates.
44. The amplification mix can be distributed in the plate before step 4. The proposed flow allows checking where samples have been filed by watching at the plate from below, which is reassuring particularly for beginners.

45. Wash by filling each well with 350 µL of wash buffer. If available, use an autowasher. After the last wash, tap the inverted plate on absorbent paper to removal remaining liquid.
46. Samples are diluted to get measures in the range of the standard curve. The dilution factor depends on several parameters (time of stimulation, volumes used, cell type, cytokine of interest, …) and can be determined empirically in a small pilot experiment (testing a few presumed positive samples and a negative sample).
47. TMB (3, 3′, 5, 5′-tetramethyl benzidine) is a chromogenic substrate. TMB oxidized by HRP in the presence of H_2O_2 forms a water-soluble blue product. Addition of stop solution (sulfuric acid) stops the enzymatic reaction and turns TMB to yellow.
48. MTT is a pale yellow compound that is cleaved by living cells through NADPH-dependent cellular oxidoreductase enzymes to yield a dark blue formazan product. The process requires metabolically active mitochondria of live cells.
49. Experimental entotoxemia is a severe condition during which mice die of overwhelming inflammation.
50. Labeling with a permanent marker should be repeated every 2–3 days. Chip-based identification systems can also be used if allowed by the veterinary office for short-term experiments. Weak septic animals have to have access to food and water; it is therefore mandatory to place food and gel water inside the cage.
51. Drug concentration has to be adapted to its efficacy. In the example given in Fig. 3, 250 µL of cambinol (0.8 mg/mL) corresponds to 200 µg, i.e., 10 mg/Kg (8–12-week-old mice weight around 20 g).
52. To draw blood from mice exactly 1 h after LPS challenge, prepare all the materials needed in advance and collect blood in the same order as mice were challenged. One hour post-challenge is normally the optimal timing to detect circulating TNF by ELISA or Luminex (*see* Subheading 3.7) [31]. A 1/4 dilution of the serum is usually then used but should be adapted.
53. If the operator is not comfortable with mouse holding, a restraint system can be used. Our experience is that mice left free on a grid during the bleeding procedure are less stressed and aggressive.
54. We have established a severity score graded from 1 to 5 as follows: grade 1, ruffled fur; grade 2, ruffled fur plus either mobility disturbance, conjunctivitis or diarrhea; grade 3, ruffled fur, plus mobility disturbance, plus either conjunctivitis or

diarrhea; grade 4, moribund; and grade 5, death [32]. The score, and more generally the whole follow-up of in vivo study, should be performed blinded. Humane endpoints based on severity score may be applied when you have standardized your model. Measurement of animal weight and temperature are giving additional criteria of follow-up.

[AU1]

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3.6 Sirtuin 2 deficiency increases bacterial phagocytosis by macrophages and protects from chronic staphylococcal infection

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Abstract

Sirtuin 2 (SIRT2) is one of the seven sirtuins that constitute the family of NAD^+-dependent histone deacetylases. Through epigenetic and non-epigenetic mechanisms mediated by protein deacetylation, sirtuins impact on many biological processes and are involved in the pathogenesis of age related pathologies such as metabolic, cardiovascular, neurodegenerative and oncologic diseases. Studies of the impact of sirtuins during sterile inflammation have led to controversial observations. Moreover, whether SIRT2 modulates antimicrobial host defenses remains mostly unknown. SIRT2 was the most expressed sirtuin in myeloid cells with strong expression in macrophages and dendritic cells (DCs). Using SIRT2 deficient mice, we showed that SIRT2 deficiency did not impair intracellular signaling and cytokine production by splenocytes, macrophages and DCs exposed to a broad panel of immunological and microbial stimuli and in mice challenged with toxic shock syndrome toxin-1 and lipopolysaccharide. Yet, SIRT2 deficiency enhanced bacterial phagocytosis by macrophages. In agreement with these data, SIRT2 deficiency neither sensitized nor protected mice from endotoxemia, TNF-induced shock, fulminant lethal *Escherichia coli* peritonitis and non-lethal *Klebsiella pneumonia* pneumonia, but powerfully reduced morbidity parameters and increased survival in a model of chronic infection by *Staphylococcus aureus*. Altogether, these data suggest that SIRT2 modulates antimicrobial host defense mechanisms and support the development of sirtuin inhibitors as possible adjunctive therapies to treat chronic bacterial infections.

Keywords (3-10): Sirtuin, Histone deacetylase, Epigenetics, Innate immunity, Cytokine, Macrophage, Endotoxemia, Sepsis

Introduction

Innate immune cells are at the vanguard of host defenses against microbial infections. Monocytes/macrophages and dendritic cells (DCs) sense microbial or danger-associated molecular patterns (MAMPs and DAMPs released by injured or stressed cells) through pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), NOD-like receptors, c-type lectins, scavenger receptors, RIG-I-like receptors and intra-cytosolic DNA sensors (1-3). The interaction between MAMPs or DAMPs with PRRs activates mitogen-activated protein kinases (MAPKs), nuclear factor-κB (NF-κB) and interferon (IFN) response factor (IRF) signaling pathways that coordinate the expression of cytokines and adhesion, major histocompatibilty complex (MHC) and co-stimulatory molecules. The cellular and soluble mediators mobilized upon infection tightly regulate the development of the inflammatory response, the establishment of antimicrobial cellular and humoral responses and the restoration of homeostasis once the pathogen have been contained or eradicated.

Sirtuins (SIRTs) belong to the superfamily of histone deacetylases (HDACs) that comprises eleven Zn-dependent HDACs (HDAC1-11) and seven NAD^+-dependent sirtuins (SIRT1-7). HDACs are epigenetic erasers catalyzing histone deacetylation, leading to chromatin compaction and transcriptional repression. HDACs deacetylate numerous proteins other than histones, among which transcription regulators. In agreement, HDACs impact on diverse biological processes with well recognized effects on metabolism, circadian rhythm and cell cycle (4-8). Sirtuins have been proposed to promote longevity and to represent therapeutic targets for age related pathologies such as type 2 diabetes and cardiovascular, neurodegenerative and oncologic diseases (9-11). Our knowledge about the impact of sirtuins on innate immune responses is fragmentary with the majority of studies focusing on SIRT1 and SIRT6, and with an overall complex picture attributing mainly anti-inflammatory, but also proinflammatory properties to these enzymes (12).

SIRT2 is mainly cytoplasmic but can translocate to the nucleus (7). SIRT2 was recently reported to possess proficient demyristoylation activity, the physiological relevance of which remaining to be established (13). SIRT2 deacetylates many proteins such as histones, α-tubulin and transcription regulators, and is involved in the regulation of cell cycle, metabolic pathways, neurodegeneration and tumorigenesis (14-16). With relevance to inflammation, SIRT2 decaetylates at lysine 310 NF-κB p65, resulting in the expression of a subset of p65-dependent genes in response to TNF stimulation in mouse embryonic fibroblasts (17). In agreement with this initial observation, SIRT2 deficiency sustains brain inflammation in models of intra-cortical LPS administration and traumatic brain injury, and increases severity of collagen-induced arthritis and colitis (18-20). Yet, SIRT2 deficiency decreases LPS-induced, NF-κB p65-mediated, inflammatory responses by macrophages and renal tubular inflammation (21, 22). Supporting of a possible anti-inflammatory role, SIRT2 promotes the degradation of HIF-1α, a critical regulator of metabolism and inflammation (23, 24). Finally, in a model of experimental stroke, SIRT2 deficiency preserves neurological functions without affecting inflammatory parameters (25). Overall, SIRT2 seems to impact on inflammation in a context-dependent manner, but whether it influences innate immune responses to infection is mostly unknown.

To fill in that gap, we analyzed the response of macrophages, dendritic cells (DCs) and splenocytes to immunoregulatory compounds and bacteria and used a panel of preclinical mouse models of toxic shock, endotoxemia and gram-negative and gram-positive bacteria infection. Our results indicate that SIRT2 deficiency does not modulate cytokine production by innate immune cells but enhances bacterial phagocytosis by macrophages. In agreement with these data, SIRT2 deficiency neither sensitizes nor protects mice from TNF and LPS-induced shock, fulminant peritonitis and non-lethal pneumonia, but powerfully reduces morbidity and mortality parameters of chronic staphylococcal infection. Therefore, SIRT2 inhibitors might represent adjunctive therapies to treat chronic bacterial infections.

Results

SIRT2 is the main sirtuin expressed by myeloid cells.

SIRT1-7 expression was quantified by RT-PCR in bone marrow (BM) and bone marrow-derived macrophages (BMDMs), conventional dendritic cells (BMDCs) and Flt3L-derived DCs (**Fig. 1 A**). SIRT2 was the most expressed sirtuin in all populations. SIRT2 was also the predominant sirtuin expressed in RAW 264.7 and J774.1 macrophage cell lines and in spleen, liver and kidney from BALB/c mice (data not shown). Western blot analyses confirmed higher expression levels of SIRT2 over SIRT1 in BMDMs (34). To define whether macrophages express particularly high levels of SIRT2 when compared to other immune or myeloid-derived cells, we extracted expression data from the BioGPS resource (**Fig. 1 B**). Osteoblasts, osteoclasts, macrophages and mast cells expressed SIRT2 mRNA at higher levels (830-2'000 A.U.) than granulocytes, NK cells, thymocytes, T cells and B cells (280-320 A.U.). Overall, the expression pattern of SIRT2 suggested it could play a role in the control of immune responses mediated by macrophages and DCs. To address that question, SIRT2 deficient mice were generated (**Fig. 1 C** and (302)).

SIRT2 deficiency has no major impact on the development of immune cells and host response to TSST-1.

$SIRT2^{-/-}$ mice born at the expected Mendelian ratio and developed without any obvious abnormality. The proportions and absolute numbers of immune cell subpopulations in the thymus (**Table 1**) and spleen (**Table 2**) were similar in $SIRT2^{+/+}$ and $SIRT2^{-/-}$ mice, including CD4/CD8 double negative (DN), double positive (DP) and single positive (SP) thymocytes and splenic T cells (DN and SP, naïve and memory), B cells (immature and mature), $CD11c^{+}$ DCs ($B220^{-}$ cDCs and $B220^{+}$ pDCs), and $Foxp3^{+}$ T regulatory cells. Therefore, SIRT2 had no major impact on immune cell development.

As a first step to address whether SIRT2 affects immune responses, $SIRT2^{+/+}$ and $SIRT2^{-/-}$ splenocytes were exposed to a range of microbial and immunological stimuli (LPS, CpG, concanavalin A, anti-CD3/CD28, PHA, TSST-1 and SEB). The proliferation and production of IL-2 and IFNγ by splenocytes were not affected by SIRT2 deficiency (**Fig. 2 A-B**). Moreover, blood concentrations of IFNγ were similar in $SIRT2^{+/+}$ and $SIRT2^{-/-}$ mice injected with TSST-1 (**Fig. 2 C**).

SIRT2 deficiency does not affect cytokine response of BMDMs and BMDCs exposed to microbial ligands and sensitivity to endotoxemia.

Sensing of microbial ligands through TLRs initiate intracellular MAPK signaling leading to the production of cytokines by innate immune cells. Western blot analyses revealed no differences in the rate of LPS-induced phosphorylation of p38 and JNK between $SIRT2^{+/+}$ and $SIRT2^{-/-}$ BMDMs, and only a slight and late reduction of ERK1/2 phosphorylation in $SIRT2^{-/-}$ BMDMs (30% reduction at 1 hour) (**Fig. 3 A**). TNF and IL-6 mRNA levels were up-regulated to a similar extend in $SIRT2^{+/+}$ and $SIRT2^{-/-}$ BMDMs exposed for 1 hour to LPS, Pam_3CSK_4 and CpG (*i.e.* TLR4, TLR1/2 and TLR9 ligands) (**Fig. 3 B**). Tlr1, Tlr2, Tlr4 and Tlr9 mRNA were modulated likewise in $SIRT2^{+/+}$ and $SIRT2^{-/-}$ BMDMs (**Fig. 3 B**). In line with these results, $SIRT2^{+/+}$ and $SIRT2^{-/-}$ BMDMs secreted comparable levels of TNF and IL-6 in response to stimulation with LPS, Pam_3CSK_4, CpG, *E. coli* and *S. aureus* (**Fig. 3 C**). Finally $SIRT2^{+/+}$ and $SIRT2^{-/-}$ BMDCs showed a similar cytokine expression profile upon exposure to LPS, Pam_3CSK_4, CpG, *E. coli* and GBS (**Fig. 3 D**). These results argued against an important role of SIRT2 in controlling cytokine response by innate immune cells exposed to TLR ligands.

To confirm these observations *in vivo*, we developed models of endotoxemia titrated to cause either mild or severe shock (**Fig. 4 A-D**). In the mild model, TNF and IL-12p40 concentrations in blood and survival (83% *vs* 100%, P = 0.3) of $SIRT2^{+/+}$ and $SIRT2^{-/-}$ mice were comparable (**Fig. 4 A-B**). In the severe model, TNF, IL-6 and IL-12p40 concentrations in blood and mortality rates (88% in both groups, P = 0.69) were strongly increased, but remained similar in $SIRT2^{+/+}$ and $SIRT2^{-/-}$ mice (**Fig. 4 C-D**). In line with these observations and the concept that TNF is one of the main mediators of the lethal effect of endotoxin (37), $SIRT2^{+/+}$ and $SIRT2^{-/-}$ mice were equally sensitive to fulminant TNF-induced shock (P = 0.6; **Fig. 4 E**). Overall, SIRT2 neither impaired nor protected from endotoxemia.

SIRT2 deficiency increases phagocytosis by macrophages.

Phagocytosis is a major mechanism of anti-microbial host defenses mediated primarily by professional phagocytes, *i.e.* macrophages, DCs and neutrophils. Therefore, we tested whether SIRT2 deficiency affects phagocytosis. $SIRT2^{+/+}$ and $SIRT2^{-/-}$ BMDMs, BMDCs and neutrophils were incubated with fluorescent beads and analyzed by flow cytometry (**Fig. 5 A-C**). Interestingly, $SIRT2^{-/-}$ BMDMs phagocytized beads better than $SIRT2^{+/+}$ BMDMs (32.4±1.9 *vs* 24.5±1.2 percent positive cells, P = 0.002), whereas no differences were observed using BMDCs (13.5±1.2 *vs* 15.1±0.7) and neutrophils (4.6±0.6 *vs* 4.7±0.2) (**Fig. 5 A-B**). The increased phagocytosis by $SIRT2^{-/-}$ BMDMs was evident whether (**Fig. 5 C**) or not (**Fig. 5 A**) fluorescent beads were opsonized before usage. Furthermore, $SIRT2^{-/-}$ BMDMs phagocytized better than $SIRT2^{+/+}$ BMDMs a panel of fluorescently labeled heat-killed bacteria (percent of $SIRT2^{-/-}$ *vs* $SIRT2^{+/+}$ BMDMs ingesting bacteria: *E. coli* J5: 53.6% *vs* 43.6%, *E. coli* O111: 31.6% *vs* 23.0%, *Salmonella* Typhimurium: 24.1% *vs* 17.6%, *Neisseria meningitis*: 46.1% *vs* 37.2%, *S. pneumonia*: 49.6% *vs* 34.7%). To extend these observations in a more realistic situation, BMDMs were exposed to live *E. coli*, *S. aureus* and GBS for 1 hour before measuring the number of bacteria taken up by plating cell lysates and enumerating colonies. Confirming the observations obtained using inert beads and bacteria, for all the bacterial strains tested, the

numbers of bacteria phagocytized were 1.3-1.6-fold higher using SIRT2$^{-/-}$ when compared to SIRT2$^{+/+}$ BMDMs (**Fig. 5 D**).

SIRT2 deficiency does not impact on the expression of phagocytic receptors.

Professional phagocytes express a panel of phagocytic and opsonic phagocytic receptors including macrophage scavenger receptor 1 (Msr1/SR-AI/CD204), CD14, CD36, C-type lectins such as dectin-1 (encoded by Clec7a) and members of the integrin superfamily (such as integrin α5/Itga5/CD49e, integrin αM/Itgam/CD11b, integrin αX/Itgax/CD11c, integrin β2/Itgb2/CD18). SIRT2$^{-/-}$ and SIRT2$^{+/+}$ BMDMs expressed similar mRNA levels of a large panel of phagocytic receptors (*i.e.* Itga5, Itga6, Itgal, Itgam, Itgax, Itgb1, Itgb2, Cd14, Cd36, Msr1 and Clec7a) at baseline (**Fig. 5 E**) and upon stimulation with LPS for 4 hours (**data not shown**). Moreover, flow cytometry analyses confirmed similar membrane-bound expression of CD11b (Itgam), CD11c (Itgax), CD14 and Msr1 by resting SIRT2$^{-/-}$ and SIRT2$^{+/+}$ BMDMs (**Fig. 5 F**). Therefore, SIRT2 deficiency improved phagocytosis by BMDMs, presumably without affecting phagocytic receptor expression.

SIRT2 deficiency protects from chronic staphylococcal infection.

E. coli, *S. aureus* and *K. pneumonia* are three of the most frequent causes of bacterial sepsis in humans (38). Considering that SIRT2 deficiency had no impact in models of fulminant septic shock during which overwhelming cytokine response has a deleterious role (**Fig. 4**), we elected to test the impact of SIRT2 in rapidly lethal, sub-lethal and chronic models of bacterial infections; hypothesizing that increased phagocytosis in SIRT2-deficient mice might provide some benefit during non-stringent, chronic, infection.

Supporting our assumptions, in a model of fulminant, rapidly lethal peritonitis induced by *E. coli* (**Fig. 6 A**), body weight losses, bacterial dissemination into the blood, and survival rates (12.5% in both groups, P = 0.7) were comparable in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice. Moreover, in a less fulminant model of sepsis induced by systemic infection with *S. aureus* in which mortality occurred 3 to 16 days after infection (**Fig. 6 B**), severity scores, body weight loss and survival (SIRT2$^{+/+}$ *vs* SIRT2$^{-/-}$: 33.3 *vs* 69.2%; P = 0.04) were all markedly improved in SIRT2$^{-/-}$ mice. Finally, in a non-severe model of *K. pneumoniae* pneumonia (**Fig. 6 C**), body weight loss and survival (85.7% in both groups, P = 0.9) were not affected by SIRT2 deficiency.

Discussion

In the present study, we identified a novel role of SIRT2 in host-pathogen interaction. SIRT2 deficiency promotes bacterial phagocytosis by macrophages, without impacting on cytokine secretion. These observations were corroborated in a panel of preclinical mouse models demonstrating that SIRT2 deficiency protects from chronic staphylococcal infection while not influencing the course of endotoxemia, TNF-induced shock, fulminant peritonitis and non-severe pneumonia. These observations are particularly relevant in light of the development of pharmacological modulators of sirtuins for clinical applications (17-19, 39).

SIRT2 was the most expressed sirtuin in bone marrow, BMDMs, BMDCs and Flt3L-DCs, with highest mRNA levels observed in BMDMs. In agreement, SIRT2 was detected in microglial cells *in vivo* (19,

20), and expression studies in primary cells confirmed stronger SIRT2 expression in macrophages than in $CD8^+$ and $CD8^-$ conventional DCs and $B220^+$ plasmacytoid DCs (**Fig. 1B**). Greatest SIRT2 expression was disclosed in mast cells, suggesting that SIRT2 may play a particular role in this cell type.

SIRT2 deficiency had no major impact on LPS-induced MAPK activation in macrophages. Considering that SIRT2-deficient macrophages, DCs and splenocytes produced cytokines like their wild-type counterparts, and that cytokine blood levels were similar in $SIRT2^{+/+}$ and $SIRT2^{-/-}$ endotoxemic mice, it is most likely that activation of the NF-κB pathway was not or negligibly affected by SIRT2. These observations are in line with studies showing that inflammatory parameters were comparable in $SIRT2^{+/+}$ and $SIRT2^{-/-}$ mice with experimental stroke and *Mycobacterium tuberculosis* infection (25, 40). Yet, contradictory findings have been reported about the impact of SIRT2 on inflammatory responses. While SIRT2 deficiency favored NF-κB p65 acetylation and activity, increasing p65-depedent gene expression (17-19, 39); SIRT2 deficiency also reduced NF-κB and p38 and JNK activation through IκB phosphorylation and acetylation-dependent increased activity of MAPK phosphatase-1, respectively (21, 39). Moreover, SIRT2 deficiency sustained brain inflammation, colitis and collagen-induced arthritis but protected from renal inflammation (18, 19, 21, 39).

The discrepant outcomes of SIRT2 on inflammatory responses mirror conflicting results observed for other sirtuins. For example, SIRT1 protects from experimental autoimmune encephalomyelitis, arthritis, lung inflammation, hepatic steatosis and insulin resistance, but plays a role in the pathogenesis of lupus, arthritis, allergic airway disease and allograft rejection (41-49). Similarly, SIRT6 protects from liver fibrosis, atherosclerosis, osteoarthritis and arthritis, but promotes TNF production and the development of autoimmune encephalomyelitis and cerebral ischemia (50-57). This overall complex picture may arise from different experimental conditions used to decipher the role of sirtuins, with a possible impact of qualitative and quantitative differences in caloric input or subtle variations of NAD^+ availability impacting on sirtuin activity. Another important parameter to take into account is that SIRT1 and SIRT6 modulate circadian function and are themselves affected by circadian oscillation in the abundance of NAD^+ (58).

Two studies examined SIRT2 in the context of infection by intracellular bacteria. In a first model, *Listeria monocytogenes* was shown to promote SIRT2-dependent histone H3 deacetylation and redirect host gene expression to promote infection (59). Whether other microorganisms subvert SIRT2 or other sirtuins at their own benefit is presently unknown. In a second model, SIRT2 deletion in the myeloid compartment had no noticeable impact on host defenses against *Mycobacterium tuberculosis* infection as revealed by cellular infiltrates, cytokine expression and long term bacterial burden in lungs (40).

We analyzed host responses to extracellular bacteria most frequently isolated from septic patients (38). Strikingly, SIRT2 deficiency enhanced the engulfment of gram-positive and gram-negative bacteria by macrophages without affecting the expression of phagocytic receptors. These observations could be reconciled by the fact that SIRT2 deacetylates α-tubulin at lysine 40, thereby destabilizing the microtubule network (60, 61). Indeed, during the process of phagocytosis, cells undergo a massive rearrangement of the cytoskeleton involving microtubule dynamics and requiring microtubule stabilization (62, 63). Paclitaxel, that enhances tubulin polymerization and stabilizes microtubules, promotes phagocytosis; while microtubule

depolymerizating agents such as colchicine and nocodazole reduce phagocytosis by macrophages (64-66). The reason why SIRT2 deficiency impacted on phagocytosis by macrophages but not DCs and neutrophils remains unclear, and may reflect cell-specific differences regarding SIRT2 functions.

Besides being a central mechanism controlling nutrient homeostasis, autophagy plays a role in host defenses by facilitating the clearance of bacteria from the cytoplasm (67). Interestingly, hyperacetylation of tubulin stimulated autophagy by nutrient deprivation and SIRT2 deficiency increased autophagy in a colorectal cancer cell line (68, 69). By regulating tubulin acetylation, SIRT2 may contribute to modulate phagocytosis and autophagy defense mechanisms, although the latter has not been formally demonstrated.

From a translational standpoint, it was important to define the impact of SIRT2 in preclinical models of infection. A main observation of this study is that SIRT2 deficiency confers protection from chronic staphylococcal infection, but neither protects nor sensitizes to toxic shock, mild and severe endotoxemia, rapidly lethal *E. coli* peritonitis and non-severe *K. pneumoniae* pneumoniae. So far, these results comfort the clinical development of SIRT2 inhibitors with a safety profile in terms of susceptibility to infections, which contrasts with inhibitors of HDAC1-11 that increased susceptibility to infection in preclinical mouse models and the administration of which was reported to be associated with episodes of severe infection in patients (30, 36, 70-72). Nonetheless, the panel of sepsis conditions should be enlarged to warranty definite conclusions.

Overall, SIRT2 has a subtle impact on host defense response to bacterial infections. Our results are encouraging in the perspective of developing therapies directed at SIRT2 for treating metabolic and neurodegenerative diseases. As an example, SIRT2 inhibitors mediated neuroprotective effects in models of Parkinson's disease and Huntington's disease (73, 74). Additionally, SIRT2 inhibitors might be viewed as an adjunctive therapy for treating chronic bacterial infection.

Materials and methods

Ethics statement

Animal experimentations were approved by the Office Vétérinaire du Canton de Vaud (authorizations n° 876.8 and 877.8) and performed according to Swiss guidelines and ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines).

Mice, cells and reagents

Eight to 12-week-old female BALB/cByJ and C57BL/6J mice (Charles River Laboratories, Saint-Germain-sur-l'Arbresle, France) were housed under specific pathogen-free conditions. SIRT2 knockout mice (18) were backcrossed 12 times on a C57BL/6J background. Splenocytes were cultured in RPMI 1640 medium containing 2 mM glutamine, 50 µM 2-ME, 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen, San Diego, CA) and 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich St. Louis, MO) (26). Bone marrow (BM) cells were cultured in IMDM (Invitrogen) containing 50 µM 2-ME, penicillin, streptomycin and 10% FCS. Medium was supplemented with 20 ng/ml M-CSF or 20 ng/ml GM-CSF plus 20 ng/ml IL-4 (ProSpec, East

Brunswick, NJ) or 200 ng/ml FMS-like tyrosine kinase 3 ligand (Flt3l, Shenandoah biotechnology, Warwick, PA) to generate BM-derived macrophages (BMDMs), or BM-derived dendritic cells (BMDCs) or Flt3L-DCs, respectively. BMDCs were collected after 6 days. BMDMs and Flt3L-DCs were collected after 7 days of culture. Cells were seeded in 96-well (1 x 10^5 cells), 24-well (5 x 10^5 cells) or 6-well (2 x 10^6 cells) plates in medium without growth factor and antibiotics unless specified. Bone marrow neutrophils were isolated using the Neutrophil Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and short-term cultured in PBS containing 2% FCS.

Salmonella minnesota ultra pure LPS was from List Biologicals Laboratories (Campbell, CA), Pam_3CSK_4 from EMC microcollections (Tübingen, Germany), CpG ODN 1826 (CpG) from InvivoGen (San Diego, CA), toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxin B (SEB) from Toxin Technology (Sarasota, FL), concanavalin A and phytohemagglutinin (PHA) from Sigma-Aldrich, and anti-CD3ε and anti-CD28 antibodies (clones 145-2C11 and 37.51) from eBioscience (San Diego, CA). Clinical strains of *Escherichia coli* (*E. coli*) O18, *E. coli* J5, *E. coli* O111, *Salmonella enterica* serovar Typhimurium C5 (*Salmonella* Typhimurium) *Klebsiella pneumonia* caroli (*K. pneumonia*), *Neisseria meningitis*, *Streptococcus pneumonia*, *Staphylococcus aureus* AW7 (*S. aureus*) and Group B Streptococcus (GBS) were grown in brain heart infusion broth (BD Biosciences, Erembodegem, Belgium) (27, 28). Microorganisms were washed in PBS and adjusted to 10^{10} CFU/ml. For *in vitro* stimulation experiments, bacteria were heat-inactivated for 2 h at 56°C.

RNA analyses

Total RNA was isolated RNeasy kit and reverse transcribed using the QuantiTect reverse transcription kit (Qiagen, Hilden, Germany). Real-time PCR were conducted with a QuantStudio™ 12K Flex system (Life Technologies, Carlsbad, CA). Reactions, tested in triplicates, consisted of 1.25 µl cDNA, 1.25 µl H_2O, 0.62 µl primers and 3.12 µl Fast SYBR® Green Master Mix (Life Technologies). Primer pairs for amplifying Tnf, Il6, Tlr1, Tlr2, Tlr4, Tlr9 and hypoxanthine guanine phosphoribosyl transferase (Hprt) cDNA were as published (29, 30). Gene specific expression was normalized to Hprt expression. Sirt1-7 expression levels were extracted from the BioGPS resource (http://biogps.org). For microarray analyses, RNA concentration and quality were assessed using a NanoDrop®ND-1000 spectrophotometer, RNA 6000 NanoChips and the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, USA). One hundred ng RNA were amplified using the Ambion® WT Expression Kit (Life Technologies). cDNA was fragmented and labelled with GeneChip® WT Terminal Labeling kit (Affymetrix, Santa Clara, CA). Affymetrix mouse gene 2.0ST arrays were hybridized for 16 hours at 45°C with 3.7 µg of fragmented targets, washed and stained according to the protocol of the Affymetrix GeneChip® Expression Analysis Manual (Fluidics protocol FS450_0002). Arrays were scanned with the GeneChip® Scanner 3000 7G (Affymetrix). Normalized expression signals were calculated from Affymetrix CEL files by the Robust Multi-array Average algorithm (RMA), using the Affymetrix Expression Console Software, version 1.3.1.187. Hybridization quality was assessed using Expression Console Software as well. Statistical analyses were performed using the free high-level interpreted statistical language R, version 3.0.1. Differential hybridized features were identified using the R Bioconductor package "limma" that implements linear models for microarray data (31). *P* values were adjusted for multiple testing

using the Benjamini and Hochberg's method to control the false discovery rate (FDR). Probe sets with a FDR < 0.05 were considered significant. Microarray data were generated and analyzed by the Genomic Technologies Facility at the University of Lausanne (Lausanne).

Western blot analyses

Protein extracts were submitted to PAGE and transferred onto nitrocellulose membranes as decribed (32). Membranes were incubated with primary antibodies specific of SIRT2 (Abcam, Cambridge, UK), tubulin (Sigma-Aldrich) and total and phosphorylated ERK1/2, p38 and JNK (Cell Signaling Technology), and then with a secondary horseradish peroxidase-conjugated antibody (Sigma-Aldrich) (33). Blots were revealed with the enhanced chemiluminescence Western blotting system (GE Healthcare, Little Chalfont, Royaume-Uni). Images were recorded using a Fusion Fx system (Viber Lourmat, Collégien, France).

Flow cytometry

Single cell suspensions from thymus and spleen or BMDMs were enumerated and incubated with 2.4G2 monoclonal antibody (mAb). Immune cell subpopulations were tracked by staining performed using mAbs described in **supplementary Table 1**. Splenic $CD4^+$ $CD25^+$ $Foxp3^+$ cells were detected using The Mouse Regulatory T Cell Staining Kit (eBioscience). Data were acquired using a LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo Version 8.5.3 software (FlowJo LLC, Ashland OR).

Proliferation assay

The proliferation of 1.5×10^5 splenocytes cultured for 48 hours in 96-well plates was quantified by measuring ^{3}H-thymidine incorporation over 18 hours (34).

Cytokine measurements

Cytokine concentrations were quantified using DuoSet ELISA kits (R&D Systems, Abingdon, UK) or Luminex assays (Affimetrix eBioscience, Vienna, Austria) (35).

***In vivo* models**

Eight to 12-week-old female $SIRT2^{+/+}$ and $SIRT2^{-/-}$ mice matched for age were used. To analyze the response to TSST-1, mice were challenged intraperitoneally (i.p.) with 10 µg of the toxin. Sub-lethal and lethal models of endotoxic shock were performed by challenging mice i.p. with 200 µg and 500 µg LPS, respectively. To induce TNF shock, mice were sensitized with an i.p. injection of D-galactosamine (30 mg/kg, Sigma-Aldrich) followed immediately after with an i.p. challenge with TNF (25 mg/kg, Preprotech, Rocky Hill, NJ). Bacterial sepsis was induced by challenging mice i.p. with 10^5 CFU *E. coli* O18, intravenously (i.v.) with 10^7 CFU *S. aureus*, or intranasally (i.n.) with 30 CFU *K. pneumoniae*. Blood was collected 0, 1, 6, 8 or 24 hours post-challenge to quantify cytokines and bacteria (29). Body weight losses, severity scores and survival were registered at least once daily. The severity score was graded from 1 to 5 (28). Animals were euthanized when they met a severity score of 4.

Phagocytosis assays

Fluoresbrite® Yellow Green Microspheres (Polysciences Inc, Warrington, PA) or FITC-labeled bacteria were added to cells at a ratio of 10 beads or bacteria/cell. After 1 hour, cells were collected, washed, incubated for 1 minute with trypan blue (0.25 mg/ml), washed and analyzed by flow cytometry. In some experiments, beads were opsonized with serum for 30 min at 37°C. Phagocytosis of live *E. coli* O18, *S. aureus* and GBS was performed essentially as described (36). Briefly, BMDMs were incubated for 1 hour with bacteria at a multiplicity of infection of 5 bacteria/cell. Non-adherent bacteria were removed by washing and extracellular bacteria killed by a 30-minute exposure to 100 µg/ml gentamicin (Essex Chemie, Luzern, Switzerland; for *E. coli* and GBS) or 10 µg/ml ciprofloxacin (Fresenius Kabi, Oberdorf, Switzerland; for *S. aureus*). Serial dilutions of cell lysates were distributed on agar plates. Colonies were enumerated to calculate the number of phagocytosed bacteria.

Statistical analyses

Comparisons between the different groups were performed by analysis of variance followed by two-tailed unpaired Student's t-test. The Kaplan-Meier method was used for building survival curves and differences were analyzed by the log-rank sum test. All analyses were performed using PRISM (GraphPad Software). *P* values were two-sided, and values < 0.05 were considered to indicate statistical significance.

Authorship

EC, TH, MM, JL and BT performed *in vitro* experiments. EC, TH and DLR performed *in vivo* experiments. HAO and JA contributed to reagents. TR conceived the project, designed the experiments and wrote the paper. EC, HAO, JA revised the paper.

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Conflict of Interest Disclosure

The authors declare no competing financial interests

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Table 1. Thymic populations in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice

	SIRT2$^{+/+}$	SIRT2$^{-/-}$
CD4^{+} CD8^{+}	82.3±3.1	82.9±0.4
CD4^{-} CD8^{-}	2.0±0.6	1.8±0.2
CD25^{+} CD44^{+}	1.8±0.6	1.6±0.5
CD25^{-} CD44^{+}	0.2±0.01	0.2±0.01
CD25^{+} CD44^{-}	1.4±0.6	1.3±0.4
CD25^{-} CD44^{-}	96.6±1.2	96.9±1.0
CD4^{+} CD8^{-}	12.0±2.3	12.1± 0.7
CD4^{-} CD8^{+}	3.6±0.3	3.3±0.8

Data are means ± SD of 4 animals per group and expressed as the percentage of total cells (CD4^{+} CD8^{+}, CD4^{-} CD8^{-}, CD4^{+} CD8^{-} and CD4^{-} CD8^{+}) or percentage of CD4^{-} CD8^{-} parental cells (CD25^{+} CD44^{+}, CD25^{-} CD44^{+}, CD25^{+} CD44^{-} and CD25^{-} CD44^{-}). Total cell numbers were 49.2±15.4 and 55.2±5.7 millions in the thymus of SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice, respectively.

Table 2. Splenic populations in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice

	SIRT2$^{+/+}$	SIRT2$^{-/-}$
Cell number (x10^{-6})	74.2± 5.6	67.4±8.7
CD3^{+} T cells (%)	27.3±4.6	36.3±4.8
CD4^{+}	62.3±2.7	61.5±4.3
CD4^{+} CD44^{low} CD62L^{high} (naive)	46.0±2.9	43.7±6.8
CD4^{+} CD44^{high} CD62L^{low} (memory)	16.3±2.9	17.8±6.9
CD8^{+}	31.5±2.0	32.0±2.9
CD8^{+} CD44^{low} CD62L^{high} (naive)	23.1±0.5	23.5±0.3
CD8^{+} CD44^{high} CD62L^{low} (memory)	8.4±0.5	8.5±0.3
CD4^{-} CD8^{-}	1.3±0.2	1.6±0.2
B220^{+} B cells (%)	52.2±7.4	51.3±3.1
B220^{+} IgD^{+} CD23^{+} (mature)	45.6±1.2	44.1±0.9
B220^{+}, non-IgD^{+}/CD23^{+} (immature)	6.6±1.2	7.2±0.9
CD11c^{+} DCs (%)	6.6±0.2	6.4±0.6
B220^{-}	62.8±2.5	62.8±4.7
B220^{+}	37.2±2.5	37.2±4.7
CD4^{+} CD25^{+} Foxp3^{+} Tregs (%)	4.5±0.4	4.7±0.2

Data are means ± SD of 4 animals per group and expressed as total cell number (x10^{-6}) per spleen, percentage of splenic cells (CD3^{+}, B220^{+}, CD11c^{+} and CD4^{+} Foxp3^{+}) or percentage of CD3^{+}, B220^{+} or CD11c^{+} parental populations. Total cell numbers were 74.2± 5.6 and 67.4±8.7 millions in the spleen of SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice, respectively.

Supplementary Table 1. Antibodies used for flow cytometry analyses

Target	Clone name	Coupling
B220	RA3-6B2	eFluor® 450
CD3	145-2C11	PE, eFluor® 450
CD4	RM4-5	PE, FITC
CD8	53-6.7	APC-eFluor® 780, APC-Cy7
CD11b	M1/710	PE, APC
CD11c	HL3	APC
CD14	Sa2-8	PE-Cy7
CD23	B3B4	PE
CD25	PC61.5	APC
CD36	72-1	PE
CD44	IM7	APC, eFluor® 450
CD62L	MEL-14	FITC
CD93	AA4.1	APC
IgD	AMS 9.1	FITC
MHC-II	114.15.2	FITC

PE: phycoerythrin; FITC: fluorescein isothiocyanate; APC: allophycocyanin. All antibodies were from eBioscience, except the anti-IgD-FITC that was from BD Biosciences (Pharmingen).

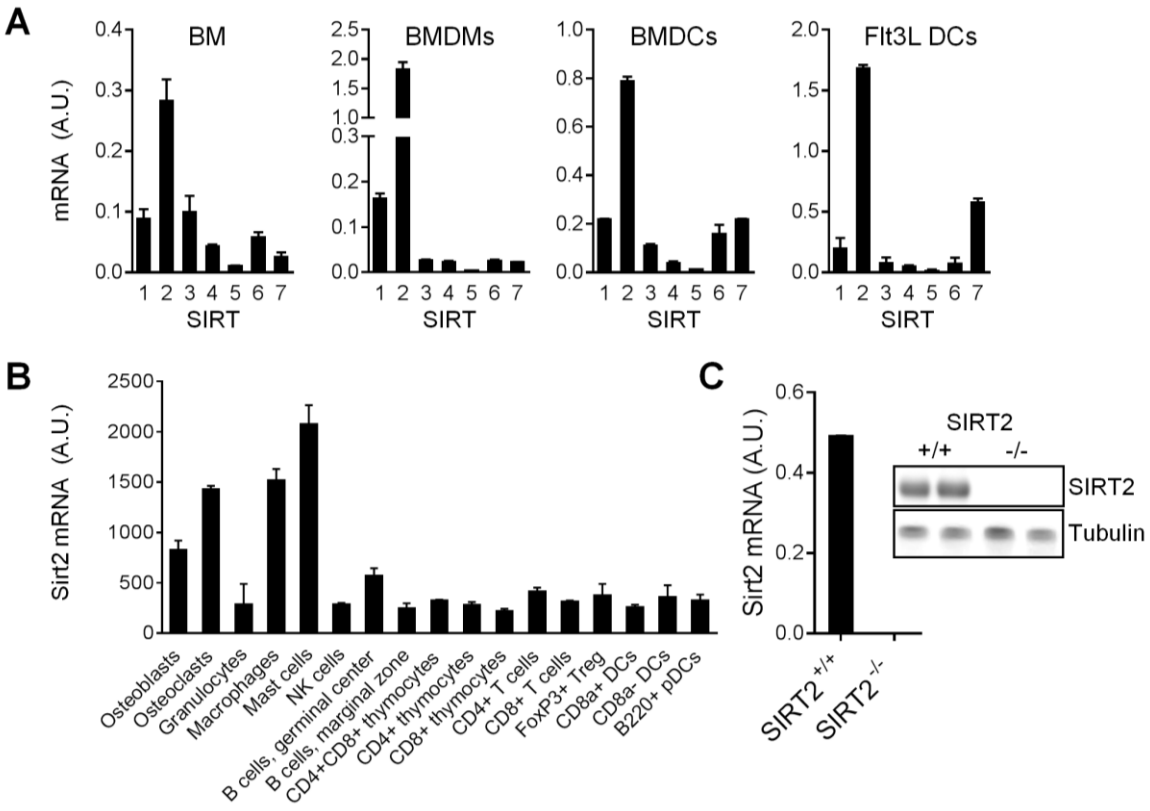


Figure 1. Sirt2 is the main sirtuin expressed in myeloid cells. (**A**) SIRT1-7 mRNA levels were quantified in bone marrow (BM), BM-derived macrophages (BMDMs) and dendritic cells (BMDCs) and Flt3L-derived DCs by RT-PCR. SIRT1-7 mRNA levels were normalized to Hprt mRNA levels. Data are means ± SD of triplicate samples from one experiment performed with 4 BALB/c mice and are representative of 2 experiments. (**B**) SIRT2 mRNA expression levels in a panel of myeloid-derived cells (http://biogps.org). (**C**) SIRT2 mRNA and SIRT2 protein expression levels in $SIRT2^{+/+}$ and $SIRT2^{-/-}$ BMDMs assessed by RT-PCR and Western blotting, respectively. A.U.: arbitrary units.

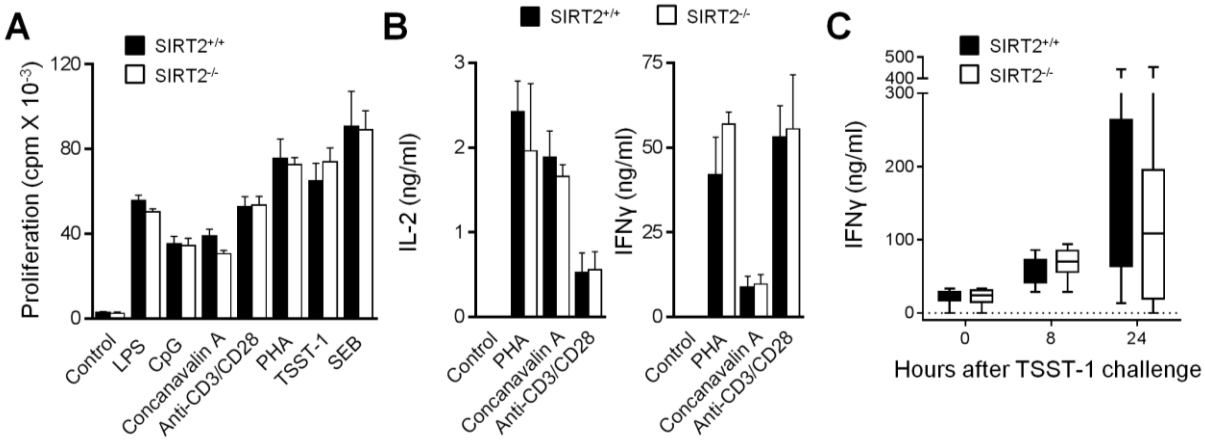


Figure 2. SIRT2 deficiency does not affect proliferation and cytokine response of splenocytes nor the production of IFNγ in mice challenged with TSST-1. (**A-B**) SIRT2$^{+/+}$ and SIRT2$^{-/-}$ splenocytes were incubated for 48 hours with LPS (5 µg/ml), CpG (2 µg/ml), concanavalin A (5 µg/ml), anti-CD3/CD28 antibodies (1µg/ml), TSST-1 (2 µg/ml) and SEB (5 µg/ml). (**A**) Proliferation was measured by ^{3}H-thymidine incorporation. (**B**) IL-2 and IFNγ concentrations in cell culture supernatants were quantified by ELISA. Data are means ± SD of triplicate samples from one experiment performed with 4 mice and are representative of 2 experiments. (**C**) SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice (n = 8 per group) were injected with TSST-1 (10 µg i.p.). Blood was collected after 0, 8 and 24 hours to quantify IFNγ concentrations by Luminex. Data are means ± SD. P > 0.5 for all conditions.

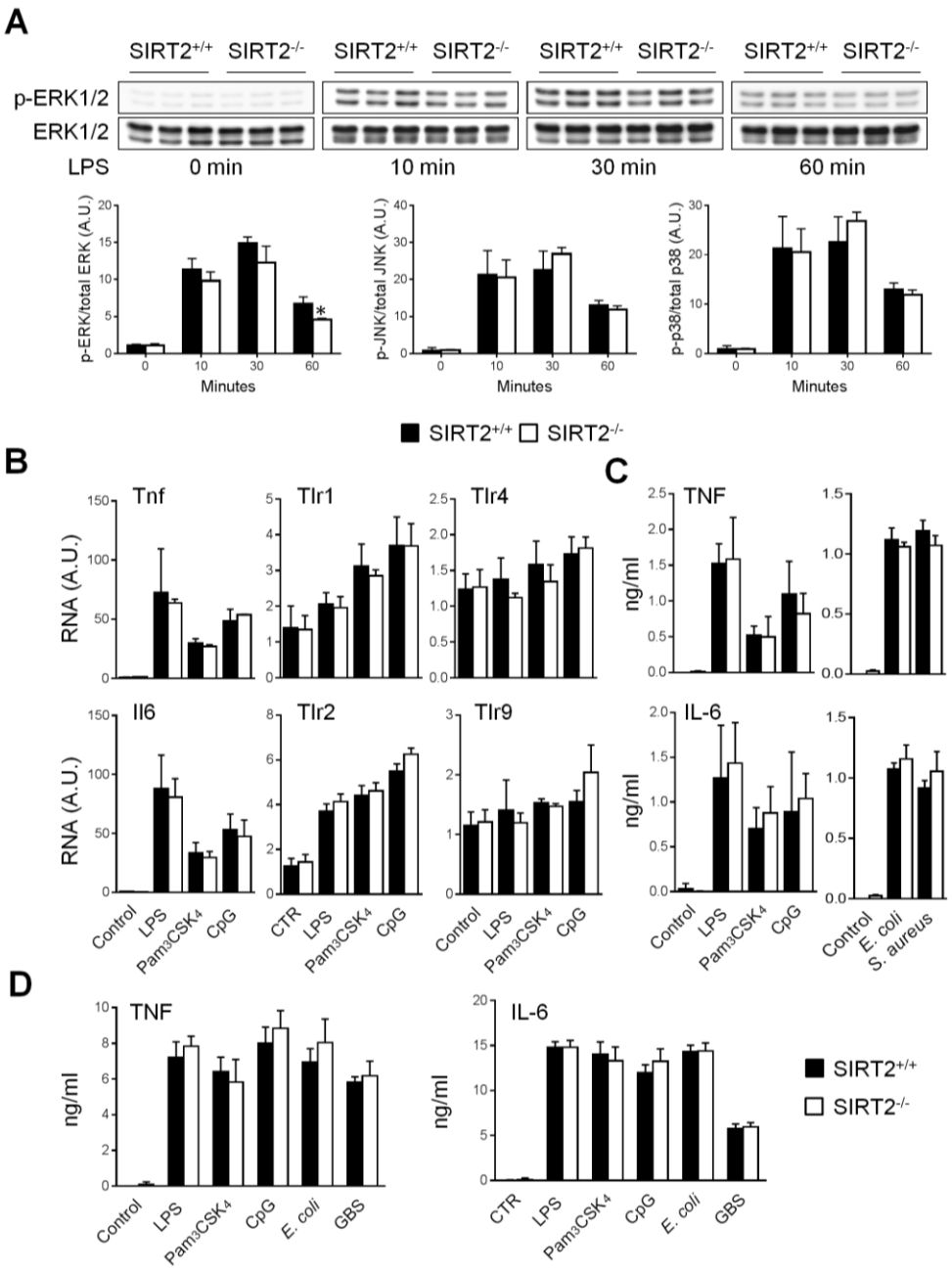


Figure 3. SIRT2 deficiency does not affect the response of macrophages and DCs to microbial stimulation. SIRT2$^{+/+}$ and SIRT2$^{-/-}$ BMDMs (**A-C**) and BMDCs (**D**) were exposed to LPS (10 ng/ml), Pam_3CSK_4 (10 ng/ml) and CpG (2 µg/ml), *E. coli* (10^6 CFU/ml), *S. aureus* and GBS (2.5 x 10^6 CFU/ml). (**A**) Expression levels of phosphorylated (p) and total ERK1/2, p38 and JNK were analyzed by Western blotting and quantified by imaging. Data are means ± SD obtained with three mice. (**B**) Tnf, Il6, Tlr1, Tlr2, Tlr4 and Tlr9 mRNA levels were analyzed by RT-PCR. mRNA levels were normalized to Hprt mRNA levels. Data are means ± SD of triplicate samples from one experiment performed with 3 mice. A.U.: arbitrary units. (**C**, **D**) TNF and IL-6 concentrations in cell culture supernatants collected 8 hours after stimulation. Data are means ± SD of triplicates samples from one experiment performed with 6 mice (**C**) or 3 mice (**D**). *, P = 0.02.

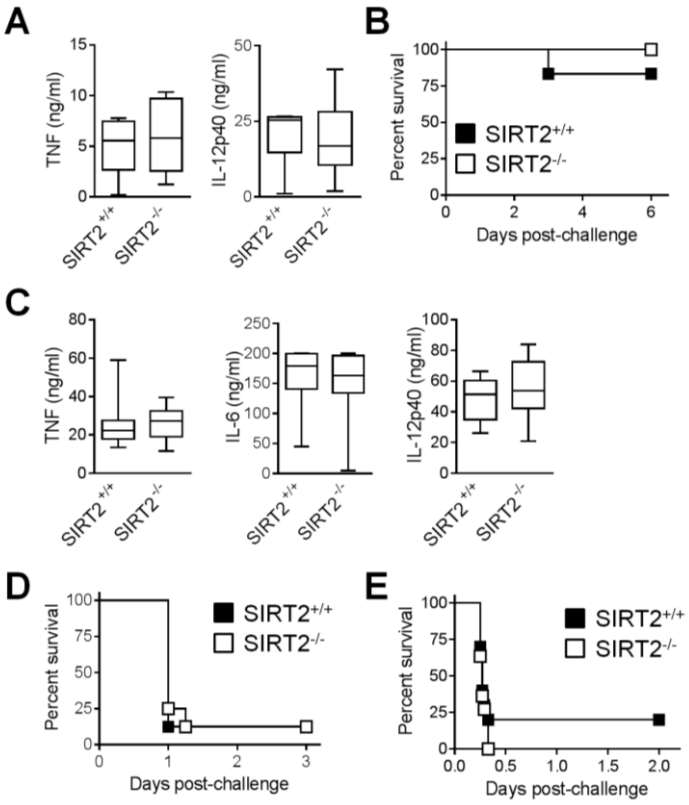


Figure 4. SIRT2 deficiency does not affect endotoxemia or TNF-induced shock. SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice were injected i.p. with 200 µg LPS (**A**-**B**, n = 6 per group) and 500 µg LPS (**C**-**D**, n = 8 per group). (**A and C**) TNF, IL-6 and IL-12p40 concentrations in blood collected 1hour (TNF) and 6 hours (IL-6 and IL-12p40) after LPS challenge. (**B**-**D**) Survival of mice. P = 0.3 and 0.6. (**E**) Survival of SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice (n = 11 per group) sensitized with 30 mg/kg D-galactosamine and challenged i.p. with 25 mg/kg TNF. P = 0.6.

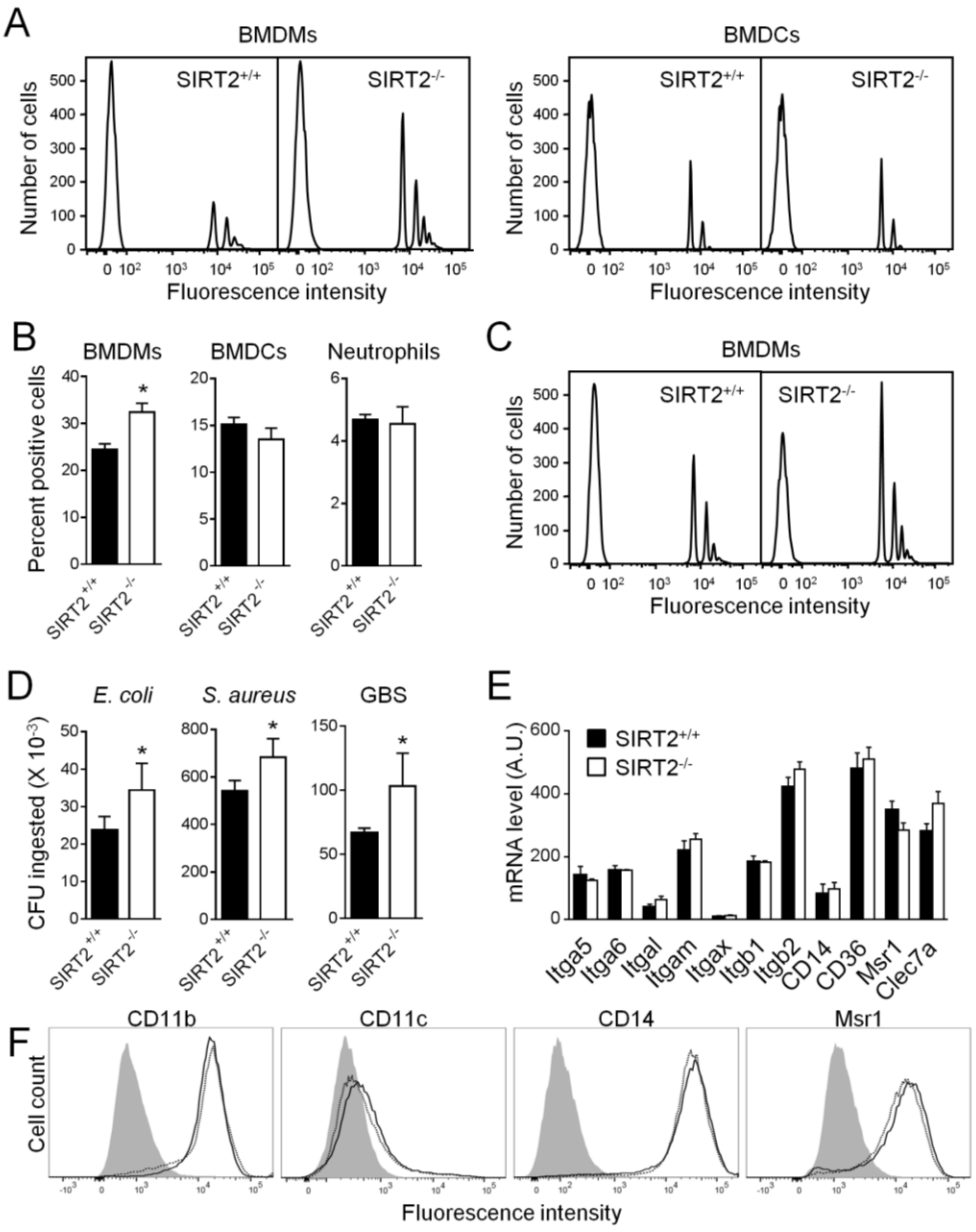


Figure 5. SIRT2 deficiency increases bacterial phagocytosis by macrophages. SIRT2$^{+/+}$ and SIRT2$^{-/-}$ BMDMs (**A-F**), BMDCs (**A-B**) and neutrophils (**B**) were incubated with Fluoresbrite® Yellow Green Microspheres (10 beads/cell, not opsonized in **A** and **B**, opsonized in **C**) or live *E. coli*, *S. aureus* and GBS (10 bacteria/cell, **D**). (**A-C**) After 1 hour of incubation, cells were analyzed by flow cytometry and results expressed as percent fluorescent cells. Data are means ± SD from one experiment performed with 4 mice. *, P = 0.002 (**B**). (**D**) BMDMs were collected 1 hour after infection to assess phagocytosis. Data are means ± SD from one experiment performed with 4 (*E. coli* and GBS) or 8 (*S. aureus*) mice. *, P = 0.03, 0.006 and 0.03 for *E. coli*, *S. aureus* and GBS. (**E**) Itga5, Itga6, Itgal, Itgam, Itgax, Itgb1, Itgb2, Cd14, Cd36, Msr1 and Clec7a mRNA expression levels were determined by DNA array analyses. Data are means ± SD of one experiment performed with 3 mice. (**F**) CD11b, CD11c, CD14, Msr1 expression levels were analyzed by flow cytometry.

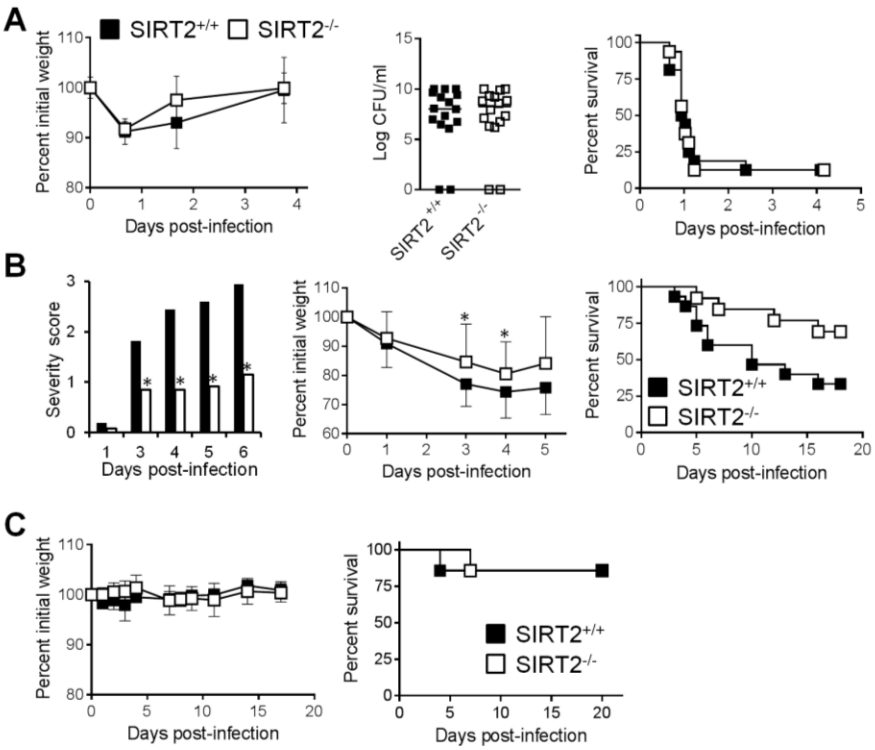


Figure 6. SIRT2 deficiency protects from *S. aureus* infection. (**A**) Body weight losses, bacteria in blood 24 hours post-infection (P = 0.9) and survival (P = 0.7) of $SIRT2^{+/+}$ and $SIRT2^{-/-}$ mice challenged i.p. with 10^5 CFU *E. coli* (n = 16 per group). (**B**) Severity scores, body weight losses and survival (P = 0.04) of $SIRT2^{+/+}$ and $SIRT2^{-/-}$ mice challenged i.v. with 10^7 CFU *S. aureus* (n = 13 and 9). Severity scores: *, $P \leq 0.01$. Body weight losses: *, P = 0.03 and 0.04. (**C**) Body weight losses and survival (P = 0.9) of $SIRT2^{+/+}$ and $SIRT2^{-/-}$ mice challenged i.n. with 30 CFU *K. pneumoniae* (n = 7 per group).

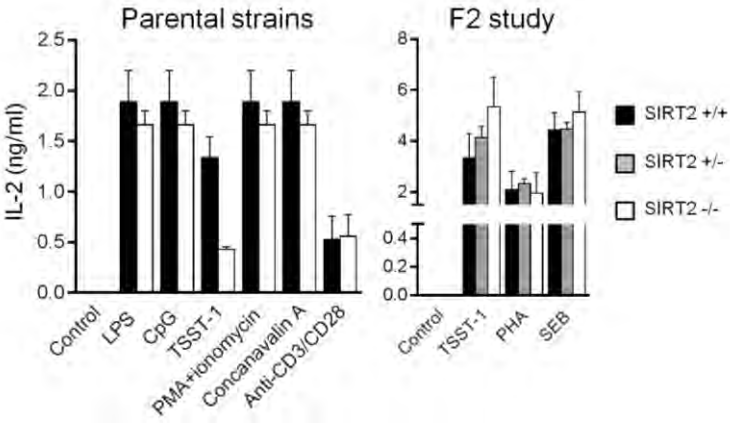
Additional data

Evidences for a genetic drift of SIRT2$^{-/-}$ mice

When we first screened SIRT2$^{+/+}$ and SIRT2$^{-/-}$ splenocytes for their proliferation and production of IL-2 and IFNγ, we noticed a reduced response of SIRT2$^{-/-}$ when compared with SIRT2$^{+/+}$ splenocytes in response to TSST-1 stimulation, whereas SIRT2$^{+/+}$ and SIRT2$^{-/-}$ splenocytes behave similarly in response to LPS, CpG ODN 1826, PMA and ionomycin, concanavalin A and anti-CD3/CD28 (**Additional Figure 1** and data not shown).

TSST-1 is a staphylococcal exotoxin that acts as an exogenous superantigen (SAg) by bridging MHC-II molecules on the surface of antigen presenting cells (APCs) with conserved regions of T cell receptor (TCR) Vβ3, Vβ15 and Vβ17 chains expressed by T cells (377, 378). Thus, impaired response to TSST-1 by SIRT2$^{-/-}$ splenocytes could result from an impaired expression (possibly SIRT2-dependent) of MHC-II, Vβ3, Vβ15, Vβ17 or co-stimulatory molecules (*i.e.* CD28) necessary for optimal response to TSST-1. We also envisaged possible genetic background differences between SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mouse lines that would determine the phenotype observed. For example, endogenous mouse mammary tumor viruses (MMTVs) express endogenous SAg that in the context of I-E molecules, and for some of them I-A molecules, induces the deletion of SAg-reactive T cells through negative selection in the thymus.

To test this last possibility, we performed an F2 study in which we compared proliferation and IL-2 and IFNγ production by SIRT2$^{+/+}$, SIRT2$^{+/-}$ and SIRT2$^{-/-}$ splenocytes. Cells were stimulated with TSST-1, PMA and ionomycin, concanavalin A, anti-CD3/CD28, PHA and SEB, another staphylococcal toxin acting as a SAg. Unfortunately, the phenotype previously observed in response to TSST-1 was lost in the F2 progeny (**Additional Figure 1** and data not shown). Moreover, flow cytometry analyses of TCR-Vβ repertoire expressed by thymocytes and splenocytes, and of I-A^{b} expression by DCs, B cells and monocytes/macrophages did not reveal significant differences between SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice (data not shown).



Additional Figure 1. Comparison of the response of SIRT2$^{+/+}$, SIRT2$^{+/-}$ and SIRT2$^{-/-}$ splenocytes in an F2 study. Splenocytes from SIRT2$^{+/+}$ and SIRT2$^{-/-}$ parental mice (left) and from SIRT2$^{+/+}$, SIRT2$^{+/-}$ and SIRT2$^{-/-}$ mice of F2 study (right) were incubated for 48 hours with 5 µg/ml LPS, 2 µg/ml CpG ODN 1826, 2 µg/ml TSST-1, 50 ng/ml PMA and ionomycin, 5 µg/ml concanavalin A (ConA), 1 µg/ml anti-CD3/CD28, 5 µg/ml phytohemagglutinin (PHA) and 5 µg/ml SEB. IL-2 production was quantified by ELISA. Data are means ± SD of triplicates determinations with 4-6 mice per group. Comparisons between the different groups were performed by unpaired t test using PRISM (GraphPad Software).

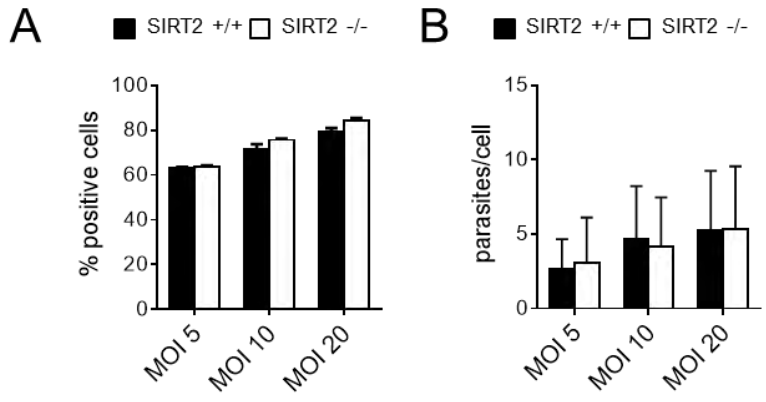
We conclude from these experiments that the observed impaired response to TSST-1 by SIRT2$^{-/-}$ splenocytes was due to some genetic drift rather than to a SIRT2-dependent specific effect. Therefore, new SIRT2$^{+/+}$ and SIRT2$^{-/-}$ breeders were obtained from our collaborators (Prof J. Auwerx, EPFL, Lausanne, Switzerland) to start new colonies. Additionally, animals were backcrossed twice before using them in all the experimental settings reported in this manuscript.

Impact of SIRT2 on *Leishmania Mexicana* Phagocytosis by BMDMs

Given the observation that SIRT2$^{-/-}$ BMDMs phagocytose bacteria better than SIRT2$^{+/+}$ BMDMs, we questioned whether a similar phenotype exists for other kinds of pathogens. To investigate this area, we collaborated with the laboratory of Prof Fabienne Tacchini-Cottier (Department of Biochemistry, University of Lausanne, Switzerland) that is specialized in studying the pathogenesis of leishmaniasis.

SIRT2$^{+/+}$ and SIRT2$^{-/-}$ BMDMs were incubated for 4 hours with constitutive red fluorescent protein (mCherry)-expressing *Leishmania mexicana* at a multiplicity of infection (MOI) of 5, 10 and 20 before determining by flow cytometry the percentage of BMDMs that phagocytosed *Leishmania*. Cells were also seeded onto poly-lysine coated coverslip and stained with Diff Quick to quantify by microscopy the number of parasites ingested per BMDMs. As summarized in **additional Figure 2**, no significant differences were observed between SIRT2$^{+/+}$ and SIRT2$^{-/-}$ BMDMs. The reason why SIRT2$^{-/-}$ BMDMs do not phagocyte more parasites than SIRT2$^{-/-}$ BMDMs while they phagocyte better bacteria might be due to differences in size of the

microorganisms and processes involved in their uptake. Of note, the above experiments were performed with parasites collected in the stationary phase. In the late stationary phase, *Leishmania* differentiates into a more rare and difficult to obtain metacyclic form, considered to be the infectious form. Additional tests could use metacyclic parasites.



Additional Figure 2. SIRT2 knockout does not affect phagocytosis of *Leishmania mexicana* by BMDMs. SIRT2 $^{+/+}$ and SIRT2$^{-/-}$ BMDMs were exposed for 4 hours to *L. Mexicana*, expressing the mCherry red-fluorescent protein, at MOI 5, 10 and 20. **A**, The percentage of positive cells was determined by flow cytometry. Data are mean ± SD of quadruplicate measures from one experiment. **B**, BMDMs were seeded onto poly-lysine coated coverslip, fixed and stained with Diff Quick. The number of parasite/cell was counted in randomly selected 100 cells. Data are mean ± SD of quadruplicate measures from one experiment.

4 CONCLUSIONS AND PERSPECTIVES

4.1 Propionate, short chain fatty acids, microbiome, and innate immune defenses

Symbiosis between the microbiota and the host is a complex phenomenon, whose relevance in human health and diseases just starts being appreciated. Multiple studies have been conducted to unravel the mechanisms underlying the benefits of host-flora interactions and the relationship between dysbiosis and pathological conditions such as cancer, inflammatory bowel diseases, asthma, obesity and malnutrition related complications (379-386). It has been proposed to target the microbiota either directly or through the diet to ameliorate disease conditions. Indeed, diet contributes to determine the composition of the gut flora (387-389). Additionally, commensal species produce in different proportions metabolites that in turn promote or inhibit the expansion of some microbial species (390).

The human gut flora is composed of bacteria from three main phyla: Bacteriodetes, Firmicutes and Actinobacteria (391, 392). SCFAs are produced in the large bowel through fermentation of non-digestible fibers and from unabsorbed carbohydrates and proteins. Propionate is one of the most abundantly produced SCFA, together with butyrate and acetate. Propionate is produced via at least three biochemical pathways: 1) the succinate pathway performed by Bacteriodetes and Firmicutes that represents the main source of propionate, 2) the acrylate pathway carried out by few strains of Firmicutes (Veillonellaceae and Lachnospiraceae families) and 3) the propanediol pathway that is more widespread and achieved by several strains of Firmicutes (393). SCFAs have trophic, antioxidant and anticarcinogenic activities, but also anti-inflammatory properties, an aspect especially well characterized mainly for butyrate (96, 394-399).

Two studies reported the inhibition by propionate of the LPS response of human THP-1 and murine RAW 264.7 macrophage cell lines (400, 401). More recently evidence arose in BMDMs and BMDCs (402). We show that propionate inhibits cytokine and chemokine production in a stimulus and cell type specific manner in innate immune cells. Previous studies reported similar cell-dependent differences for the effects of SCFAs (96, 395). Additionally, the impact of propionate on LPS-induced chemokine and cytokine production is stronger on mature than on immature human moDCs (403). The reasons why similar SCFAs mediate different effects using identical stimulatory conditions are unknown. Discrepancy may result from the fact that SCFAs proceed through distinct mechanisms. SCFAs act via the metabolite sensing receptors (GPCRs)

GPR41, GPR43 and GPR109A and diffuse into cells to inhibit class I and II HDACs. GPCRs are unequally expressed by immune cells, and a comprehensive analysis of SCFA sensing by GPCRs is missing. For instance, GPR109A is expressed at higher level than GPR41 and GPR43 by human moDCs (403), while GPR43 is more expressed than GPR41 in THP-1 cells and primary human neutrophils and monocytes (400). One may speculate that the expression patterns of GPCRs underlie differential effects of SCFAs. Additionally, different GPCRs may lead to differential intracellular signaling due to the coupling to different G proteins. Trimeric G proteins are composed of α, β and γ subunits and are classified into four subtypes based on the structure and function of α subunits: G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ (404). GPR43 is coupled to G_q and $G_{i/o}$, while GPR41 and GPR109A are coupled only to $G_{i/o}$ (405). Although signaling downstream of G proteins depends on several factors, like associated receptors and ligands, $G_{i/o}$ is commonly believed to inhibit the activity of adenylate cyclase, the enzyme that forms cyclic AMP (cAMP) from ATP. G_q interacts with the phospholipase Cβ (PLCβ) that, once activated, hydrolyzes the cytosolic phosphatidylinositol 4,5-bisphosphate (PIP2). This leads to the production of inositol-1,4,5-trisphosphate (IP3) that promotes Ca^{2+} flux, and of 1,2-diacylglycerol (DAG) that activates the protein kinase C (406, 407). A further level to be considered is the affinity of different SCFAs for GPCRs. *In vitro* cell-based analyses have demonstrated that propionate similarly binds to GPR41 and GPR43 and more potently than acetate (408).

Modulation of innate cell response by propionate could be partially or fully mediated by inhibition of HDACs. Propionate nicely inhibits the production of IL-6 and IL-12p40, but not TNF, by murine macrophages. A similar differential impact on TNF versus other cytokines has been observed with other HDACi (96, 105, 409, 410). Moreover, despite the evidence that propionate activates signaling (cAMP and Ca^{2+} flux) downstream GPR41 and GPR43 stronger than any other SCFAs (408), propionate is less effective than butyrate at reducing cytokine production by macrophages, DCs and whole blood, as shown here and by others (402, 403, 411). Further supporting the idea that propionate acts primarily as a HDACi is the observation that propionate induces Ca^{2+} influx (as consequence of GPR43 activation) in neutrophils but not in monocytes, while strongly inhibiting MCP-1 and IL-10 production (412). Running experiments using GPR41, GPR43, GPR109A knockout mice or cells would definitely answer whether or not these receptors participate in mediating the anti-inflammatory activity of propionate reported in our models. Of note, bone marrow chimera experiments between wild type mice and GPR41 and $GPR43^{-/-}$ mice revealed that the role of these receptors in immune responses against *Citrobacter rodentium* is more relevant in non-bone marrow derived cells that in bone marrow derived cells (413).

While butyrate and valproate protect mice from lethal sepsis and septic shock (96, 100, 414, 415), propionate has no impact on morbidity parameters and mortality in a panel of preclinical lethal models including endotoxemia and *K. pneumoniae*, *S. aureus* and *C. albicans* sepsis. Moreover, propionate has no impact on models of mild infection with *E. coli* and *C. albicans*. These data are reassuring in the perspective of developing therapies based on propionate or diet. Still, propionate should be tested in additional, non-stringent models of infections taking place at other anatomical sites (gut, kidney, skin..). Indeed, propionate dampens inflammation during airway allergy, and other SCFAs such as acetate and butyrate protect from colitis (371, 372, 395, 416).

The discrepancy between the clear anti-inflammatory activity of propionate *in vitro* and the absence of effect *in vivo* might be due to opposite effects on different cell types. Indeed, *in vitro* studies cannot recapitulate the complex response of a whole organism. We mainly focus on macrophages and DCs. Yet, propionate promotes neutrophil rolling, adherence, chemotaxis, migration, superoxide production and cell death (408, 417-419).Therefore, the impact of propionate on neutrophils should be addressed in our models of infection.

Another important aspect that should be considered is the influence of propionate on the adaptive immune system. Mice that survived *K. pneumonie* and *S. pneumonia* infection had lower anti-bacteria IgG titers. To address the relevance of this observation, a model of re-infection, in which mice with or without propionate treatment are re-exposed to a sublethal dose of pathogen, is planned. Another approach could be to collect serum or T-cells from propionate treated and untreated mice infected with a non-lethal dose of bacteria (or with inactivated bacteria), and test whether transferring serum or T-cells from propionate treated mice is less efficient at protecting naïve mice from a lethal infection with the same bacteria.

The reduced production of anti-bacteria antibodies by propionate treated mice reises questions about the impact of propionate on adaptive immune parameters. Many experiments can be envisaged such as addressing the migratory and antigen presentation abilities of APCs, the skewing of the T helper response and the interaction between APCs and T cells or T cells and B cells. It is known that intestinal microbiota and their metabolites influence T cell subset development in the gut and systemically. For example, propionate and butyrate, but not acetate, promote the generation of extrathymic Tregs and butyrate induces Tregs in the colon and confers protection from colitis (370, 374, 416). High levels of propionate in the gut are also associated with hematopoietic generation of macrophage and DC precursors that colonize lungs, hamper the generation of a Th2 response and protect from airway allergies (375). Yet, propionate, butyrate and acetate

can trigger the differentiation of naïve T cells into either Th1, Th17 or Treg cells according to the inflammatory environment (373, 413). Thereby, it would be interesting to test whether in our models of infection propionate treatment skews a particular T cell program. This could be achieved by tracking, through intracellular flow cytometry, cytokine expression by spleen and lymph node $CD4^+$ and $CD8^+$ T cells in order to calculate the proportions and absolute numbers of Th1, Th2, Th17 and Treg cells.

Finally, we analyzed the effect of propionate exclusively from the host point of view. We do not know whether propionate favors or disadvantages in any way the pathogens used in our experiments. For example, it is known that enterohemorrhagic *E. coli* upregulates virulence factors and adherence in the presence of SCFAs (420) and that *Nesseria meningitidis* expresses a gene cluster that allows the uptake and usage of propionate, thus conferring *Nesseria meningitidis* a selective growth advantage in the mouth which is rich in propionate (421). Propionate also positively (up to 3 mM) or negatively (3-50 mM) affects biofilm formation by *Actinomyces naeslundi* (422).

4.2 Cambinol and innate immune responses

Cambinol is a powerful anti-inflammatory drug *in vitro* and *in vivo* that protects from lethal sepsis in preclinical models. The anti-inflammatory properties of cambinol have been confirmed in independent studies. Cambinol reduces NF-κB activation and TNF, IL-6 and RANTES production by LPS-stimulated J77.4 mouse macrophages (423). Of interest, no effect on p38 phosphorylation at 30 min was observed in J77.4 macrophages, while we observed that cambinol inhibits p38, ERK1/2 and JNK activation in RAW 264.7 macrophages. Cambinol and its analog sirtinol reduce airway allergic inflammation by impairing DC migration to the bronchial lymph nodes and Th2 response. Additionally, cambinol reduces platelet aggregation and granule release (424).

In our study, the immunomodulatory properties of cambinol were not recapitulated using SIRT1-specific (CHIC-35 and EX-527) and SIRT2-specific (AGK-2 and AK-7) inhibitors used alone or in combination. We therefore speculate that cambinol acts by targeting more than just SIRT1 and SIRT2. The broader than supposed specificity of cambinol, and of inhibitors in general, may be due to the fact that drug discovery uses R&D approaches far from physiological conditions. In the case of cambinol, sirtuin genes were cloned into expression plasmids, expressed and purified in bacteria. The deacetylation assays were performed in a cell free system, incubating a chemically acetylated peptide with purified sirtuins in the presence of NAD^+ (179). Interestingly, a target of cambinol in primary neurons has been recently identified as neutral

sphingomyelinase 2 (nSMase2). The inhibitory activity of cambinol for nSMase2 in neurons is 10-fold stronger than that for SIRT1 and SIRT2. (425). While cambinol remains a promising drug for treating oncologic and inflammatory diseases, the analysis of the impact of sirtuins on immune responses requires more specific systems. Therefore, we used SIRT2 full knockout mice to analyze the impact of this sirtuin on innate immune responses.

4.3 Impact of sirtuin 2 deficiency on innate immune responses

We demonstrate that SIRT2 deficiency increases the phagocytosis of inert polystyrene beads, heat-inactivated bacteria and live bacteria by macrophages. SIRT2 targets microtubules and more specifically deacetylates α-tubulin on lysine 40 (123). Although there is evidence that acetylation of α-tubulin is associated with microtubule stability, a proof of concept is missing. Moreover acetylation status of α-tubulin impacts on the recruitment of motor proteins (kinesins/dyneins) and severing enzymes (katanin and members of the closely related enzyme subfamilies spastin and fidgetin), and on the conformation of the α-tubulin–β-tubulin dimers (426).

Structure and function of cytoskeleton play a key role in phagocytosis. Many studies aimed at deciphering the role of cytoskeleton in phagocytosis use cytoskeleton inhibitors. One of the first observations was that PMNs treated with colchicine or vinblastine, that prevent microtubule polymerization, phagocytize less polystyrene beads (427). During the phagocytosis of polystyrene particles of 14 µm of diameter, both microtubules, formed by tubulin, and microfilaments, composed of actin, are associated to the membrane. Microtubules extend from the membrane to the interior area of the cell after challenge (428). Yet, microtubules are highly dynamic and display a rapid turnover rate (429). Interestingly, activation of human monocytes and macrophages with LPS augments microtubule stability by increasing the levels of tubulin and microtubules associated proteins (MAPs) (430).

The fact that SIRT2 deacetylates α-tubulin goes well with the observed decreased phagocytosis by SIRT2-deficient BMDMs. To further characterize the impact of SIRT2 during phagocytosis, it would be interesting to compare microtubule stability, acetylation status and subcellular localization dynamics in $SIRT2^{+/+}$ and $SIRT2^{-/-}$ macrophages undergoing phagocytosis. This could be performed using confocal and time-lapse microscopy, and by measuring phagocytosis in the presence of cytoskeleton and/or SIRT2 inhibitors. Yet, one may envisage that microtubules are not directly involved in the phagocytic process. There is evidence that microtubules are present throughout the cytoplasm except in those regions where pseudopodia rich in

actin microfilaments are engulfing particles. This is because microfilament distribution is regulated by microtubules (431). Furthermore as actin is also involved in phagocytosis (432, 433), we might test whether SIRT2 is capable of deacetylating actin and whether this impacts on phagocytosis. Other interesting avenues of research can be proposed. Since the endolysosome maturation requires complex interactions with microtubules (431, 434), killing efficiency of bacteria should be analyzed. Microtubules are important for the formation of lipid rafts (435), and an additional hypothesis would be that destabilization of microtubules in SIRT2-deficient cells reduces lipid raft formation, clustering of phagocytic receptors and phagocytosis, regardless of the equal expression of phagocytic receptors observed in $SIRT2^{+/+}$ and $SIRT2^{-/-}$ macrophages.

Increased phagocytosis was observed in SIRT2-deficient BMDMs but not in BMDCs or neutrophils, suggesting that SIRT2 has cell specific functions. The fact that HDAC6 deacetylates α-tubulin and co-immunoprecipitates with SIRT2 puts forward that these 2 enzymes cooperate to deacetylate tubulin (436). Hence, differences in HDAC6 expression or activity in different cell types might influence the impact of SIRT2 deficiency. Therefore, we will make a comparative expression study of HDAC6 and SIRT2 in phagocytic cells.

Interestingly, we demonstrate that impaired phagocytosis has consequences *in vivo*, since SIRT2-deficiency protects mice from chronic infection by *S. aureus*. Similar results in other models of chronic infection, such as pneumonia or candidiasis, would strengthen our finding. "*In vivo*" phagocytosis could be performed by delivery of fluorescent beads in the airways of mice and subsequent measurement of their uptake by macrophages recovered from bronchial alveolar lavages. A system allowing the real time follow-up of bacterial dissemination *in vivo* might give more insights into the impact of SIRT2 deficiency. In that context, we just installed in our P2 animal facility an In-Vivo Xtreme II Optical/X-ray small animal imaging system (Bruker). We are currently setting-up conditions for noninvasively imaging in mice infected with a luminescent strain of *S. pneumoniae* engendered with lux transposon cassette, Tn4001 luxABCDE Kmr (437).

4.4 SIRT2 deficiency and formation of neutrophils extracellular traps (NETs)

Neutrophils are specialized in microbial phagocytosis and killing (438). In 2004 neutrophils were shown to produce NETs that bind, neutralize and kill extracellular pathogens (38). NETs are formed by neutrophil granular and nuclear components (elastase, myeloperoxidase, DNA and histones). NETs interact with a variety of gram-positive and gram-negative pathogens and prevent the dissemination of microorganisms (439). The production of NETs has been associated with cell death (NETosis). Nevertheless, whether NET production provokes NETosis or is compatible with viability is not completely clear as both phenomena have been described (440).

Considering that the extrusion of NETs might require important cytoskeleton dynamics, we hypothesized that SIRT2 deficiency could affect this process. To test our hypothesis, we purified neutrophils from $SIRT2^{+/+}$ and $SIRT2^{-/-}$ bone marrow and analyzed LPS-induced NETs. As shown in **Figure 1**, $SIRT2^{-/-}$ neutrophils produce less NETs when compared with $SIRT2^{+/+}$ neutrophils.

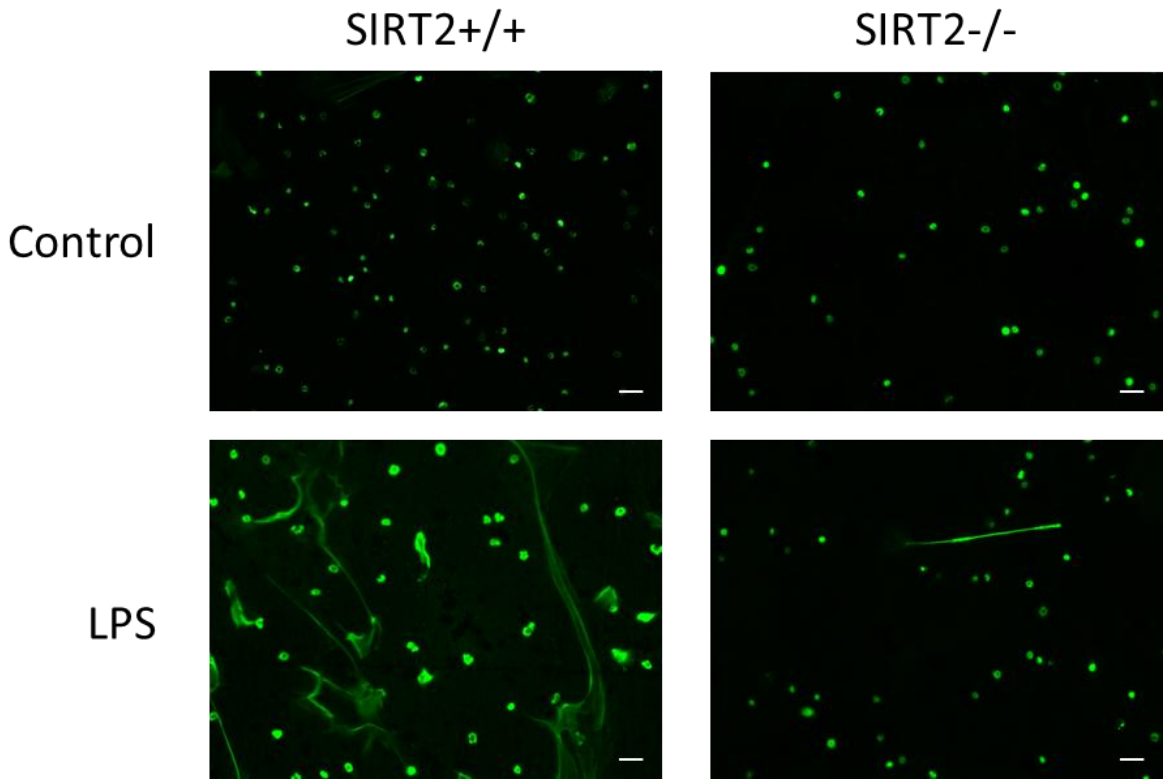


Figure 1. SIRT2 deficiency impairs the formation of extracellular trap by neutrophils. $SIRT2^{+/+}$ and $SIRT2^{-/-}$ neutrophils were purified using the Neutrophil Isolation Kit from Miltenyi Biotech, seeded onto poly-lysine coated coverslips and stimulated for 4 hours with 10 ng/ml LPS. Cells were fixed with 4% PFA. NETs were stained with SYTOX® and pictures acquired with a Zeiss Axio Imager Z1 Microscope. Original magnification 63 ×. Scale bar indicates 100 µm. Data are from one representative of three neutrophil preparations.

To confirm our data, we then detected the production of NETs by SIRT2$^{+/+}$ and SIRT2$^{-/-}$ live neutrophils by differential staining of dead cells and NETs with SYTOX® Orange (**Figure 2A**) and live neutrophils with SYTO® green (**Figure 2B**). This experiment confirmed that, upon LPS stimulation, SIRT2$^{-/-}$ neutrophils produce less NETs when compare with SIRT2$^{+/+}$ neutrophils. Further investigations are required to confirm these promising preliminary data, to identify the underlying mechanisms and to address the *in vivo* relevance of this observation.

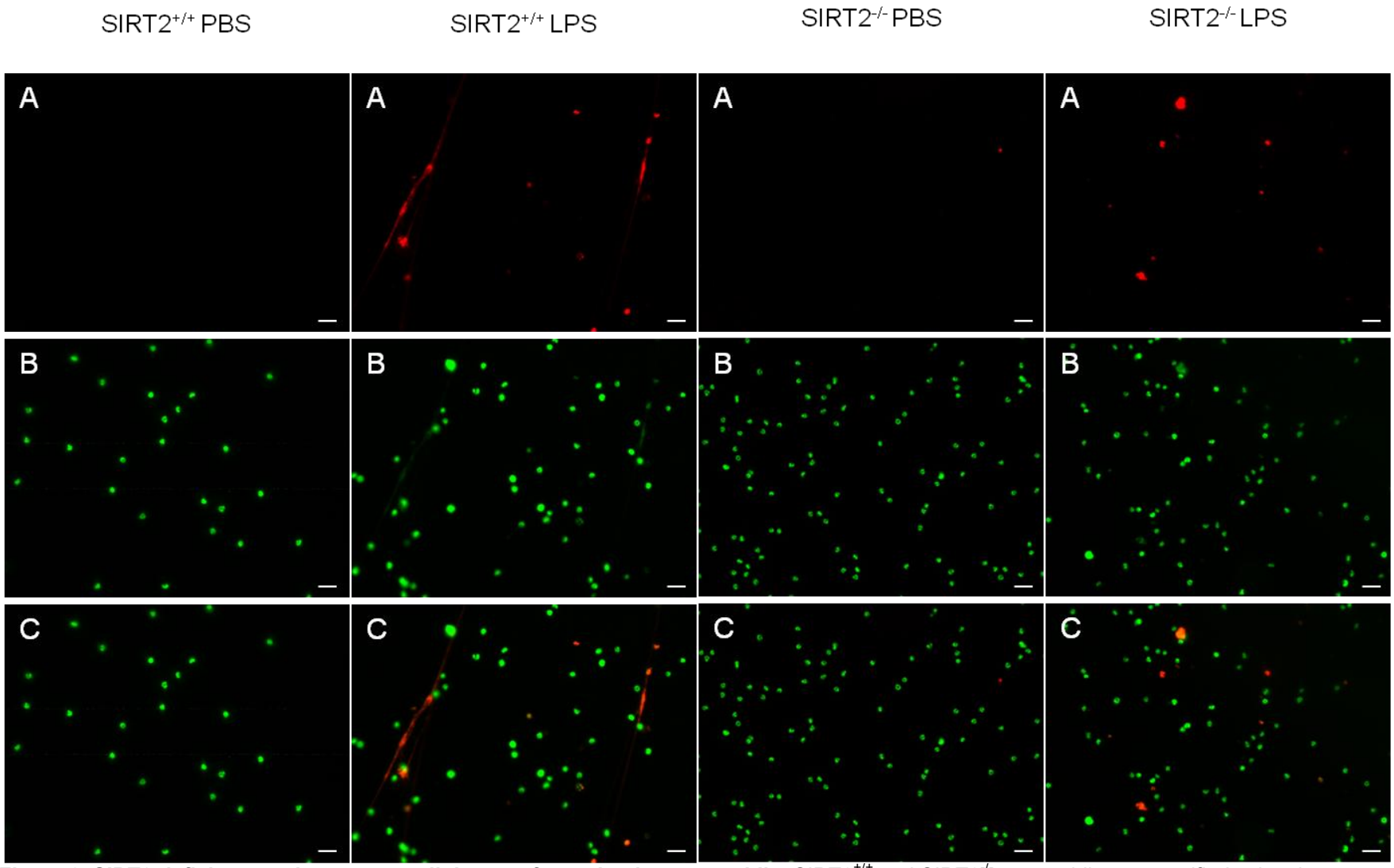


Figure 2. SIRT2 deficiency reduces extracellular trap formation by neutrophils. $SIRT2^{+/+}$ and $SIRT2^{-/-}$ neutrophils were purified using the Neutrophil Isolation Kit from Miltenyi Biotech, seeded onto poly-lysine coated coverslip and stimulated for 4 hours with 10 ng/ml LPS. **A**, Dead cells and NETs were stained with SYTOX® Orange. **B**, Live neutrophils were stained with SYTO® green. **C**, Overlay of **A** and **B**. Pictures were acquired with a Zeiss Axio Imager Z1 Microscope. Original magnification 63 ×. Scale bar indicates 100 µm. Data are from one experiment.

4.5 SIRT3 knockouts and the development of SIRT2/SIRT3 double knockout mice

Oxidative stress and immune responses are interconnected through ROS and reactive nitrogen species (RNS) generated by immune cells. ROS and RNS are not only toxic for pathogens, but also activate MAPK and NF-κB pathways that direct the expression of immune genes, a role more specifically attributed to mitochondrial ROS (mtROS) (441, 442). Moreover ROS are involved in the activation of the NLRP3 inflammasome that controls IL-1β and IL-18 processing (442-444).

SIRT3 is the main mitochondrial deacetylase. SIRT3 has been shown to be involved in the maintenance of mitochondrial homeostasis and to play an important role in dampening oxidative stress and ROS production (445). SIRT3 reduces cellular ROS levels dependent on superoxide dismutase 2 (SOD2), a major mitochondrial antioxidant enzyme that is acetylated at two critical lysines: K53 and K89 (376). Moreover, inducers of the NLRP3 inflammasome break mitochondrial homeostasis and diminish the concentration of NAD^+. This leads to the activation of SIRT2 and to the accumulation of acetylated α-tubulin. Acetylated α-tubulin mediates the recruitment of the adaptor protein ASC and NLRP3 inflammasome complex formation (446). Thus, we hypothesized that SIRT3 controls ROS production influencing inflammasome activation and IL-1β secretion, possibly in collaboration with SIRT2.

We studied the impact of SIRT3 deficiency on oxidative stress, generation of mtROS and inflammatory and immune responses of macrophages. Although extensive studies have not be performed yet, we obtained some encouraging results showing increased production of active IL-1β, yet normal expression of CASP-1, by $SIRT3^{-/-}$ BMDMs exposed to nigericin and ATP. Should our observations be confirmed, additional experiments will be designed to investigate whether non-canonical NLRP3 inflammasome-associated or inflammasome-independent caspases, such as CASP-8 and CASP-11, are involved (447). Furthermore, we plan to quantify mtROS and total ROS, inflammasome assembly and cytokine production (with a focus on IL-1β and IL-18) by $SIRT3^{+/+}$ $SIRT3^{-/-}$ BMDMs, BMDCs and granulocytes. An *in vivo* approach would be to compare IL-1β levels in the peritoneal cavity of $SIRT3^{+/+}$ and $SIRT3^{-/-}$ mice injected with uric acid.

As mentioned above SIRT3 is the main mitochondrial sirtuin. Moreover, SIRT2 is the most expressed sirtuin in myeloid cells (see section **5.6**). Considering that sirtuins might have compensatory effects and that both SIRT3 and SIRT2 are involved in inflammasome activation, we decided to generate a double

SIRT2$^{-/-}$ and SIRT3$^{-/-}$ knockout mouse line. To that end, we crossed SIRT2$^{-/-}$ and SIRT3$^{-/-}$ mice. F1 animals were crossed to obtain SIRT2$^{-/-}$/SIRT3$^{-/-}$ mice in the F2 population. The expected Medelian ratio of double knockout in F2 is 1/16 (**Figure 3**) and 1/32 for a specific sex. We genotyped 312 F2 mice and identified 7 female and 3 male double knockouts. We used these mice to establish a new mouse line that we are currently breading in SPF conditions in our animal facility. These animals will be used to test the impact of SIRT2/SIRT3 deficiency on innate immune responses and inflammasome activity.

	SIRT2 $^{+/+}$ SIRT3 $^{+/+}$	SIRT2$^{+/+}$ SIRT3 $^{-/-}$	SIRT2 $^{-/-}$ SIRT3 $^{+/+}$	SIRT2 $^{-/-}$ SIRT3 $^{-/-}$
SIRT2 $^{+/+}$ SIRT3 $^{+/+}$	SIRT2 $^{+/+}$ SIRT3 $^{+/+}$	SIRT2 $^{+/+}$ SIRT3 $^{+/-}$	SIRT2 $^{+/-}$ SIRT3 $^{+/+}$	SIRT2 $^{+/-}$ SIRT3 $^{+/-}$
SIRT2 $^{+/+}$ SIRT3 $^{-/-}$	SIRT2 $^{+/+}$ SIRT3 $^{+/-}$	SIRT2 $^{+/+}$ SIRT3 $^{-/-}$	SIRT2 $^{+/-}$ SIRT3 $^{+/-}$	SIRT2 $^{+/-}$ SIRT3 $^{-/-}$
SIRT2 $^{-/-}$ SIRT3 $^{+/+}$	SIRT2 $^{+/-}$ SIRT3 $^{+/+}$	SIRT2 $^{+/-}$ SIRT3 $^{+/-}$	SIRT2 $^{-/-}$ SIRT3 $^{+/+}$	SIRT2 $^{-/-}$ SIRT3 $^{+/-}$
SIRT2 $^{-/-}$ SIRT3 $^{-/-}$	SIRT2 $^{+/-}$ SIRT3 $^{+/-}$	SIRT2 $^{+/-}$ SIRT3 $^{-/-}$	SIRT2 $^{-/-}$ SIRT3 $^{+/-}$	SIRT2 $^{-/-}$ SIRT3 $^{-/-}$

Figure 3. Punnett square of F1 gametes and expected frequencies of F2 genotypes. Any SIRT2$^{-/-}$ SIRT3$^{-/-}$ should appear with 1/16 of frequency. For a chosen sex the frequency is 1/32.

4.6 Impact of SIRT2 on immune responses under metabolic stress and model of high fat diet

Sirtuins require NAD^{+} for their enzymatic activity and therefore are intrinsically coupled to the metabolic status of the cell. Sirtuins are involved in the control of energy homeostasis and glucose and lipid metabolic pathways (**Table 6**, **section 1.6.10**). Considering that metabolism plays a central role in controlling immune responses and that metabolic changes occur during immune cell activation, we hypothesized that sirtuins represent a bridge between metabolism and immune response. Therefore, we started to study the role of SIRT2 knockout under metabolic stress conditions.

Weaned mice were fed *ad libitum* with normal diet (ND; 10% of Kcal provided by fat mass) or high fat diet (HFD; 60% of Kcal provided by fat mass) for 20 weeks and weight was monitored regularly. HFD induced a significant increase of weight of SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice compared with ND (**Figure 4A**). SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice gain weight at a similar extent as shown by similar HFD/ND ratios (**Figure 4B**). Not

surprisingly, mice under HFD had increased blood levels of cholesterol and triglycerides (**Figure 4C**, left panels). Interestingly, hepatic transaminases were significantly more elevated in HFD compared with ND in SIRT2$^{+/+}$ mice but not in SIRT2$^{-/-}$ mice (**Figure 4C**, right panels). When we analyzed the spleens from SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice, we did not observe any significant differences in the proportions and absolute numbers of T cells and B cells (**Figure 5A and 5B**), except for Foxp3^{+} Tregs. Indeed, the percentage of Tregs was decreased by HFD in SIRT2$^{+/+}$ mice but not in SIRT2$^{-/-}$ mice (**Figure 5C**).

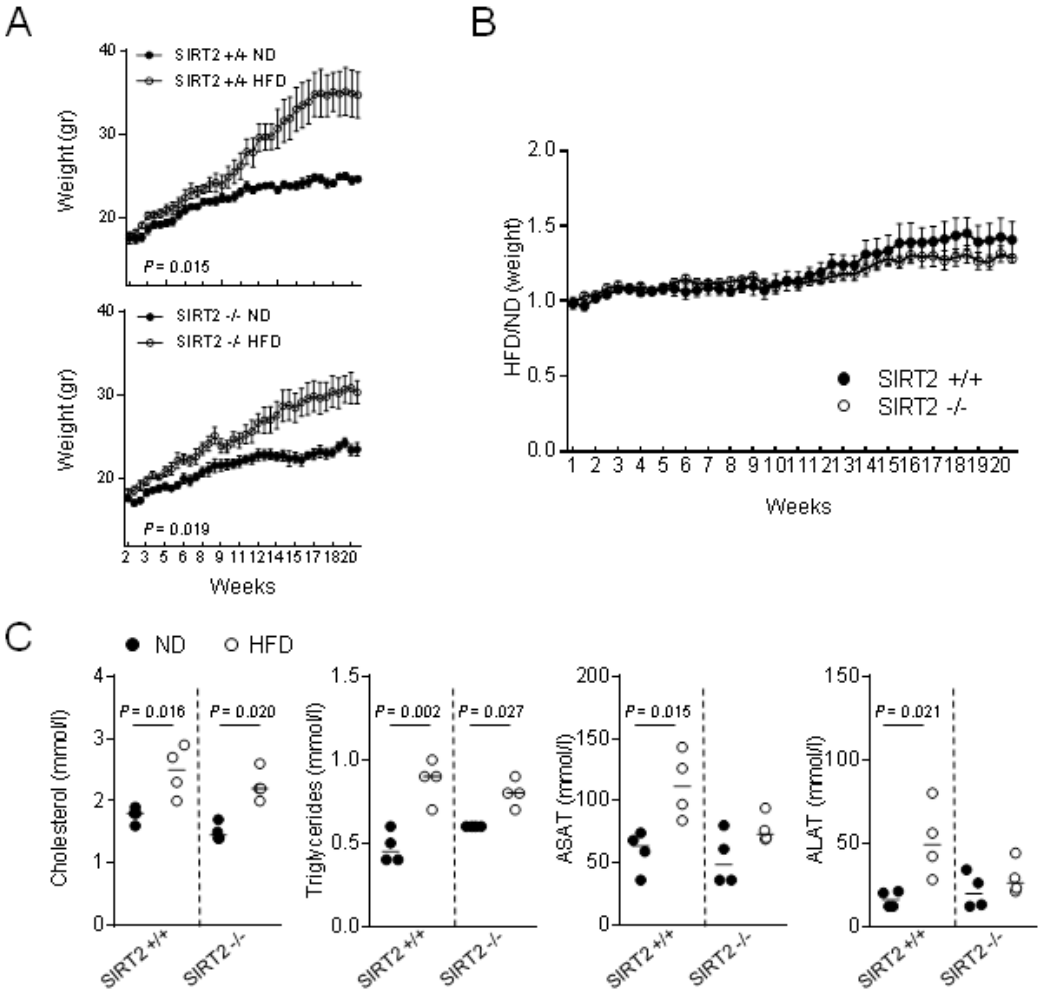


Figure 4. High fat diet regimen increases body weight and alters blood parameters. A, Weight of SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice fed under normal diet (ND) or high fat diet (HFD) for 20 weeks (n = 4 per group). Data are mean ± SEM. **B**, The HFD/ND ratio was calculated. Statistical significance was assessed by unpaired two-way ANOVA using PRISM. **C**, Mice were sacrificed after 20 weeks of diet. Blood levels of cholesterol, triglycerides, ASAT and ALAT were measured. Data are single values from one experiment. Comparisons between the different groups were performed by unpaired t test using PRISM.

The study of the impact of SIRT2 on immune responses under metabolic stress condition is particularly relevant considering the close and complex relationships between sirtuins, metabolism and immunity. Moreover, it may reveal immune phenotypes otherwise hidden under normal diet conditions. In the first experiment presented here, splenic Tregs were reduced in HFD $SIRT2^{+/+}$ mice, but not in their $SIRT2^{-/-}$ counterparts. Inhibition of Treg accumulation in the spleen of HFD-fed mice was previously reported (448). Moreover, HFD induces the depletion of hepatic Tregs, promoting nonalcoholic, endotoxin-induced steatohepatitis (449). Whether SIRT2 deficiency prevents liver injury and steatosis to steatohepatitis transition by maintaining Treg cells and dampening liver inflammation would certainly be an interesting subject of research. We have conserved samples of liver and other organs of the different experimental groups that will allow us to quantify proinflammatory and anti-inflammatory parameters in ND and HFD $SIRT2^{+/+}$ and $SIRT2^{-/-}$ mice. Of note, SIRT2 promotes adipolysis, by deacetylating FOXO1 in adipocytes, (327) and microtubule remodeling during adipocyte maturation (450), both mechanisms that may also participate to steatosis. To further investigate the role of SIRT2 in immune responses under metabolic stress conditions, we will concentrate on *in vivo* models of infection post-diet. Considering that sirtuins were pointed by several studies as mediators of the beneficial effect of calorie restriction and that SIRT2 expression increases in the liver and adipose tissue of mice under calorie restriction (451), it would be particularly relevant to assess the impact of SIRT2 knockout in sepsis models in mice under calorie restriction. Finally, considering that SIRT3 expression in several tissues is increased during calorie restriction and decreased in models of HFD (334, 452), we would also like to study the role of SIRT3 in immune responses and inflammasome activation under metabolic stress conditions (calorie restriction and HFD models).

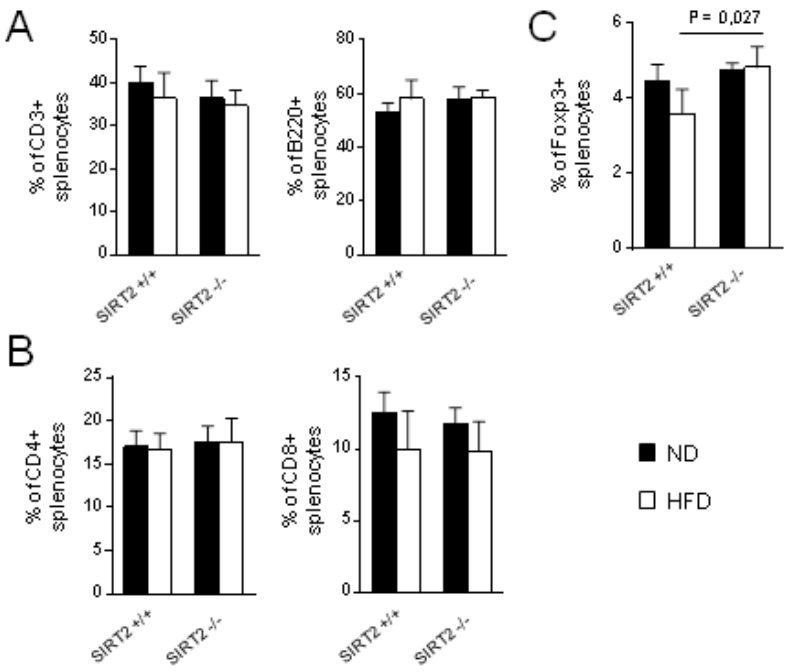


Figure 5. Foxp3^{+} regulatory T cells (Tregs) are increased in the spleen of SIRT2$^{-/-}$ mice under HFD but not under ND conditions. Spleens of SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice fed under ND or HFD were collected, splenocytes isolated and the proportions of CD3^{+}, B220^{+} (**A**), CD8^{+}, CD4^{+} (**B**) and Foxp3^{+} (**C**) cells were determined by flow cytometry. Data are mean ± SD (n = 4). Comparison between the different groups was performed by unpaired t test using PRISM.

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6 APPENDIX

I participated to the appendixed article by performing stimulation of BMDCs and analyzing cytokine production by Luminex (**Figure 4**).

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INNATE IMMUNE SENSING OF *Fusarium culmorum* BY MOUSE DENDRITIC CELLS

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Chronic inhalation of grain dust is associated with asthma and chronic bronchitis in grain worker populations. Exposure to fungal particles was postulated to be an important etiologic agent of these pathologies. *Fusarium* species frequently colonize grain and straw and produce a wide array of mycotoxins that impact human health, necessitating an evaluation of risk exposure by inhalation of *Fusarium* and its consequences on immune responses. Data showed that *Fusarium culmorum* is a frequent constituent of aerosols sampled during wheat harvesting in the Vaud region of Switzerland. The aim of this study was to examine cytokine/chemokine responses and innate immune sensing of *F. culmorum* in bone-marrow-derived dendritic cells and macrophages. Overall, dendritic cells and macrophages responded to *F. culmorum* spores but not to its secreted components (i.e., mycotoxins) by releasing large amounts of macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, monocyte chemoattractant protein (MCP)-1, RANTES, and interleukin (IL)-12p40, intermediate amounts of tumor necrosis factor (TNF), IL-6, IL-12p70, IL-33, granulocyte colony-stimulating factor (G-CSF), and interferon gamma-induced protein (IP-10), but no detectable amounts of IL-4 and IL-10, a pattern of mediators compatible with generation of Th1 or Th17 antifungal protective immune responses rather than with Th2-dependent allergic responses. The sensing of *F. culmorum* spores by dendritic cells required dectin-1, the main pattern recognition receptor involved in β-glucans detection, but likely not MyD88 and TRIF-dependent Toll-like receptors. Taken together, our results indicate that *F. culmorum* stimulates potently innate immune cells in a dectin-1-dependent manner, suggesting that inhalation of *F. culmorum* from grain dust may promote immune-related airway diseases in exposed worker populations.

Chronic inhalation of environmental microscopic fungal particles at high concentrations is associated with persistent lung inflammation and respiratory disorders in different occupational populations (Poole et al., 2012; Vacher et al., 2014). In particular, grain workers who are regularly exposed to grain dust (a complex mixture of fungal particles, bacteria, insect compounds, animal wastes, inorganic compounds–silicates, chemicals, gases, and fumes) are known to have reduced lung volume and display symptoms commonly associated with chronic bronchitis, asthma, and asthma-like disorder (Linaker and Smedley, 2002; Rask-Andersen, 2011; Eduard et al., 2009).

One fungal genus in grain dust is of particular concern for human health: *Fusarium*. This genus includes a number of economically important plant pathogenic species that are responsible for crown rot, root rot, and *Fusarium* head blight (FHB), a

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Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/uteh

destructive disease of cereal grain crops worldwide (Bottalico and Perrone, 2002; Lanoue et al., 2010; Stephens et al., 2008). Among the *Fusarium* species, *F. graminearum, F. culmorum, F. avenaceum,* and *F. poae* produce a range of mycotoxins that are a serious human health concern when ingested (Bottalico and Perrone, 2002; Foroud and Eudes, 2009; Grenier and Applegate, 2013). The trichothecenes such as deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV), T-2 toxin, and fusarenon-X and the estrogenic metabolite zearalenone (ZEA) are mycotoxins for whose cytotoxicity and immunosuppressive activities are the best described (Bondy and Pestka, 2000; Antonissen et al., 2014). Curiously, although *Fusarium* species are likely to be present in grain dusts, little is known regarding their frequencies in grain aerosols or their capacity to elicit innate immune responses and impact human health upon inhalation.

The development of microbial-induced airway pathologies is largely conditioned by the initial local immune response. Innate immune cells such as alveolar macrophages lining the respiratory tract and dendritic cells (DC) located within the epithelium and interstitium of the lungs form the primary line of defenses against invading microorganisms. These cells sense microorganisms via pattern recognition receptors (PRR) that are specialized in the recognition of so-called microbial associated molecular patterns (MAMP), in the case of fungi principally β-glucans, mannans, and chitin. The most important families of PRR involved in the sensing of fungal MAMP are C-type lectin receptors (CLR, such as dectin-1, dectin-2, DC-SIGN, the mannose receptor, the mannose binding lectin, mincle, and galectin-3), Toll-like receptors (TLR, mainly TLR2, TLR4, and TLR9), and Nod-like receptors (NLR) (Romani, 2011; Brown, 2011). Dectin-1 is the primary receptor involved in sensing of β-glucans, whereas TLR2 senses phospholipomannans, TLR4 O-branched mannans and glucoronoxylomannan, and TLR9 fungal DNA (Brown and Gordon, 2001; Jouault et al., 2003; Shoham et al., 2001; Netea et al., 2006). Upon microbial sensing, innate immune cells produce a panel of cytokines, chemokines, and other immunomodulatory molecules that play a key role in initiation, amplification, and regulation of innate and adaptive immune responses. Dendritic cells (DC) orchestrate these events by shaping the polarization of naive CD4+ T helper (Th) cells into Th1, Th2, or Th17 effector T cells that exert different impacts on antifungal responses. Th1 and Th17 cells promote protective immunity and fungal clearance, while Th2 cells favor development of fungal allergic responses (Romani, 2011).

Although an increasing number of environmental fungal species are associated with allergic and nonallergic respiratory disorders when inhaled, the innate immune responses triggered and mechanisms underlying their recognition by innate immune cells remain poorly investigated. Given this context, the aim of this study was to characterize the response of innate immune cells, in particular DC, to *F. culmorum,* identified as the most common *Fusarium* species present in wheat-associated aerosols from the Vaud area of Switzerland. This investigation also undertook to identify the PRR involved in the sensing of *F. culmorum* by DC.

MATERIALS AND METHODS

Sampling of Fungi From Wheat Dust

Aerosols were collected on gelatin filters (25 mm, Sartorius AG, Gottingen, Germany) during wheat harvesting between July 15 and August 15, 2010, in 44 wheat fields in the Vaud region of Switzerland. Sampling was performed at a flow rate of 2 L/min using pocket pumps (MSA Escort Elf, Mine Safety Appliance Company, Pittsburgh, PA, or SKC pocket pump 210-1002, SKC, Inc., Eighty-four, PA) and clear styrene cassettes with 3 sections (25 mm diameter, SKC, Inc.). The pumps were placed on the combine harvester, 3 m above the soil surface. The temperature, barometric pressure, and relative air humidity were measured at each sampling site with a thermo-hygrometer and

barometer PCE-THB 40 (PCE Group Iberica, Albacete, Spain).

DNA Extraction and *Fusarium* Species Identification

The gelatin filters were placed in 6 ml 0.9% NaCl, shaken at room temperature for 15 min, and centrifuged for 30 min at 8500 × g. The pellet was mechanically disrupted with a Tissue Lyser (Qiagen, Hombrechtikon, Switzerland) in the first buffer of the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH). Then DNA was extracted according to the manufacturer's instructions. Amplification of the four most frequent *Fusarium* species associated with FHB (*F. graminearum, F. culmorum, F. avenaceum,* and *F. poae*) was performed with validated species-specific primers pairs, respectively MGB-GRA, MGB-Cul, MGB-Ave, and Fp82 (Moradi et al., 2010). Polymerase chain reaction (PCR) reactions were performed using the peqGOLD Taq DNAPolymerase (PeqLab, Axon Lab AG, Baden-Dättwil, Switzerland), 1× PCR Buffer S, and 1× Enhancer Solution P using 5 µl DNA. The amplification conditions were described in Moradi et al. (2010).

Fungal Strain and Spores Suspension Preparation

Fusarium culmorum 2156 strain was kindly provided by the Agroscope, the Swiss Center of Excellence for Agricultural Research (Changins, Switzerland). *Fusarium culmorum* 2156 spores were grown on potato dextrose agar (PDA) plates (Oxoid, Basingstoke, UK) and cultured for 7 d at 23°C. Spores were collected by flushing PDA plates with 3 × 10 ml of sterile phosphate-buffered saline (PBS). The solution was filtered through large-mesh gauze to eliminate residual agar and centrifuged 10 min at 3500 × g. The pellet was resuspended in PBS and spores were enumerated. Spore suspensions were used immediately or kept for 24 h at 4°C or 22°C. Each spore suspension was divided into two equal parts: One was left untouched (called total preparation), whereas the other was centrifuged for 10 min at 3500 × g to separate the supernatant (SN) from spores (SP), which were resuspended in an equal volume of PBS. Inactivated spores were obtained by heating fresh suspensions of *F. culmorum* 2156 for 10 min at 100°C or 2 h at 56°C or by treatment for 10 min with 4% paraformaldehyde (PFA) and extensive washings.

Mycotoxin Extraction

Spores from three independent cultures were used. Mycotoxins were extracted from spores or supernatants using a methanol (MeOH)/Milli-Q water (70:30, v/v) solution by shaking for 10 min and sonication for 15 min with a Branson 5210 apparatus (Branson Ultrasonics S.A., Carouge, Switzerland). Extracts were filtered with a 10-kD, 15-ml, Vivaspin 4 Centrifugal Concentrator (Sartorius Stedim Biotech, Aubagne, France) and evaporated for 90 min using 10 psi nitrogen with a TurboVap LV system (Capiler LifeSciences, Moutain View, CA). The residue was dissolved in 950 µl MeOH/water (20:80, v/v).

LC-MS/MS Analyses

Analytical standards of DON, 3-ADON, 15-ADON, NIV, and ZEA at 100 µg/ml in acetonitrile were purchased from Fluka (Sigma-Aldrich, St Louis, MO). Internal standards of U-[$^{13}C_{15}$]-DON, U-[$^{13}C_{17}$]-3-ADON, U-[$^{13}C_{15}$]-NIV, and U-[$^{13}C_{18}$]-ZEA at 25 µg/ml in acetonitrile were obtained from ROMER Labs (Tulln, Austria). Mycotoxins were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a Varian 1200l mass spectrometer (Varian, Walnut Creek, CA). Twenty-five microliters of each sample, including ^{13}C internal standards (each at 1 µg/ml), was loaded onto an Ascensis Express F5 column (100 × 3.0 mm, 2.7 µm) joined to an Ascensis Express F5 Guard column (5 × 3.0 mm, 2.7 µm; SUPELCO Bellefonte, PA) using an automatic injector. Mycotoxins were eluted at a flow rate of 0.4 ml/min with a water/methanol mobile phase as follows (percent water/methanol): 0–5 min 80/20,

5–18.5 min 4/96, and 18.5 min 80/20. Eluents (LC-MS quality, Carlo Erba reagents, Val-de-Reuil, France) were buffered with 5 μ*M* ammonium acetate, pH 7.1. LC-MS/MS interface conditions for ionization of the mycotoxins in the negative mode were needle voltage –4500 V, nebulizing gas (compressed air) 51 psi, drying gas N_2 270°C and 19 psi, and shield voltage –600 V. MS parameters and collision cell energies are given in Table 1. Standard curves were generated based on six different concentrations of pure mycotoxins. The limits of detection (LOD) of DON, 3-ADON, 15-ADON, NIV, and ZEA were calculated based on signal-to-noise (S/N) ratios of 3:1 and were 0.43, 1.91, 2.01, 1.75, and 0.67 pg/mg spore, respectively. The limits of quantification (LOQ) of DON, 3-ADON, 15-ADON, NIV, and ZEA were calculated based on S/N ratios of 10:1 and were 1.43, 6.36, 6.71, 5.84, and 2.24 pg/mg spore, respectively.

Mice and Cells

Eight- to 10-wk-old female C57BL/6 mice weighing 20–25 g (Charles River Laboratories, L'Arbresle, France) were housed under specific-pathogen-free conditions. MyD88 and dectin-1 knockout mice ($MyD88^{-/-}$ and dectin-$1^{-/-}$) (Taylor et al., 2007; Kawai et al., 1999) on a C57BL/6 background were obtained from Professor Shizuo Akira (Osaka University, Japan) and Professor Gordon Brown (University of Aberdeen, UK). All animal procedures were approved by the "Office Vétérinaire du Canton de Vaud," Lausanne, Switzerland (authorization numbers 876.7 and 876.8) and performed according to our institution guidelines for animal experiments. Bone marrow cells were obtained from femurs and tibias and cultured for 6 d in IMDM medium (Invitrogen, San Diego, CA), pH 7.4, supplemented with 10% heat-inactivated fetal calf serum (FCS; Seromed, Berlin, Germany), 50 μ*M* 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and granulocyte–macrophage colony-stimulating factor to generate bone-marrow-derived DC (BMDC) or macrophage colony-stimulating factor to generate bone marrow-derived macrophages (BMDM) (Lugrin et al., 2013; Roger et al., 2007).

Cell Stimulation With *F. culmorum*

Loosely adherent immature BMDC were recovered by flushing with Versene (Invitrogen). BMDM were detached by incubation for 10 min at 4°C with Versene. Cells were washed with IMDM, counted, and seeded in 96-well cell culture plates (Corning Costar, Sigma-Aldrich) at a density of 2.5×10^5 cells per well. BMDC and BMDM obtained from three independent mice were pooled before seeding. Cells were exposed for 24 h to an equivalent multiplicity of infection (MOI, i.e., a spore/cell ratio) of 0.01, 0.1, 1, or 10 of *F. culmorum* 2156 preparations. As controls of cell responsiveness, cells were exposed to 10 ng/ml *Salmonella minnesota* ultrapure lipopolysaccharide (LPS; List Biologicals Laboratories, Campbell, CA),

TABLE 1. Mass Spectrometry Parameters Used for Mycotoxin Detection

Mycotoxin	Q1 (m/z)	Q3 (m/z)	Capillary voltage (V)	Collision energy (eV)
NIV	311	281	−55	10
^{13}C NIV	326	295	−53	10
DON	295.2	279	−60	10
^{13}C DON	310.1	279	−60	10
3-ADON	336	306	−55	10
^{13}C 3-ADON	354	322	−50	9
15-ADON	337	149.1	−50	12
ZON	317	131	−50	32
^{13}C ZON	335	140	−55	50

Note. Q1: precursor ion; Q3: main product ion.

2 μg/ml CpG motif containing oligonucleotide 1826 (CpG; InvivoGen, San Diego, CA), 10 μg/ml polyinosinic:polycytidylic acid (poly(I:C); InvivoGen), and 10 μg/ml *Saccharomyces cerevisiae* hot alkali-treated zymosan (InvivoGen). LPS, CpG, poly(I:C), and zymosan are TLR4, TLR9, TLR3, and dectin-1 agonists, respectively. Images from cell cultures were recorded at 40× magnification using an Eclipse TS 100 inverted microscope (Nikon, Tokyo, Japan) coupled to a Digital Sight DS-Fi1 Nikon camera (Nikon).

Cell Viability Assay

Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell proliferation and viability assay (Mombelli et al., 2011; Tawadros et al., 2013). Briefly, at the end of incubation with *F. culmorum* 2156, cells were incubated for 2 h with 1 mg/ml MTT. Medium was removed and replaced by 100 μl solution containing 10% sodium dodecyl sulfate (SDS), 5 m*M* HCl, and 66% (v/v) 2-propanol to lyse cells and dissolve formazan crystals. The absorbance was read at 570 nm using a Synergy H1 microplate reader (BioTek, Luzern, Switzerland). On each 96-well cell culture plate, serial quantities (0.3×10^4 –5×10^5) of cells left untreated were processed in parallel to establish a standard curve.

Quantification of Cytokines and Chemokines

Tumor necrosis factor (TNF), interleukin (IL)-6, and IL-12p40 concentrations in cell-culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Abingdon, UK) (Roger et al., 2011). A screening of mediators produced by *F. culmorum* 2156-infected BMDCs cells was performed with a mouse cytokine Bioplex assay (Bio-Rad, Hercules, CA) using Luminex technology (Luminex Corporation, Austin, TX) (Delaloye et al., 2009). Twenty-one mediators were tested: TNF, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-33, interferon (IFN) γ, interferon gamma-induced protein (IP-10; CXCL10), KC (CXCL1), macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), MIP-2 (CXCL2), MIP-3α (CCL20), monocyte chemoattractant protein (MCP)-1 (CCL2), RANTES (CCL5), granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and matrix metallopeptidase (MMP)-9.

Statistical Analyses

Comparisons between groups were performed by an analysis of variance (ANOVA) followed by Student's *t*-test. Two-sided *p* values less than .05 were considered to indicate statistical significance. All analyses were performed using PRISM (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Frequency of *Fusarium* Species in Bioaerosols From Wheat Harvesting

Aerosols were sampled during wheat harvesting in 44 wheat fields in the Vaud region of Switzerland. Species-specific PCR revealed that *F. culmorum, F. poae,* and *F. graminearum* were present in 54, 40, and 36% of aerosols, respectively. *Fusarium avenaceum* was not detected. Considering these frequencies, subsequent experiments were performed using *F. culmorum* 2156, a *Fusarium* strain highly aggressive on Swiss wheat cultivars, from the collections of the Swiss Center of Excellence for Agricultural Research (Agroscope, Changins).

Cytotoxic Effect of *Fusarium culmorum* Spores on Dendritic Cells

Dendritic cells play a critical role in sensing and processing of inhaled microorganisms and release of immunomodulatory mediators upon triggering by fungi (Roy and Klein, 2012). To establish the conditions for immune cell stimulation by *F. culmorum* 2156, mouse DC (i.e., BMDC) were exposed to increasing concentrations of freshly prepared suspensions of *F.*

culmorum 2156 spores and used as an indicator for production of IL-12p40, a cytokine that is usually abundantly secreted by DC exposed to microbial products. *Fusarium culmorum* 2156 was incubated with BMDC at ratios ranging from 1 to 1,000 spores per 100 cells (i.e., MOI 0.01 to MOI 10). IL-12p40 concentrations in cell culture supernatants collected after 24 h were measured by ELISA. IL-12p40 production followed a concentration-dependent bell-shaped response curve, with concentrations increasing between MOI 0.01 to MOI 1 and markedly decreasing using MOI 10 of *F. culmorum* 2156. Similar results were obtained for TNF and IL-6 production by BMDC or when using macrophages such as BMDM (data not shown) as responding cells, indicating that the effect was not specific to IL-12p40 or BMDC. Taken together, these results suggested that high concentrations of *F. culmorum* 2156 spores exert a toxic effect on innate immune cells. To test this hypothesis, the viability of BMDC was measured using the MTT assay. A significantly reduced cell survival was observed only when BMDC were exposed to *F. culmorum* 2156 at MOI 10 (36 ± 8% of the signal obtained with BMDCs cultured in control medium; Table 2).

The observed cell death might be attributed to toxic molecules contained in, or secreted by *F. culmorum* spores. Hence, cell death induced by spore (SP) and supernatant (SN) fractions that were obtained after centrifugation of *F. culmorum* 2156 suspensions was monitored. Moreover, to assess whether nongerminated and germinated *F. culmorum* 2156 behaved differently, spore and supernatant fractions were prepared from either fresh suspensions of *F. culmorum* 2156 or suspensions kept at 4°C or 22°C for 24 h before use (germination was confirmed by microscopic examination). Total, spore, and supernatant fractions exerted no marked cytotoxic effect on BMDC when used up to MOI 1 (Table 2, and data not shown). At MOI 10, the spore fraction reduced cell viability (39 ± 8% of the control) to an extent similar to the total preparation, whereas supernatants showed no cytotoxicity, suggesting that *F. culmorum* 2156 did not release cytotoxic mediators at effective biological concentrations under the conditions tested. Similar results were also obtained using nongerminated and germinated preparations of *F. culmorum* 2156.

TABLE 2. Cytotoxic effects of *Fusarium culmorum* on BMDCs

	Cell viability (% of signal in control medium)		
	MOI 0.1	MOI 1	MOI 10
Total preparation	111 ± 15	96 ± 5	36 ± 8*
Spores	116 ± 18	104 ± 4	39 ± 8*
Supernatant	124 ± 1	128 ± 4	103 ± 6

*$p < .05$.

Mycotoxins are present at high concentrations in agricultural dusts (Hardin et al., 2009). *Fusarium culmorum* produces trichothecene mycotoxins and the estrogenic metabolite ZEA, which are cytotoxic for mammalian cells in part because they inhibit protein synthesis (Bin-Umer et al., 2011; Zinedine et al., 2007; Behm et al., 2012). To determine whether *F. culmorum* 2156 spores release mycotoxins that might impact immune cell behavior, DON, 3-ADON, 15-ADON, NIV, and ZEA were extracted and quantified by LC-MS/MS from *F. culmorum* 2156 spore and supernatant fractions. DON was detected in supernatants (8 ± 2.4 ng/ml), 3-ADON in spores and supernatants (4 ± 1.5 pg/mg spores and 2.9 ± 1.2 ng/ml supernatant), and ZEA in spores (12.3 ± 6.6 pg/mg spores). No other mycotoxins were identified in the different fractions. Taking into consideration the cytotoxic effects induced by *F. culmorum* 2156 at MOI 10, the following experiments were performed using *F. culmorum* 2156 preparations at MOI 0.01–1.

Dendritic Cells (DC) Ingest *Fusarium* Spores and Produce Large Amounts of Cytokines and Chemokines

DC are professional phagocytic cells. Indeed, careful microscopic examination clearly showed that BMDC ingested *F. culmorum* 2156 spores (Figure 1). In order

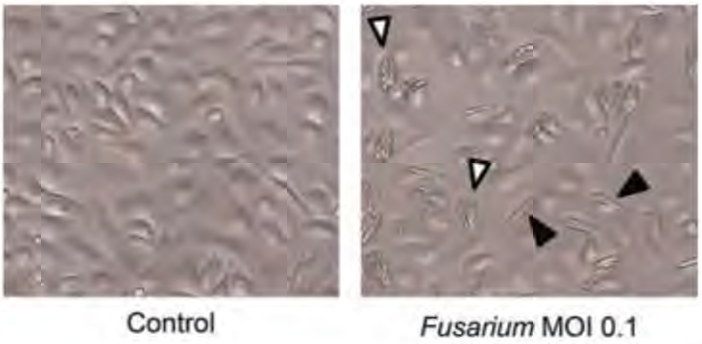

FIGURE 1. Ingestion of *Fusarium culmorum* spores by BMDCs. BMDCs were exposed for 24 h to freshly prepared *F. culmorum* 2156 spores equivalent to MOI 0 and 0.1. Images were recorded at 40× magnification. Close and open arrows point to BMDCs having ingested one and two spores, respectively. Note that the compartments of macroconidia exhibit extensive swelling, indicating germination, whereas no germ tube are visible.

to determine whether *Fusarium* spores or its secreted products stimulated production of polarizing cytokines by BMDC, total, spore, and supernatant fractions were tested. As shown in Figure 2, the total and spore but not supernatant fractions of both nongerminated and germinated *F. culmorum* 2156 concentration-dependently stimulated release of high amounts of IL-12p40 by BMDC. Total suspensions induced quantitatively more IL-12p40 than spore suspensions when using germinated preparations, which may result from shedding of immunoreactive cell wall components or intracellular components during germination. Low levels of IL-12p40 were eventually produced by BMDC exposed to supernatant fractions, which was associated with a few contaminating spores observed by microscopy.

To determine whether *Fusarium* spores required being alive to stimulate BMDC, the IL-12p40-inducing activity of live, heat-inactivated (100°C for 10 min and 56°C for 2 h) and PFA-inactivated spores was compared with PFA-inactivated spores. The inactivation process fully abrogated spore germination (Figure 3A), and markedly affected IL-12p40 production (Figure 3B). Indeed, heat- and PFA-inactivated *F. culmorum* 2156 spores stimulated the release of 2- to 18-fold lower levels of IL-12p40 than live spores. Of note, *Fusarium* spores germinated with

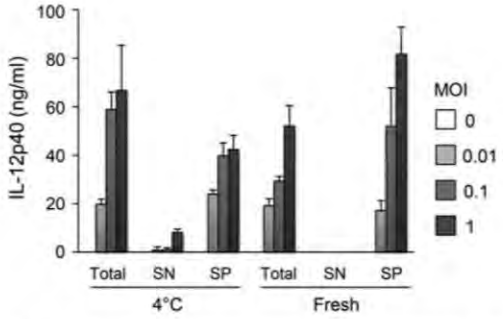

FIGURE 2. *Fusarium culmorum* spores induce the production of IL-12p40 by BMDCs. BMDCs were exposed for 24 h to increasing concentrations (equivalent to MOI 0, 0.01, 0.1, and 1) of *F. culmorum* 2156 fractions prepared as described in Materials and Methods. IL-12p40 concentrations were quantified by ELISA. Data are means ± SD of triplicate samples from one experiment performed with a pool of BMDCs obtained from three mice. Similar results were obtained in a second experiment. Total: whole spore suspension; SN: supernatant; SP: spores; Fresh: fractions were prepared from fresh suspensions of *F. culmorum* 2156; 4°C: fractions were prepared from suspensions of *F. culmorum* 2156 kept for 24 h at 4°C in PBS. Significant at $p = .02$ when comparing 4°C and fresh total vs. 4°C and fresh SP at MOI 0.1.

signs of swelling but essentially no germ tube production when incubated with BMDC (Figure 1). Thus, live spores appear to be more immunostimulatory than inactivated spores.

The cytokines produced by DC play a key role in shaping development of naive CD4+ T cells into Th1, Th2, or Th17 effector T cells that affect differentially antifungal

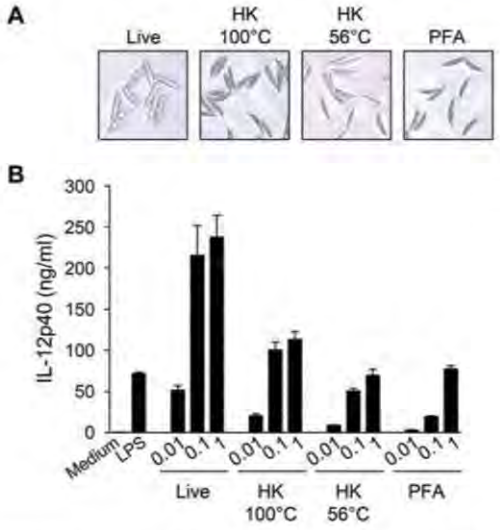

FIGURE 3. Inactivated *Fusarium culmorum* spores induce the production of reduced levels of IL-12p40 by BMDCs. (A) *Fusarium culmorum* 2156 spores were inactivated by heating (by incubation for 10 min at 100°C or 2 h at 56°C) or PFA treatment. Live and inactivated spores were cultured in IMDM for 24 h. Images were recorded at 40× magnification. BMDCs were exposed for 24 h to increasing concentrations (equivalent to MOI 0, 0.1, and 1) of freshly prepared live and inactivated spores or LPS (10 ng/ml). (B) IL-12p40 concentrations were quantified by ELISA. Data are means ± SD of triplicate samples from one experiment performed with a pool of BMDMs obtained from 3 mice. HK: heat killed.

responses. Th1 and Th17 responses facilitate fungal clearance, whereas Th2 responses are involved in development of allergic reactions. To have a general overview of the mediators secreted by BMDC stimulated with *F. culmorum* 2156, the profile of cytokines (TNF, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-33, IFNγ, GM-CSF, and G-CSF), chemokines (IP-10/CXCL10, KC/CXCL1, MIP-1α/CCL3, MIP-1β/CCL4, MIP-2/CXCL2, MIP-3α/CCL20, MCP-1/CCL2, and RANTES/CCL5) and MMP-9 was analyzed using the Luminex technology (Figure 4). This panel included cytokines involved in skewing or amplifying Th1 (IL-12p70, IFNγ, TNF), Th2 (IL-4, IL-10), and Th17 (IL-6, CCL20) responses and powerful neutrophil chemoattractants (IP-10 and KC). Eleven of 21 mediators were detectable in cell-culture supernatants collected 24 h after stimulation. As previously observed with IL-12p40 (Figure 2), total and spore preparations stimulated cytokine/chemokine release to a similar extent, while supernatants were mostly inactive. Interestingly, *F. culmorum* 2156 spores induced abundant quantities (20–90 ng/ml) of MIP-1α, MIP-1β, MIP-2, MCP-1, and RANTES, and lower but still appreciable quantities (0.8–2.5 ng/ml) of TNF, IL-6, IL-12p70, IL-33, G-CSF, and IP-10.

Fusarium culmorum Stimulates BMDCs Through Dectin-1 but Not MyD88

Dectin-1, TLR2, TLR4, and TLR9 are major receptors involved in the sensing of fungi by innate immune cells (Romani, 2011; Brown, 2011). All TLR except TLR3 trigger intracellular signaling and cytokine/chemokine production through recruitment of adaptor molecule MyD88 (Savva and Roger, 2013). The involvement of these TLR in immune responses may be indirectly monitored by assessing the impact of MyD88 deficiency. TLR3, but also TLR4, triggers signaling through the adaptor molecule TRIF involved in the production of type-I IFN and IFN-inducible genes (Savva and Roger, 2013). To investigate whether TLR and dectin-1 participated in the detection of *F. culmorum* 2156 by innate immune cells, the production of IL-12p40 by wild-type, MyD88$^{-/-}$, TRIF$^{-/-}$, and dectin-1$^{-/-}$ BMDCs exposed was compared (Figure 5). The TLR4 and TLR9 agonists LPS and CpG were used as controls of MyD88 deficiency, the TLR3 agonist poly(I:C) as a control of TRIF deficiency, and zymosan (β1,3-glucans) as a control of dectin-1 deficiency. *Fusarium culmorum* 2156 induced IL-12p40 release by wild-type and knockout BMDCS in a concentration-dependent manner. Dectin-1$^{-/-}$ BMDCs produced approximately twofold less IL-12p40 than wild-type BMDC, whereas MyD88$^{-/-}$ and TRIF$^{-/-}$ BMDC generated amounts of IL-12p40 similar to wild-type BMDC (Figures 5A, 5B, and 5C). Thus, evidence indicates that dectin-1 but not MyD88 or TRIF-dependent TLRs was necessary for recognition *F. culmorum* 2156 by BMDC.

DISCUSSION

Results demonstrated that *F. culmorum* spores activate innate immune cells at least in part through a dectin-1-dependent pathway to release significant quantities of chemokines and pro-inflammatory cytokines, with a pattern of mediators more likely compatible with generation of Th1 or Th17 antifungal responses than with Th2 allergic responses. Interestingly, *F. culmorum* was the most represented of *Fusarium* spp. in aerosols sampled during wheat harvesting in the Vaud region of Switzerland, followed by *F. poae* and *F. graminearum*, whereas *F. avenaceum* was not detected. To our knowledge, no prior study analyzed the spectrum of *Fusarium* species in wheat dust aerosols using molecular techniques. However, the same *Fusarium* species were previously isolated using a culture-based method in a limited number of settled grain dust samples collected from farms in eastern Poland (Krysinska-Traczyk et al., 2001).

Exposure of DC and macrophages to high concentrations of *F. culmorum* 2156 spores (i.e., MOI 10) was associated with increased rate of cell death. Considering that *Fusarium* spp. may secrete mycotoxins with cytotoxic effects, the concentrations of mycotoxins expressed by *F. culmorum* 2156 were quantified by LC-MS/MS. These analyses revealed that DON was detected in supernatants, ADON in spores and supernatants, and ZEA in spores of *F. culmorum* 2156 preparations. Yet the concentrations of DON, 3-ADON, and ZEA were far lower than concentrations of mycotoxins previously reported to inhibit proliferation of innate immune cells in vitro. Indeed, the IC_{20} of DON, 3-ADON, 15-ADON, and NIV ranged from 150 to 1500 ng/ml, whereas the IC_{50} values of ZEA and its metabolites α-zearalenol and β-zearalenol were approximately 3200 ng/ml against mouse macrophages (Pestka, 2008; Yu et al., 2011; Lu et al., 2013). With respect to DC, the IC_{50} of NIV against BMDC was 200 ng/ml. BMDC were not sensitive to DON (up to 1200 ng/ml), contrary to porcine monocyte-derived DC (IC_{50} DON = 800 ng/ml) (Luongo et al., 2010; Bimczok et al., 2007). Although there are species- and cell-type-specific differences in the cytotoxic effects of mycotoxins, and mycotoxins derived from complex mixtures may exert synergistic effects (Alassane-Kpembi et al., 2013), it appears that the decreased viability of BMDC observed here was unrelated to production of DON, 3-ADON, 15-ADON, NIV, and ZEA by *F. culmorum* 2156. Although one cannot exclude that *F. culmorum* 2156 produced mycotoxins that were not analyzed in the present study (e.g., fusarenon-X), a more likely explanation is that innate immune cells underwent cell death due to overstimulation by fungal spores used at high MOI, as previously observed upon overstimulation of DC with microbial ligands (Zanoni et al., 2009). In agreement with this assumption, BMDC exposed to lower MOI released elevated amounts of cytokines.

Fungal toxins, particularly trichotecens, may possess immunomodulatory functions (Bondy and Pestka, 2000; Antonissen et al., 2014). However, our observations suggested that *F. culmorum* 2156 did not release mycotoxins in sufficient quantities to alter cytokine production by BMDC. Indeed, BMDC and BMDM exposed to nontoxic MOIs of *F. culmorum* 2156 produced large amounts of cytokines. Further, higher concentrations of DON, 3-ADON, and ZEA than the concentrations reached in our system were required to modulate cytokine production by human Jurkat T cells and porcine epithelial cells (Pestka et al., 2005; Taranu et al., 2014).

Activation of naive Th cells by DC requires three signals: (1) stimulation of T cells through recognition of antigenic peptides presented by major histocompatibility complex (MHC) molecules on DC by the T-cell receptor (TCR) expressed by T cells, (2) co-stimulation of T cells primarily by interaction of CD28 on T cells with CD80 or CD86 expressed by DCs, and (3) polarization of T cells by cytokines. Endocytosis is a prerequisite for antigen processing by antigen-presenting cells (APC) such as DC and macrophages. The fact that BMDC ingested *Fusarium* spores supports the hypothesis that APC present *Fusarium*-derived antigenic

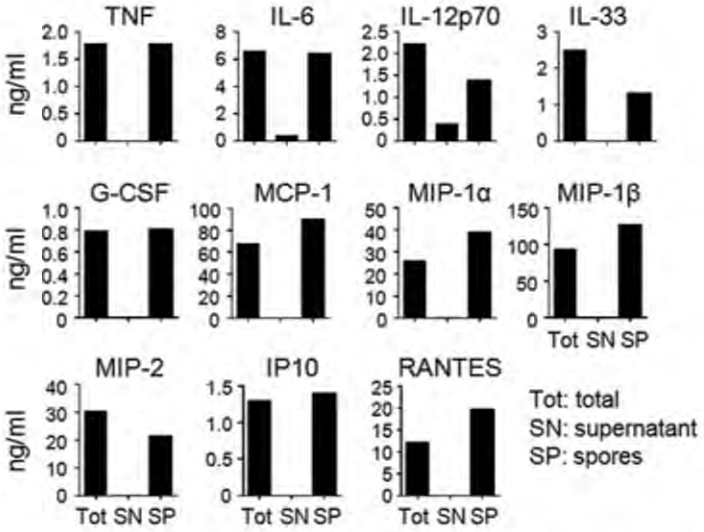

FIGURE 4. *Fusarium culmorum* spores induce the production of a large panel of cytokines and chemokines by BMDCs. BMDCs were exposed for 24 h to fresh preparations of *F. culmorum* 2156 used at an equivalent MOI 1. The concentrations of TNF, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-33, IFNγ, IP-10 (CXCL10), KC (CXCL1), MIP-1α (CCL3), MIP-1β (CCL4), MIP-2 (CXCL2), MIP-3α (CCL20), MCP-1 (CCL2), RANTES (CCL5), MMP-9, GM-CSF, and G-CSF were determined by Luminex. Results are single measurements of the 11 mediators that were produced at detectable levels in cell culture supernatants upon *F. culmorum* 2156-stimulation. Tot: whole spore suspension; SN: supernatant; SP: spores.

peptides to T cells. Although analysis of whether DC increased expression of co-stimulatory molecules upon infection was not determined, this is likely occurring considering the potent immunogenicity of *Fusarium*. Indeed, innate immune response induced by *F. culmorum* in BMDC was characterized by marked chemokine production and a less abundant cytokine generation.

Overall, the pattern of cytokines and chemokines secreted by *F. culmorum*-infected BMDC suggested that BMDC favored antifungal protective immune responses rather than allergic responses, since IL-12p70, TNF, IL-6, and CCL20 were readily detected whereas IL-4 and IL-10 were not measurable. In agreement with our results, IFNγ and IL-17 expression was elevated and IL-4 not detected in human corneal tissues infected with *F. solani* (Karthikeyan et al., 2011). Grain workers frequently develop asthma or asthma-like symptoms. Asthma has long been considered as a Th2-mediated pathology, yet recent studies also involved Th17 cells in asthma development (Manni et al., 2014). The mechanisms underlying predominant Th2, Th17, or Th2/Th17 mixed responses are still to be defined. Grain workers also suffer from chronic bronchitis-like symptoms. Chronic bronchitis develops after prolonged exposure to sufficient concentrations of fungal spores and is characterized by excessive inflammation and neutrophil influx in the airways. Although extrapolating our observations to pathologies prevalent in grain workers needs to be considered with caution, cytokines and chemokines induced by *F. culmorum* might be pathophysiologically relevant.

BMDC sensed *F. culmorum* 2156 at least partly through dectin-1, but not through MyD88 and TRIF-dependent pathways. β-Glucans, the primary dectin-1 agonists, are usually not exposed to surface of resting conidia, but the fungal cell wall is a dynamic structure changing during morphological transitions with influence on immune responses. For example, *Aspergillus fumigatus* swollen and germinating conidia stimulated more innate immune cells than dormant spores, which correlated

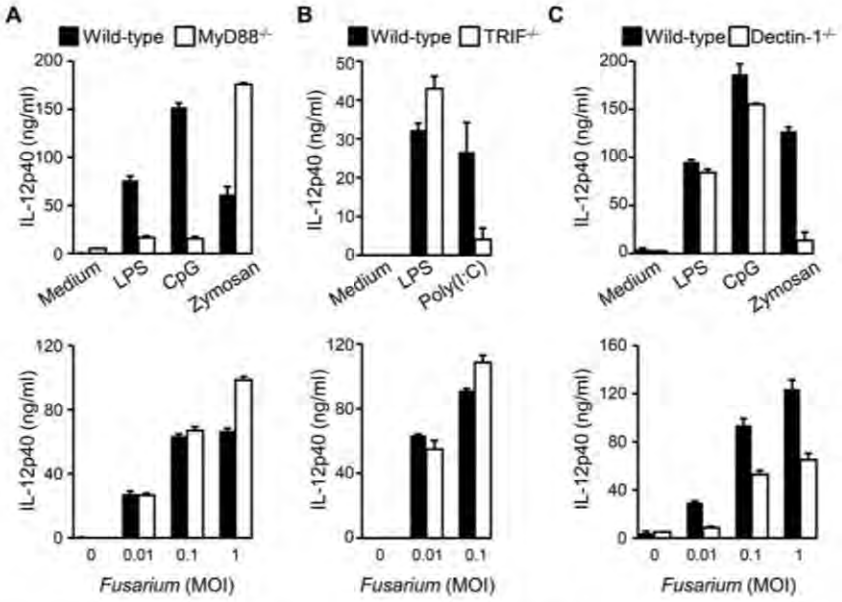

FIGURE 5. *Fusarium culmorum* stimulates IL-12p40 production by BMDCs in a dectin-1-dependent manner, but not MyD88 or TRIF-dependent manners. Wild-type and MyD88$^{-/-}$ BMDCs (A), wild-type and TRIF$^{-/-}$ BMDCs (B), and wild-type and dectin-1$^{-/-}$ BMDCs (C) were exposed for 24 h to LPS (10 ng/ml), CpG (2 μg/ml), poly(I:C) (10 μg/ml), zymosan (10 μg/ml), and increasing concentrations (equivalent to MOI 0, 0.01, 0.1 and 1) of freshly prepared *F. culmorum* 2156 spore suspensions. IL-12p40 concentrations were quantified by ELISA. Data are means ± SD of triplicate samples from one experiment performed with a pool of BMDMs obtained from three mice. Similar results were obtained in a second experiment (panels A and C).

with increased levels of surface-exposed β1,3-glucans (Steele et al., 2005; Hohl et al., 2005). Unfortunately, nothing is apparently known about possible differences in the cell wall structure of resting and germinating *Fusarium culmorum* spores. Yet the cell wall of *Fusarium* spp contains β1,3-glucans, α1,3-glucan, and chitin (Schoffelmeer et al., 1999), and *F. culmorum* spores germinated during the culture process used in this study. Moreover, inactivation of *F. colmurum* spores using three different protocols abrogated spore germination and reduced spore-induced cytokine production by BMDC. It is conceivable that living *F. colmurum* spores are more immunogenic than inactivated spores because they expose β-glucans during germination.

Besides C-type lectin receptors, TLR are involved in recognition of diverse fungal components (Brown, 2011; Romani, 2011). Interestingly, TLR4 deficiency impaired neutrophil recruitment to corneal stroma and worsened *Fusarium oxysporum*-induced keratitis (Tarabishy et al., 2008), indicating that different PRR are involved in the sensing of diverse *Fusarium* species or that PRR have cell-type/tissue specific roles. Differential PRR requirement may also condition the cytokine environment generated by DC and impact subsequent immune responses. Indeed, interaction of dectin-1 with TLR is required to amplify production of IL-1β, IL-6, and IL-23 and downregulate IL-12 in DC. This shift in cytokine response favors balance toward Th17 responses at the cost of Th1 responses (Vautier et al., 2010). The fact that the response of dectin-1$^{-/-}$ BMDCs to *F. culmorum* was not fully blunted suggests that additional PRR are involved in fungal detection. Among the possible candidates are complement receptor 3 and scavenger receptors (CD5, CD36, and SCARF1) that bind β-glucans, and mannose receptor, dectin-2, dectin-3, DC-SIGN, and mincle that sense mannans (Vacher et al.,

2014). It should also be mentioned that alveolar macrophages or lung DC might respond differently to *Fusarium* spores than BMDC and that species specificity might exist in host recognition of fungi (Rubino et al., 2012). Further studies are required to fully elucidate the pattern of PRR involved in the sensing of *F. culmorum* in the airways.

In summary, data showed that *F. culmorum* spores activate innate immune cells, at least in part through a dectin-1-dependent pathway, to release significant quantities of chemokines and proinflammatory cytokines. Although the pathophysiological relevance of this observation for grain worker diseases remains to be demonstrated, the cytokine/chemokine induced by *F. culmorum* may well play a role in the initial events that drive lung inflammation and promote airway pathologies upon grain dust inhalation. Further in vitro and in vivo investigations with additional fungal strains are required in order to have a better understanding of *F. culmorum* mycotoxin and allergen production, cytotoxicity, and immunomodulatory activities with a special emphasis on T helper responses and innate immune recognition by PRR other than dectin-1. In addition, epidemiologic and immunogenetic studies would greatly expand our understanding of possible associations between *Fusarium* species prevalence and genetic polymorphisms with lung diseases related to grain handling (Vacher et al., 2014). Broadening the understanding of the pathogenesis of organic dust-induced disorders may help to improve or develop diagnostic and treatment strategies.

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