Year : 2011

HISTONE DEACETYLASE INHIBITORS IMPAIR INNATE IMMUNE RESPONSES

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Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

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Lausanne 2011

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale

Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

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le Conseil de Faculté autorise l'impression de la thèse de

Monsieur Jérôme Lugrin

Master of Science Université de Lausanne

intitulée

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Lausanne, le 30 septembre 2011

pour Le Doyen
de la Faculté de Biologie et de Médecine

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SUMMARY

The innate immune system plays a central role in host defenses against invading pathogens. Innate immune cells sense the presence of pathogens through pattern recognition receptors that trigger intracellular signaling, leading to the production of pro-inflammatory mediators like cytokines, which shape innate and adaptive immune responses. Both by excess and by default inflammation may be detrimental to the host. Indeed, severe sepsis and septic shock are lethal complications of infections characterized by a dysregulated inflammatory response.

In recent years, members of the superfamily of histone deacetylases have been the focus of great interest. In mammals, histone deacetylases are broadly classified into two main subfamilies comprising histone deacetylases 1-11 (HDAC1-11) and sirtuins 1-7 (SIRT1-7). These enzymes influence gene expression by deacetylating histones and numerous non-histone proteins. Histone deacetylases have been involved in the development of oncologic, metabolic, cardiovascular, neurodegenerative and autoimmune diseases. Pharmacological modulators of histone deacetylase activity, principally inhibitors, have been developed for the treatment of cancer and metabolic diseases. When we initiated this project, several studies suggested that inhibitors of HDAC1-11 have anti-inflammatory activity. Yet, their influence on innate immune responses was largely uncharacterized. The present study was initiated to fill in this gap.

In the first part of this work, we report the first comprehensive study of the effects of HDAC1-11 inhibitors on innate immune responses *in vitro* and *in vivo*. Strikingly, expression studies revealed that HDAC1-11 inhibitors act essentially as negative regulators of basal and microbial product-induced expression of critical immune receptors and antimicrobial products by mouse and human innate immune cells like macrophages and dendritic cells. Furthermore, we describe a new molecular mechanism whereby HDAC1-11 inhibitors repress pro-inflammatory cytokine expression through the induction of the expression and the activity of the transcriptional repressor Mi-2β. HDAC1-11 inhibitors also impair the potential of macrophages to engulf and kill bacteria. Finally, mice treated with an HDAC inhibitor are more susceptible to non-severe bacterial and fungal infection, but are protected against toxic and septic shock. Altogether these data support the concept that HDAC1-11 inhibitors have potent anti-inflammatory and immunomodulatory activities *in vitro* and *in vivo*.

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine that plays a central role in innate immune responses, cell proliferation and oncogenesis. In the second part of this manuscript, we demonstrate that HDAC1-11 inhibitors inhibit MIF expression *in vitro* and *in vivo* and describe a novel molecular mechanism accounting for these effects. We propose that inhibition of MIF expression by HDAC1-11 inhibitors may contribute to the antitumorigenic and anti-inflammatory effects of these drugs.

NAD^+ is an essential cofactor of sirtuins activity and one of the major sources of energy within the cells. Therefore, sirtuins link deacetylation to NAD^+ metabolism and energy status. In the last part of this thesis, we report preliminary results indicating that a pharmacological inhibitor of SIRT1-2 drastically decreases pro-inflammatory cytokine production (RNA and protein) and interferes with MAP kinase intracellular signal transduction pathway in macrophages. Moreover, administration of the SIRT1-2 inhibitor protects mice from lethal endotoxic shock and septic shock.

Overall, our studies demonstrate that inhibitors of HDAC1-11 and sirtuins are powerful anti-inflammatory molecules. Given their profound negative impact on the host antimicrobial defence response, these inhibitors might increase the susceptibility to opportunistic infections, especially in immunocompromised cancer patients. Yet, these inhibitors might be useful to control the inflammatory response in severely ill septic patients or in patients suffering from chronic inflammatory diseases.

AKNOWLEDGEMENTS

First I would like to express my gratitude to the members of the jury, Professor François Spertini, president of the jury, Doctor Thierry Roger, thesis director, Professor Thierry Calandra, thesis co-director, Professor Regine Landmann and Professor Jacques-Antoine Haefliger both external experts.

I am grateful to Dr. Thierry Roger for giving me the opportunity to work on the hot topic that is the role of histone deacetylase inhibitors as modulator of innate immune responses. It was a great chance for me at that time to find a challenging work in a stimulating environment. I thank him for daring to hire a plant molecular biologist with a small baggage in immunology. It is appreciable being leaded by someone with a great knowledge in immunology. Thierry moreover possesses a great team spirit and natural joyousness that renders work easier.

I also would like to thank Prof. Thierry Calandra. He is always of good advice and is able to detect the problems that could be encountered with an astonishing accuracy. I thank him for the fruitful and stimulating discussions shared during the lab meetings. I am thankful to him for hosting me as young researcher in his service.

I thank all the lab members for their support. I express my best thanks to Dr. Didier Le Roy who performed the animal experiments. His friendship, good mood and his ability to listen make him precious teammate. I thank Anne-Laure Chanson and Marlies Knaup for the experiments they performed and for their friendship. I would like to express a special thank to Ivana Rubino, Julie Delaloye, Arnold Probst and Amiel Olivos with whom I share(d) daily pains and joys of being PhD students. I thank the other members of the laboratory, Laurence Guignard, Jacqueline Andrighetto, Eric Gianonni and all the members from the laboratories of Pierre-Yves Bochud and Andrej Trampuz for their support and the good advice they share during lab meetings.

Finally my family and especially my wife Isabelle have to be thanked for her patience and the strength she gives me each and every day. She is always present in my mind and my heart and is the best support that I can dream of.

ABBREVIATIONS

Ad	Adenovirus
AKT	AKR mouse T-cell lymphoma (serine/threonine protein kinase)
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
CARD	Caspase-recruiting domain
ChIP	Chromatin immunoprecipitation
CLP	Cecal ligation and puncture
CLR	C-type lectin receptor
COX-2	Cyclooxygenase 2
CpG	Cytosine-phosphate-guanosine
CRE	cAMP responsive element
CREB	CRE-binding protein
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DDT	D-dopachrome tautomerase
dsRNA	Double-stranded ribonucleic acid
DUSP	Dual-specific phosphatase
ELISA	Enzyme-linked imunosorbent assay
ERK	Extracelluar signal regulated kinase
ETS	E26 transformation specific
FOXO	Forkhead-containing protein type O
HAT	Histone acetyl-transferase
HDAC	Histone deacetylase
IC_{50}	Half maximal inhibitory concentration
IFN	Interferon
IκB	Inhibitor of κB
IL	Interleukin
IL-1R	Interleukin-1 receptor
IRAK	IL-1 receptor associated kinase
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal Kinase
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAL	MyD88 adaptor-like protein (also named TIRAP)
MAPK	Mitogen activated protein kinase
MD-2	Myeloid differentiation-2
MHC-II	Major histocompatibility complex class II molecule
MIF	Macrophage migration inhibitory factor

MKP	MAPK phosphatase
moDC	Monocyte-derived debdritic cell
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
NaB	Sodium butyrate
NAD^+/NADH	Nicotinamide adenine dinucleotide (oxidized/reduced)
NALP	NACHT domain-, leucine-rich repeat-, and PYD-containing protein
Nam	Nicotinamide
NAMPT	Nicotinamide phosphoribosyltransferase/PBEF/Visfatin
NBD	Nucleotide-binding domain
NF-κB	Nuclear factor-κB
NLR	NOD-like receptor
p38	p38 mitogen-activated protein kinase
Pam_3CSK_4	Palmitoyl-cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-OH
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PI3K	Phosphatidylinositol 3 kinase
PRR	Pattern recognition receptor
RIG-I	Retinoic acid-inducible gene-I
RLR	RIG-like receptor
RT-PCR	Real-time polymerase chain reaction
SIRT	Sirtuin (mammalian homolog of SIR2)
SIR2	Silent information regulator 2
ssRNA	Single-stranded ribonucleic acid
TIR	Toll/Interleukin-1 receptor
TIRAP	Toll/Interleukin-1 receptor domain containing adaptor (also named MAL)
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	Tumor necrosis factor receptor-associated factor
TRIF	TIR-domain-containing adaptor inducing interferon β
TSA	Trichostatin A
SAHA	Suberoylanilide hydroxamic acid
STAT	Signal transducer and activator of transcription
VPA	Valproic acid

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1 INTRODUCTION

1.1 Innate immunity

The innate immune system plays a crucial role in host defenses against invading microorganisms. The innate immune system is activated as soon as a pathogen crosses the host external defense barriers, lasts for a few hours, and usually results in the elimination of the invading microorganism. Detection of microbial pathogens is first carried out by phagocytic sentinel cells located in tissues in direct contact with the host's natural environment (monocytes/macrophages and immature dendritic cells) or that are rapidly recruited to the site of infection (neutrophils). This process involves coordinated actions of soluble and cellular molecules comprising components of the complement system, acute phase proteins such as the lipopolysaccharide (LPS) binding protein (LBP), extracellular or intracellular pattern-recognition types of molecules including the Toll-like receptors (TLRs), the nucleotide-binding oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs) and scavenger receptors. Recognition of invasive pathogens by immune cells relies on their capacity to detect microbial molecular motifs (*i.e.* microbial- or pathogen-associated molecular patterns, PAMPs) via microbial- or pathogen-recognition receptors/molecules (PRRs) (**Figure 1.1**) (1-3).

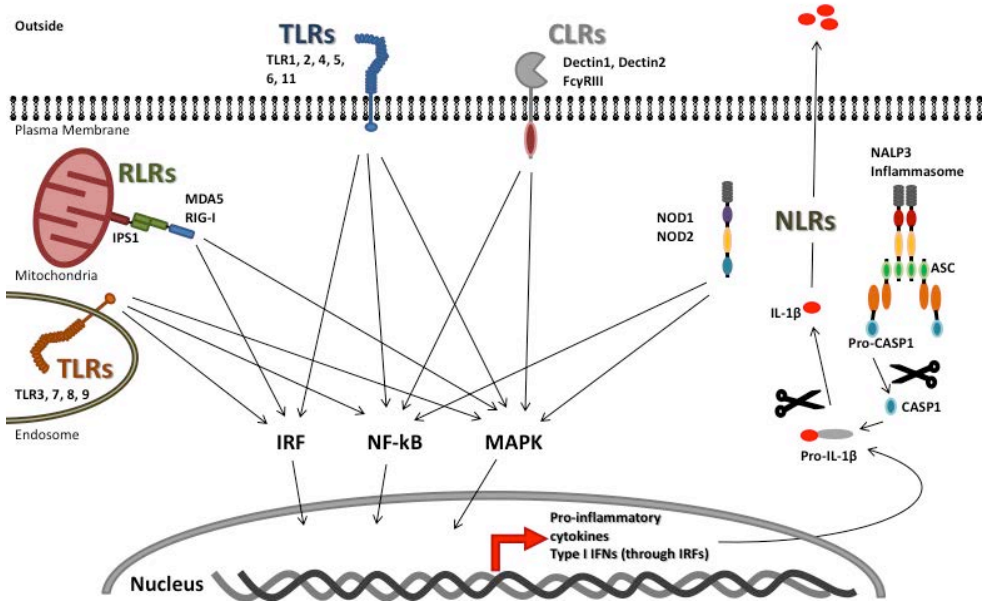

Figure 1.1. Pattern recognition receptors (PRRs). In response to microbial products, distinct families of receptors trigger intracellular signaling cascades that lead to immune responses planned to eradicate invading pathogens. Toll-like receptors (TLRs), C-type lectin receptors (CLR), RIG-I-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) recognize molecular patterns derived from bacteria, viruses, protozoa and fungi.

1.2 Toll-like receptors

TLRs recognize a broad spectrum of microbial products (**Table 1.1**). Thirteen members of the TLR family (TLR1-13) have been identified within human and mouse genomes, but only TLR1-10 are expressed in human. TLR4 was the first discovered and remains the most studied TLR. TLR4 is involved in the sensing of LPS, a pro-inflammatory component of the outer membrane of Gram-negative bacteria. TLR4 forms a complex with MD-2 on the cell surface and both of these proteins are necessary in the recognition of the lipid chains of LPS. The plasmatic LPS-binding protein (LBP) binds to the lipid A moiety of LPS and transfers LPS to CD14. CD14 is a glycophosphatidylinositol-anchored protein expressed on the surface of myeloid cells that binds to LBP and delivers the LBP-LPS complex to the TLR4-MD-2 complex. Finally, TLR4 provides the signal transduction of the LPS receptor complex.

TLR2 is involved in the sensing of a broad array of microbial products among which bacterial lipopetides and peptidoglycan sub-components and fungal mannans and glucans. TLR2 forms homodimers and heterodimers with TLR1 and TLR6. Homodimers of TLR2 recognize, for example, peptidoglycan, lipoteichoic acid (LTA) from Gram-positive bacteria. The TLR1/2 heterodimer is involved in the recognition of triacyl lipopeptides (Pam_3CSK_4) from Gram-positive bacteria and mycobacteria and the TLR2/6 heterodimer recognizes diacyl lipopetides from mycoplasma, LTA from *Streptococcus* and zymosan from *Saccharomyces* (4,5)). The recognition of certain microbial products by TLR2 involves the participation of co-receptors such as CD14, CD36 and Dectin-1 (5).

TLR5 is implicated in the detection of flagellin, a constituent of flagella from bacteria. The last TLR expressed on cell surface is TLR11. TLR11 is involved in the recognition of uropathogenic bacteria and profilin-like molecules in mice. Of note, TLR11 is thought to be non-functional in human due to a stop codon in its sequence. TLR3, TLR7, TLR8 and TLR9 reside in the intracellular vesicles (endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes) and are responsible for the recognition of microbial nucleic acids. TLR3 recognizes both viral single- and double-stranded RNA. TLR7 and TLR8 recognize viral single-stranded RNA. TLR9 detects viral double-stranded DNA, sequence specific unmethylated CpG motifs expressed in DNA from bacteria and viruses and also hemozoin from *Plasmodium* (5). TLR12 and TLR13 are expressed in mouse but their function remains

poorly defined.

Table 1.1. Toll-like receptors, their ligands and adaptor molecules. Adapted from *Kumar et al., Biochemical and Biophysical Research Communications, 2009* (5).

TLR	Location	PAMPs recognized	Origin of ligands	Signaling adaptors
TLR1/2	Plasma membrane	Triacyl-lipopeptide	Bacteria, Mycobacteria	MAL/TIRAP, MyD88
TLR2	Plasma membrane	Peptidoglycan Lipoteichoic acid Zymosan	Gram-positive bacteria Fungi	MAL/TIRAP, MyD88
TLR3	Endosome	ssRNA and dsRNA Poly(I:C)	Viruses Synthetic	TRIF
TLR4	Plasma membrane	LPS Mannan Glycoinosiolphospholipids F-protein Envelope	Gram-negative bacteria Candida Trypanosoma RSV, MMTV	MAL/TIRAP, MyD88, TRAM and TRIF
TLR5	Plasma membrane	Flagellin	Flagelated bacteria	MyD88
TLR6/2	Plasma membrane	Diacyl lipopeptides LTA Zymosan	Mycoplasma, bacteria Gram-positive bacteria *Saccharomyces*	MAL/TIRAP, MyD88
TLR7	Endosome	ssRNA Imidazoquinolines (imiquimod, R848) and nucleosides analogues	Viruses Synthetic compounds	MyD88
TLR8	Endosome	ssRNA Imidazoquinolines (R848)	Viruses Synthetic compounds	MyD88
TLR9	Endosome	Unmethylated CpG DNA	Bacteria, fungi, virus	MyD88
TLR11 (mouse)	Plasma membrane	Profilin, Uropathogenic *E. coli* Chromatin Ig-G complexes	Bacteria , yeasts, viruses Host	MyD88

1.3 Signaling through Toll-like receptors

The N-terminal extracellular domain of TLRs, which is composed 16-28 leucine-rich repeats (LRRs), is responsible for the recognition of PAMPs. The intracellular domain triggers downstream signaling and is composed of a Toll/IL-1 receptor domain (TIR). TIR domain is responsible for the recruitment of adaptor molecules that in turn will initiate intracellular signaling cascades (**Figure 1.2**). All TLRs excepted TLR3 interact with the adaptor myeloid differentiation primary response protein 88 (MyD88) (**Table 1.1**). TLR1, TLR2, TLR4 and TLR6 recruit in addition to MyD88 the linker protein TIR domain-containing adaptor protein (TIRAP also known as MAL) that bridges TIR

domains from TLRs and MyD88. Ligand binding by TLR3 and TLR4 recruits TIR domain-containing adaptor inducing interferon-β (TRIF also named TICAM-1). TLR4 recruits TRIF trough TRIF-related adaptor molecule (TRAM or TICAM-2) that bridges between TIR domains of TLRs and TRIF. MyD88 activates the IL-1 receptor associated kinase (IRAK) family that leads to the activation of TNF receptor-associated factor (TRAF) 6. TRAF6 promotes activation of TGF-beta-activated kinase (TAK) 1 that in turn activates inhibitor of κB (IκB) kinase (IKK) and mitogen-activated protein kinases (MAPKs) which lead to the activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) transcription factors, respectively (5). Activation of IRAK-1 via TLR7, TLR8 and TLR9 leads to phosphorylation and subsequent activation of interferon (IFN) regulatory factor (IRF) 1, IRF5 and IRF7. TLR3 and TLR4 signal leads in the production of type I IFNs. TRIF activates TRAF3 that serves as a linker to TBK1, which phosphorylates and activates the transcription factor IRF3. IRF3 is responsible for the induction of type I IFNs. TRIF can also activate the NF-κB and MAPK pathways via interaction with RIP1 that activates TAK1 (**Figure 1.2**) (2,5).

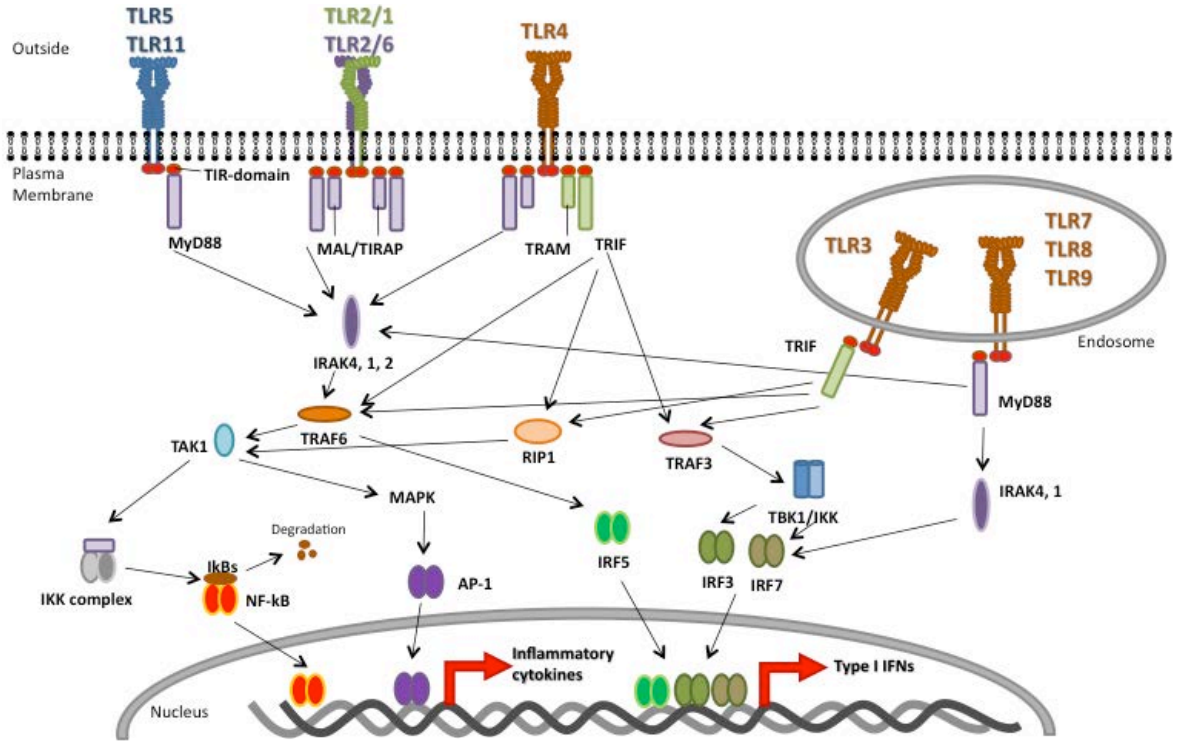

Figure 1.2. Toll-like receptor signaling. TLR1, 2, 4, 5, 6 and 11 are located on cell surface. TLR3, 7, 8 and 9 are located in endosomes. All TLRs, except TLR3, recruit the adaptor protein MyD88 through their TIR-domain. TLR3 and TLR4 also recruit TRIF.

AP-1, NF-κB and IRF transcription factors promote the rapid expression of numerous immune-related genes leading to the production and release of cytokines, among which tumor necrosis factor (TNF), interleukins (IL)-1, IL-6, IL-8, IL-10, IL-12 and IL-18, chemokines and IFNs, together with the up-regulation of cell surface molecules, such as major histocompatibility class II (MHC II), adhesins, selectins, integrins and co-stimulatory molecules. All these events are involved in the development of an inflammatory response, which is essential to coordinate the cellular and humoral responses intended to contain or eradicate invasive microorganisms (6).

1.4 RIG-like receptors, NOD-like receptors and C-type lectin receptors

RLRs are specialized in the recognition of viral RNA present in the cytoplasm, thus triggering the transcription of type I IFNs as well as pro-inflammatory cytokine genes (**Figure 1.1**). The RLR family is composed of three members: RIG-I, melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RIG-I binds 5'-triphosphate of ssRNAs and short dsRNAs whereas MDA5 detects longer dsRNAs and poly-IC. RIG-I have been involved in the sensing of paramyxoviruses (Sendai virus and Newcastle disease virus), orthomyxoviruses (influenza virus), rhabdoviruses (vesicular stomatitis virus) and flaviviruses (Japanese encephalitis virus and hepatitis C virus), and MDA-5 in the sensing of picornaviruses (encephalomyocarditis virus, Theiler's virus, and Mengo virus) family. West Nile and Dengue viruses (Flaviviruses) and reoviruses are detected by both RIG-I and MDA-5. LGP2 acts as an inhibitor of RIG-I and MDA-5 by binding to their RNA binding domain, but can also act in some circumstances as a positive regulator of MDA5 in the recognition of specific viruses (2,7).

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are located in the cytosol and detect a broad range of PAMPs and danger-associated molecular patterns (DAMPs) from both endogenous and exogenous origins. NLRs are constituted of three domains: a N-terminal protein interaction domain, a central nucleotide binding domain and a C-terminal leucine-rich repeat (LRR). Members of the NLR family are classified into subcategories according to their N-terminal protein interaction domain which are acidic transactivation domain (NLRA), baculovirus inhibitor of apoptosis protein repeat [BIR] (NLRB), CARD (NLRC), pyrin domain (NLRP) or unknown domain

(NLRX). NOD1 and NOD2 (also called NLRC1 and NLRC2, respectively) are able to recognize components of peptdioglycan (PGN). NOD1 recognizes γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and NOD2 muramyl dipeptide (MDP), which is the main constituent of PGN motif. NOD1 and NOD2 sensing of bacterial cell wall activates signaling via MAPK and NF-κB that results in the production of inflammatory cytokines and anti-microbial proteins (2) (**Figure 1.1**).

NLRs respond to PAMPs or DAMPs and promote the release of bioactive cleaved IL-1β and IL-18 after the assembly of "inflammasomes". NLRs forming inflammasomes are NALP1 (NLRP1), NALP3 (NLRP3), IPAF (NLRC4) and NAIP (NLRB) (2). Signaling occurs between PYD or CARD domains of NLRP1, NLRP3 and IPAF and the PYD domain of the adaptor molecule ASC that, through its CARD domain, interacts with and activates pro-caspase-1 into caspase-1. Finally, activated caspase-1 cleaves pro-IL-1β and pro-IL-18, leading to the secretion of bioactive IL-1β and IL-18 (**Figure 1.1**).

CLRs are another class of transmembrane PRRs (**Figure 1.1**). They are involved in the recognition of a wide range of pathogens including bacteria, fungi, viruses and parasites. Some representatives of this class are dectin-1 that recognizes β-glucans and dectin-2 that recognizes mannose from yeast and bacteria. Recognition of pathogens by CLRs induces the production of pro-inflammatory cytokines, promotes phagocytosis and stimulates the respiratory burst. Signaling occurs through the ITAM domain of dectins or their associated signaling partners. ITAM promotes phosphorylation of SYK that activates CARD9, which activates the NF-κB and MAPKs signaling pathways (8).

1.5 Innate Immunity, sepsis and septic shock

The regulation of innate immune responses has to be tightly controlled in order to coordinate the cellular and humoral responses intended to contain or eradicate invasive microorganisms. If the host fails to mount a sufficient inflammatory response, microbes will proliferate, compromising host integrity. Conversely, an overwhelming inflammatory response will lead to a cascade of events leading to shock, multiple organ failure and often death (**Figure 1.3**).

Severe sepsis and septic shock are life-threatening complications of infections. Sepsis is defined as a systemic inflammatory response syndrome (SIRS) with a known or suspected source of infection. Severe sepsis is characterized by organ dysfunction, and septic shock by vasopressor-resistant hypotension (9). Mortality associated with severe sepsis and septic shock is considerable, ranging from 20-35% in the case of severe sepsis to 50-80% for septic shock (10). Severe sepsis and septic shock are the leading cause of death in non-coronary intensive care units. It is consequently of primary interest to develop treatments blocking or controlling the overwhelming inflammatory responses in order to decrease morbidity and mortality related to the early phase of sepsis (11,12). The control of the expression of cytokines is therefore a key aspect from a therapeutic point of view.

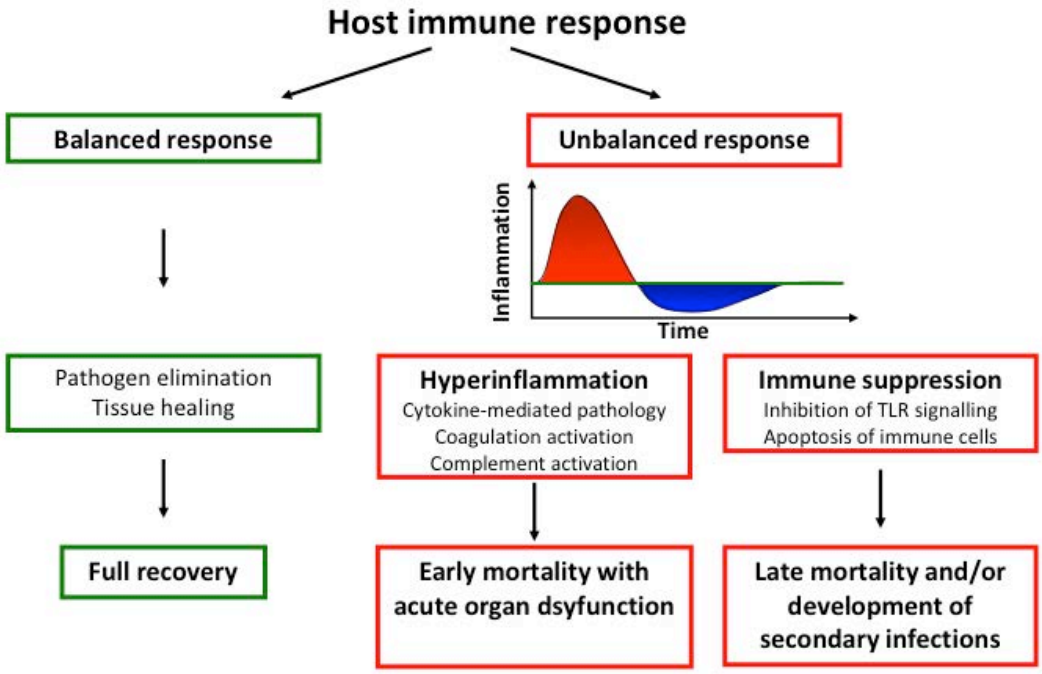

Figure 1.3. Sepsis, a dysregulated inflammatory response to infection. Host immune response to infection usually leads to pathogen elimination, tissue healing and full recovery. Sepsis is characterized in its early phase by an overwhelming inflammatory response which is involved in tissue damage, vascular collapse and multiple organ failure. Late phase of sepsis is characterized by a state of immune suppression that favors the development of secondary life-threatening infections. Adapted from *Van der Poll T & Opal SM, Lancet Infectious Diseases 2008* (11).

1.6 Chromatin dynamics and modifications

Chromatin is a highly ordered structure mainly composed of DNA and histones proteins. Condensed chromatin, also called heterochromatin, is associated with non-transcribed regions of the genome such as telomeres, centromeres and silenced genes. The condensation or packaging is a

necessity in terms of space improvement within the nucleus and it allows strengthening of DNA during mitosis and meiosis (**Figure 1.4**). On the contrary, euchromatin is defined as relaxed and accessible chromatin, essentially found in transcribed regions of the genome.

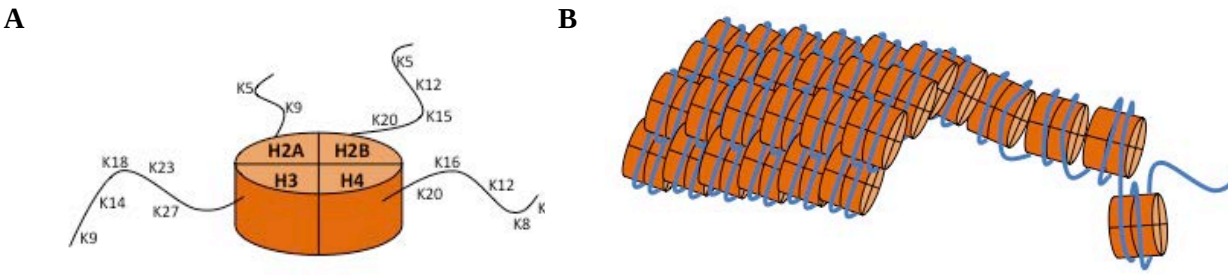

Figure 1.4. Nucleosome histone core and chromatin structure. (**A**) Histone proteins H2A, H2B, H3 and H4 compose the core of the nucleosome. Histone N-terminal tails protrude through DNA wrapped around the histone core. Histone tails contain lysine residues (K) that accommodate post-translational modifications. Here are represented the lysine residues known to be acetyated in a reversible manner. (**B**) Histone octamers around which DNA is wrapped compose a dynamic structure with the ability to compact and decompact DNA.

1.6.1 Histone proteins and nucleosomes

Histones are acidic proteins forming octamers of two histone 2A (H2A), H2B, H3 and H4 subunits, around which 147 bp DNA is wrapped in 1.67 turns. The DNA-histone octamer complex is called nucleosome and is the first scaffold for DNA compaction. H3 and H4 (and H2B to a lesser extent) from adjacent nucleosomes can interact with each other through their N-terminal tails that protrude throughout DNA (13,14). These interactions are made possible by post-translational modifications predominantly on the amino-terminal tails of histones. Post-translational modifications of histones especially on lysine, arginine and serine residues of H2B, H3 and H4 tails such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, and deimination (citrullination) mediate interactions between histone tails and control the turnover of histones. These modifications are catalyzed by pairs of enzymes with opposing activities, adding or removing functional groups (15). Histone modifications were stated about ten years ago to form an epigenetic marking system called the “histone code” (16,17) where a modification or a pattern of modifications dictate the ability of other proteins to “read” the code and promote changes in the chromatin dynamics. Combinatorial modifications of histones likely reflect a given task, but efforts made to resolve a predictable code remains largely unsuccessful. Yet, this code is now commonly

accepted to influence the chromatin-associated processes under normal and pathological situations (18).

1.6.2 Chromatin modifications

Chromatin can be modified through two major ways. First, histones can be replaced, ejected or sled along DNA. Second, histones can undergo post-translational modifications. All these events may occur at the same time in a dynamic manner. It takes only few minutes for the cell to react to a stimulus and apply these mechanisms to modify the chromatin. The major functions for these processes are the interruption of contact between nucleosomes (decompaction) and the allowance for the recruitment of transcription regulators or, conversely, the compaction of nucleosomes and undocking of transcription regulators (**Figure 1.5**). This results in the partition of the genome into heterochromatin and euchromatin in order to regulate the expression of large subsets of genes. These modifications also act at the local level, particularly on the promoter of highly regulated genes, providing a supplementary level of fine-tuning of gene transcription (15).

1.6.3 Regulation of transcription through histone modifications

The main histone modifications affecting transcription rate are methylation (lysine and arginine), acetylation (lysine) and sumoylation (lysine) of H3 and H4 and ubiquitination of H2A and H2B. Distribution of histone modifications along a given gene is not arbitrary and correlates with its transcription rate as revealed by experiments of ChIP followed by gene-array expression (ChIP on chip). For example, acetylated H3 and H4 containing nucleosomes are usually located in the promoter region of actively transcribed genes. The same correlation can be found with tri-methylated H3 at lysine 4 (H3K4me3). On the contrary, H3K27me is associated with inactive transcription (19). Histone variants are also involved in the regulation of transcription but this topic will not be dealt with in subsequent studies. Regulation of transcription by chromatin modification and remodeling is not restricted to the recruitment of transcription regulators. It has also been described to affect transcription initiation and elongation (19).

1.6.4 Acetylation of histone proteins

Acetylation of lysine residues on the tails of H3 and H4 histones is associated with relaxed transcriptionnally active chromatin (20,21), whereas hypo-acetylated histones are commonly associated with condensed chromatin of silent regions of the genome (22) (**Figure 1.5**). Acetylation of H3 and H4 brings negative charges that neutralize the positively charged core of histones, consequently hampering the interaction between histone tails and phosphate groups of DNA (23). The acetylation of histones is catalyzed by proteins that possess histone acetyltransferase (HAT) activity, such as CREB binding protein (CREBBP or CBP), E1A binding protein p300 (EP300 or p300), P300/CBP-associated factor (PCAF), general control of amino acid synthesis protein-like 5 (GCN5/KAT2A) and 60kDa Tat-interacting protein (TIP60/KAT5) (18). CBP/p300, PCAF/GCN5, and HATs in general are regulators of DNA transcription, replication, repair and condensation (15). On the contrary, histone deacetylases (HDACs) deacetylate histones H3 and H4 on lysine residues of their N-terminal tails that make them "stick" to DNA.

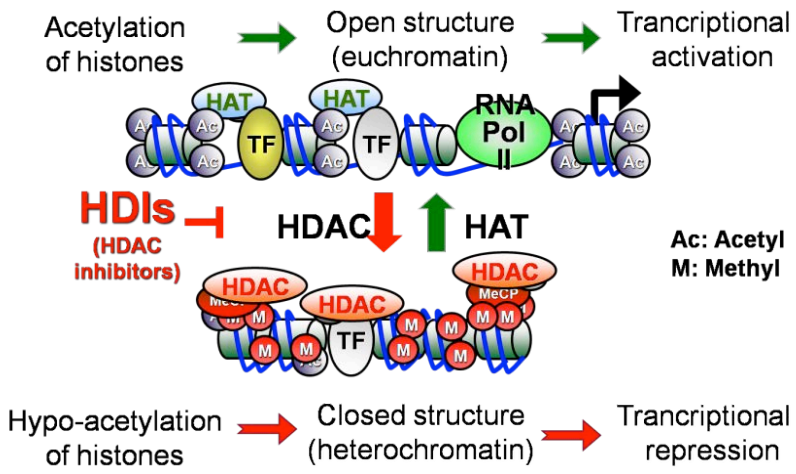

Figure 1.5. Chromatin structure and transcriptional activity. Recruitment of complexes with HAT activity allows histone acetylation and thus decompaction of DNA and recruitment of transcription regulators. Recruitment of HDACs to promoter regions promotes deacetylation of histones, DNA compaction and exclusion of transcription regulators. Ac: acetylation, HAT: histone acetyltransferase, HDAC: histone deacetylase M: methylation, TF: transcription factor.

1.6.5 Histone deacetylases (HDACs)

HDACs catalyze the cleavage of acetyl groups from lysine residues of histone H3 and H4 as well as non-histone proteins. Eighteen mammalian HDACs have been identified and classified into

four classes based on their homology with yeast HDACs (24) as well as their subcellular localization and their enzymatic activity (25) (**Figure 1.6**).

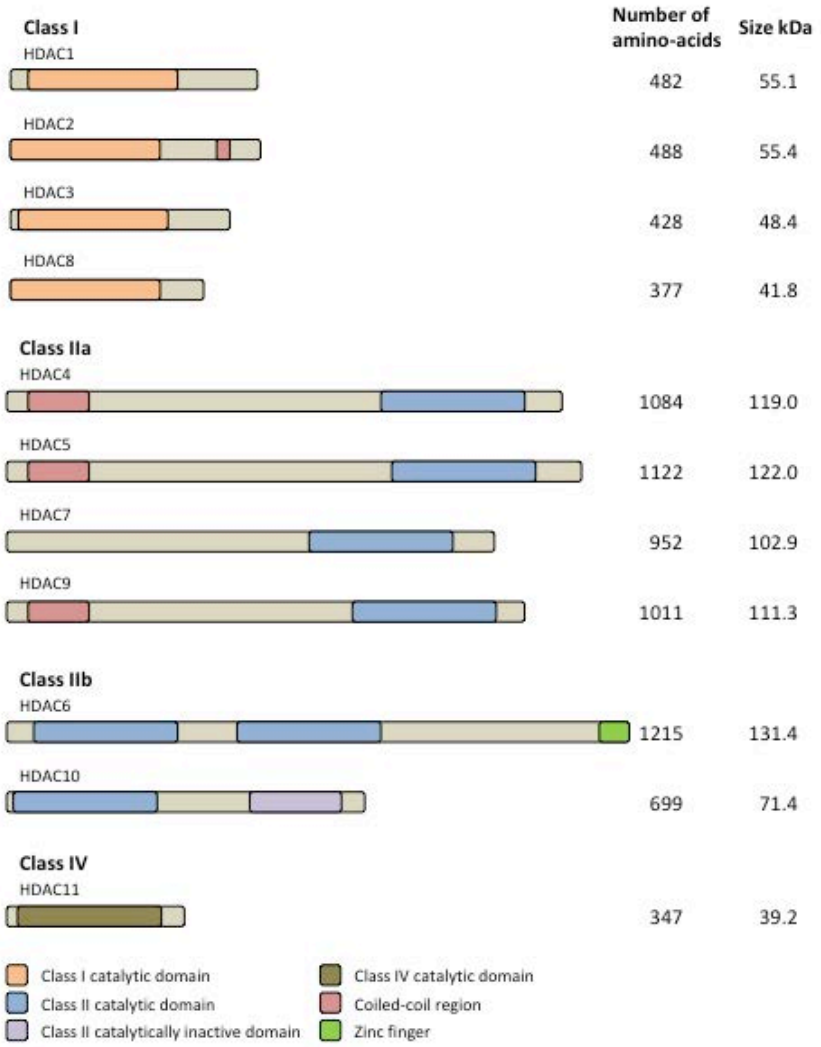

Figure 1.6. Classification of mammalian histone deacetylases (HDACs) according to their enzymatic activity and sub-cellular localization. Class I HDACs are localized in nucleus, class IIa and IIb in nucleus and cytoplasm and class IV predominantly in the nucleus. Class IIb HDACs possess two catalytic sites. Adapted from *Bolden et al., Nature Reviews Drug Discovery, 2006* (31).

Class I HDACs are homologues of yeast Rpd3 and include HDACs 1, 2, 3 and 8 and are found uniquely in the nucleus of most cell lines and tissues. Class IIa contains HDACs 4, 5, 7 and 9. Class IIb includes HDACs 6 and 10, which are closely related to class IIa enzymes, but possess two deacetylation catalytic sites (one of which is inactive in HDAC10). Classes II are related to yeast Hda1 and can be found in both the nucleus and the cytoplasm of some cell types. Classes I and II are Zn^{2+}-

dependent enzymes. Class IV, solely represented by HDAC11, has only been recently characterized (**Figure 1.6**) (26). Class III enzymes are related to the yeast Sir2p and are called sirtuins (SIRT) or sir2-like proteins (see **chapter 6**). Sirtuins are NAD^+-dependent enzymes. Of note they are not sensitive to inhibitors of classes I, II and IV HDACs. Like other HDACs, sirtuins can deacetylate lysines of non-histone proteins (27).

1.6.6 Physiology of class I, II and IV HDACs

Class I HDACs are generally found in repressive complexes such as Sin3, NuRD, CoREST, PRC2 and N-CoR-SMRT. Class I HDACs play a critical role in development (32). Eventhough their closey related structure and function might let one anticipate functional redundancy, null-phenotype of each class I HDAC results in non-viable mice (**Table 1.2**). Class IIa HDACs possess a reduced enzymatic activity and mainly function as adaptors recruiting transcriptional regulators as well as class I HDACs. Class IIa HDACs are involved in skeletogenesis and cardiovascular growth and function, regulate muscle differentiation and control endothelial function (32). As a main tubulin deacetylase, the class IIb HDAC6 regulates cytoskeleton dynamic (33). Like class IIa HDACs, class IV HDAC11 is expressed in heart, brain and muscle; however its precise function remains to be determined.

Table 1.2. HDACs, loss of function phenotypes in mice and time point of lethality of knockouts. Adapted from Haberland *et al*. 2009 (32).

Class	HDAC	Time of lethality	Phenotype
Class I	HDAC1	Embryon, day 10.5	Proliferation defects
	HADC2	Postnatal, day 1	Cardiac malformation
	HDAC3	Embryon, day 9.5	Gastrulation defects
	HDAC8	Postnatal, day 1	Craniofacial defects
Class IIa	HDAC4	Postnatal, day 7-14	Chondrocyte differentiation defect in growth plate
	HDAC5	Viable	Exacerbated cardiac hypertrophy after stress
	HDAC7	Embryon, day 11	Endothelial dysfunction
	HDAC9	Viable	Exacerbated cardiac hypertrophy after stress
Class IIb	HDAC6	Viable	Increased tubulin acetylation
	HDAC10	Not determined	-
Class IV	HDAC11	Not determined	-

Dysregulation of HDAC expression or activity has been involved in a variety of pathological disorders such as cancer, metabolic and inflammatory diseases (28-30). As it will be discussed later, modulators of HDAC activity are of great interest as they could be used to interfere with numerous diseases. For example, small molecules inhibiting class I, II and IV HDACS are among the most promising anti-cancer drugs under development.

1.6.7 Non-histone targets of HDACs

HDACs deacetylate a wide range of non-histone proteins including transcription factors, chaperones, nuclear receptors, transcription co-regulatory proteins, cell-cycle progression and cell death proteins and this list is in constant expansion (34). In fact non-histone proteins are the major targets for HDACs. Recently, a novel nomenclature has been proposed to denominate enzymes modifying histone lysines. HATs, histone methyltransferases (HMTs) and histone demethylases (HDMs) would be renamed K-acetyltransferases (KATs), K-methyltransferases (KMTs) and K-demethylases (KDM), respectively. In order to avoid confusion, HDACs and PRMTs (protein arginine N-methyltransferases) have not been renamed (35).

Like phosphorylation, protein acetylation is recognized as playing very important roles in cellular signaling at multiple levels. Directly or indirectly, acetylation regulates protein localization, stability, enzymatic activity and DNA binding activity, protein-protein interactions, gene expression and mRNA stability (36). Of note, acetylation can have dual effects on the function of a given protein (for example increased or decreased NF-κB p65 DNA binding, see **Table 1.3**). Altogether, these multiple levels of action situate acetylation as a master switch as important as phosphorylation (37).

Table 1.3. Consequences of proteins modification by acetylation. Adapted from *Spange et al., The International Journal of Biochemistry & Cell Biology, 2009* (36).

Biological process	Consequence of protein acetylation	
	Increase	**Decrease**
Protein stability	p53, p73, Smad7, c-Myc, Runx3, AR, H2A.z, E2F1, NF-E4, ER81, SREBP1a, HNF6, BACE1	GATA1, HIF-1α, pRb, SV40 T-Ag
DNA binding	p53, SRY, STAT3, GATA transcription factors, E2F1, p50 (NFκB), Er, p65 (NF-κB), c-Myb, MyoD, HNF-4, AML1, BETA2, NF-E2, KLF13, TAL1/SCL, TAF(I)68, AP endonuclease	YY1, HMG-A1, HMG-N2, p65 (NF-κB), DEK, KLF13, Fen-1
Gene expression (Transcriptional activation or inactivation)	p53, HMG-A1, STAT3, AR, ERα (basal), GATA, EKLF, MyoD, E2F1, p65 (NFκB), GR, p73, PGC1α, MEF2D, GCMa, PLAG1, PLAGL2, Bcl-6, β-catenin, KLF5, Sp1, BETA2, Cart1, RIP140, TAF(I)68	Erα (ligand-bound), HIF-1α, STAT1, FOXO1, FOXO4, RIP140
Protein interactions	STAT3, AR, EKLF, Importin A, STAT1, TFIIB, α-tubulin, actin, cortactin	p65 (RelA), Ku70, Hsp90
Localisation	→ nucleus	→ cytosol
	PCAF, SRY, CtBP2, POP-1, HNF-4, PCNA	c-Abl, p300, PAP
mRNA stability	p21, Brm	Tyrosinhydrolase (Th), eNOS
Enzymatic activity	p300, ATM	PTEN, HDAC1, Mdm2, ACS, Neil2, Polβ
Expression of viral proteins	E1A, S-HDAg, L-HDAg, HIV Tat, SV40 T-Ag	

1.7 Histone deacetylase inhibitors

1.7.1 Discovery and mechanisms of action

HDAC inhibitors from natural or synthetically origins can be classified into five groups according to their structure: hydroxamate derivatives, cyclic peptides, aliphatic acids, benzamides and ketones (**Table 1.4**). Sodium butyrate (NaB) was the first identified HDAC inhibitor (38). This aliphatic acid is a by-product of dietary fibers anaerobic fermentation. NaB suppresses deacetylation

of histones H3 and H4 and increases the sensitivity of DNA to DNase I (39). NaB inhibits the growth and promotes the differentiation and the apoptosis of cancer cells (40,41). Valproic acid (VPA) is closely related to butyrate and belongs to the same class of short-chain fatty acids HDAC inhibitors. VPA specifically inhibits the catalytic activity of class I HDACs and induces proteasomal degradation of HDAC2 (42). As NaB, VPA induces the differentiation and apoptosis of carcinoma cells. VPA inhibits cancer development and metastasis in animal models (42,43). VPA increases brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), thereby playing a neuroprotective role (44). VPA is used to treat epilepsy and bipolar disorders. Trichostatin A (TSA) is an organic compound of the hydroxamate family first isolated as an antifungal antibiotic from *Streptomyces hygroscopus*. TSA is a potent inhibitor of class I and class II HDACs *in vitro* and *in vivo* (39). Like NaB and VPA, TSA exhibits anti tumorigenic effects. It induces the differentiation, inhibits the growth and promotes and apoptosis of numerous tumors among which leukemia and glioblastoma (45,46). TSA has not reached the clinics owing to its toxicity. Yet, it remains extensively used in research as a prototypical powerful HDAC inhibitor. Moreover, the structure of TSA was used to synthesize suberoylanilide hydroxamic acid (SAHA), a hydroxamate compound which, like TSA, inhibits class I and II HDACs. The inhibitory mechanism of SAHA is similar to that of TSA: it binds directly to the Zn catalytic pocket of HDACs and mimics a lysine residue (47). SAHA has effective anticancer activity against hematologic and solid tumors. Recently, FDA has approved SAHA (vorinostat) to treat cutaneous T-cell lymphomas (48). Overall, HDAC inhibitors have shown good safety profiles in patients with solid and hematologic tumors and are amongst the most encouraging anti-cancer drugs under development (27,31,49-51). Great efforts are devoted to design novel inhibitors of HDACs with increased specificity and activity. These molecules will allow fine tuned analyses of the role of each HDAC under physiological and pathological conditions. More importantly, they will offer new therapeutic anti-cancer therapies, especially for patients with tumors resistant to conventional therapies (52).

Table 1.4. HDAC inhibitors. Adapted from *Bolden et al., Nature Reviews Drug Discovery, 2006* (31).

Class	Compound	Concentration range of activity	HDAC specificity
Short-chain fatty acid	Butyrate	mM	Class I, IIa
	Valproic acid (VPA)	mM	Class I, IIa
Hydroxamate	Trichostatin A (TSA)	nM	Class I, II
	Suberoylanilide hydroxamic acid	μM	Class I, II
	PXD101	μM	Class I, II
	LAQ824	nM	Class I, II
	LBH589	nM	Class I, II
	Pyroxamide	μM	Class I (Class II unknown)
	Tubacin	μM	Class IIb
	SK-7041	nM	HDACs 1 and 2
	SK-7068	nM	HDACs 1 and 2
Benzamide	MS-275	μM	HDACs 1, 2, 3, 8
Cyclic tetrapeptide	Depsipeptide	nM	Class I
	Trapoxin A	nM	Class I, IIa
	Apicidin	nM	HDACs 1 and 3
	CHAPs	nM	Class I
Miscellaneous	Depudecin	μM	Class I (Class II unknown)

CHAP, cyclic-hydroxamic acid containing peptide.

1.7.2 HDAC inhibitors as potential therapies for inflammatory and autoimmune disorders

Recent studies suggest that HDAC inhibitors could be used as treatment therapies for inflammatory and autoimmune disorders such as lupus, rheumatoid arthritis, autoimmune encephalitis, multiple sclerosis, graft versus host disease, asthma and colitis (**Table 1.5**) (32,49). The first model of autoimmune disease in which HDAC inhibitors have shown beneficial effects is systemic lupus erythematosus (SLE). This disease is characterized by a skewed Th2 response, which promotes the production of pathogenic auto-antibodies by B cells. TSA was shown to shift the response of T cells from SLE patients from a Th2 to a Th1 phenotype (53). Moreover TSA and SAHA decreased the expression of pro-inflammatory cytokines and disease severity in MRL-*lpr/lpr* mice that spontaneously develop an autoimmune syndrome resembling SLE (53,54). Th2 responses are characterized by the secretion of IL-4, IL-5, IL-9 and IL-13 which promote the production of allergen-specific IgE by B cells and the infiltration of inflammatory cells into the airways. Inhibition of HDAC by TSA in a mouse experimental model of asthma reduced airway hyper-responsiveness and allergic airway inflammation as well as pulmonary cytokines and IgE levels (55).

HDAC inhibitors have also been shown to impair Th1 responses. Indeed, HDAC inhibitors such as TSA, phenylbutyrate and FR901228 (depsipeptide) improved pathological scores in mouse and rat experimental models of arthritis biased for Th1 responses. The beneficial effects of HDAC inhibitors were associated with reduced expression of pro-inflammatory cytokine (among which TNF) and matrix-metalloproteinase-3 (MMP-3) and MMP-13 (responsible for matrix degradation), decreased synovial fibroblasts proliferation and reduced joint destruction (56-58). In mouse models of graft-versus-host disease (GVDH), SAHA and ITF2357 (two hydroxamate derivatives) decreased the production of Th1 cytokines (TNF, IL-1, IFNγ), increased the expression of indoleamine 2,3-dioxygenase (IDO, a suppressor of DC function) in DCs and reduced gastrointestinal tract destruction (59,60).

HDAC inhibitors were also studied as potential therapy for multiple sclerosis (MS), a demyelinating disease with a chronic inflammation of central nervous system white matter (61). In experimental autoimmune encephalomyelitis (EAE), a model of MS, TSA inhibited the activation of neuronal caspases and Th1 response of splenocytes. Of great interest, TSA also decreased spinal cord inflammation, demyelination, neuronal and axonal loss and ameliorated disability in relapsing phase of EAE (62). HDACs have also been recognized as promising therapeutic targets for many human brain or central nervous system diseases such as psychiatric (schizophrenia, drug addiction, anxiety) disorders, amyotrophic lateral sclerosis, Rubinstein-Taybi and Rett syndromes, Friedreich's ataxia and Huntington's disease (61,63).

Considering the anti-inflammatory properties of HDAC inhibitors, one may speculate that these drugs interfere with innate immune responses. When we started our research project, very few studies had addressed that aspect. NaB was reported to inhibit IL-12 and to induce IL-10 production by human monocytes stimulated with *Staphylococcus aureus* (64). SAHA inhibited LPS-induced secretion of TNF, IL-1β and IFNγ by human PBMCs, and cytokine-induced nitric oxide by RAW 264.7 cells. SAHA also reduced circulating levels of TNF, IL-1β, IL-6 and IFNγ in mouse injected with LPS (65).

Table 1.5. Beneficial effects of HDAC inhibitors therapy in experimental models of inflammatory and autoimmune diseases.

Diseases	Inhibitors	Effects	References
Systemic lupus erythematosus	TSA, SAHA	Th1 activation ↓ pro-inflammatory cytokines	(53,54)
Asthma	TSA	↓ pulmonary pro-inflammatory cytokines and IgE	(55)
Arthritis	TSA, NaB, depsipeptide	↓ pro-inflammatory cytokines in joints ↓ MMPs in joints	(56-58)
Graft-versus-host disease	SAHA, ITF2357	↓ pro-inflammatory cytokines ↑ indoleamine 2,3-dioxygenase	(59,60)
Multiple sclerosis	TSA	↓ activation of neuronal caspases ↓ pro-Th1 cytokines mRNA in splenocytes	(61)
Colitis	VPA, SAHA	↓ pro-inflammatory cytokines ↑ apoptosis of lamina propria lymphocytes	(66)

1.8 Macrophage migration inhibitory factor

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine found to play a crucial role in the control of innate immune responses, in the pathogenesis of sepsis, autoimmune diseases and cancers (67). The name MIF was coined in the early 1960s to describe the biological activity of a mediator released by activated lymphocytes inhibiting the migration of monocytes and exudate cells *in vitro* (68,69). MIF was rediscovered in the 90s as a factor released by anterior pituitary cells stimulated by LPS (70). MIF was thus proposed to be a linking factor of endocrine and immune systems.

1.8.1 *MIF* gene

Weiser and colleagues cloned a human *MIF* cDNA in the late 80s. They isolated the cDNA from a library established from a lectin-stimulated T-cell hybridoma (71). *MIF* is localized on chromosome 22 at position 22q11.2 (72). The *MIF* gene is less than 1 kb in length and is composed of three exons of 205, 173 and 183 bp separated by two introns of 189 and 95 bp. A single transcription start site, 95 bp upstream of the ATG, is situated in GC rich region lacking a TATA box. Yet, only one transcript of about 800 nucleotides could be identified by Northern blotting in several human tissues. The *MIF* gene is constitutively expressed in all organs, with high levels in the liver, kidney, brain and placenta and lower levels in the heart, skeletal muscle and pancreas (73). The *MIF* gene is structurally related to *D-dopachrome tautomerase* (*DDT*). The *MIF* and *DDT* genes are narrowly linked on human

chromosome 22 and mouse chromosome 10 (74), suggesting that they arose by duplication of an ancestral gene.

1.8.2 Regulation of *MIF* gene expression

Numerous DNA-binding sequences for transcription factors such as AP-1, NF-κB, E twenty-six (Ets), GATA, Specificity protein 1 (Sp1) and cyclic AMP response element (CRE)-binding protein (CREB) are located in the human *MIF* gene promoter region. Two Sp1 and CRE sites in the vicinity of the human *MIF* gene transcriptional start site (designated proximal Sp1 and CRE or $Sp1^{p}$ and CRE^{p}, respectively, **Figure 1.7A**) were shown to bind Sp1 and CREB and to positively regulate *MIF* gene basal and microbial product-stimulated transcriptional activity in several cell lines (75). Chromatin immunoprecipitation (ChIP) assays confirmed the binding of Sp1 and CREB to the *MIF* proximal promoter *in invo* (75).

The *MIF* gene lies within a CpG island. Interestingly, methylation of cytosine residues within CpG island is a major mechanism of epigenetic silencing. Yet, the *MIF* gene is not methylated in PBMCs and monocytic, epithelial and keratinocytic cell lines expressing different levels of MIF mRNA, suggesting that methylation of CpGs does not account for the differential expression of MIF mRNA (75).

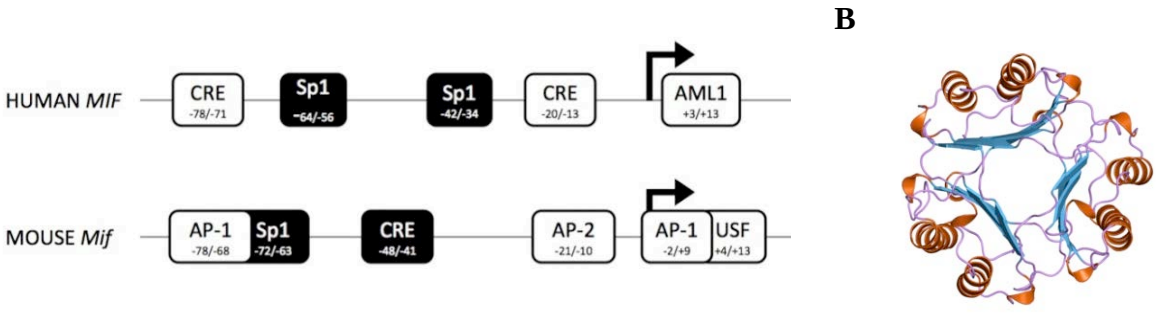

Figure 1.7. Structure of *MIF* promoter and MIF 3D view. (**A**) Comparison of the proximal promoters of the human and mouse *MIF* genes. The proximal Sp1 and CRE sites regulating the constitutive human and mouse *MIF* promoter activity are highlighted in black (adapted from *Roger et al., European Journal of Immunology, 2007* (75)). (**B**) Crystal strucure at 2.6A of human MIF trimer showing solvent accessible channel (from *Sun et al., Proceedings of the National Academy of Sciences of the United States of America, 1996* (76)).

1.8.3 MIF protein

The MIF protein is a 12.5 kDa polypeptide composed of 115 amino acids. MIF is highly conserved amongst mammalian genomes, and MIF homologues are found in plants, nematodes, arthropods, protozoan and cyanobacteria (74). Three-dimensional structure revealed by electronic microscopy of MIF crystals exposed no structural similarity with other cytokines or hormones. MIF was found to crystallize as a homotrimer (**Figure 1.7B**), which is commonly admitted to be the active form. The homotrimer structure was determined to form a β-strand barrel with an inner solvent-accessible channel whose surface is negatively charged (76,77). The channel is a potential binding site for small molecules like glutathione and dopachrome (76) or, by similarity with the enzyme 5-carboxy-methyl-2-hydroxymuconate isomerase (CHMI) from *E. coli*, to be a catalytic site (77).

MIF exerts tautomerase and thiol-protein oxydoreductase enzymatic activities. These activities were discovered because of the structure relationship between MIF and bacterial isomerase CHMI and DDT (78). Whether these enzymatic activities impact on MIF function *in vivo* is debatable. Nevertheless, ISO-1, a specific inhibitor of MIF tautomerase activity, was shown to block TNF release from LPS-stimulated macrophages and to increase mouse survival in models of septic shock (79). Overall, the molecular mechanisms linking the enzymatic and cytokine activities of MIF remain largely to be described.

MIF is stored in secretory granules and is released upon stimulatory conditions. Given that MIF lacks the classical N-terminal glycosylation necessary for a classical ER/golgi export to plasma membrane, MIF secretion is mediated through a non-classical secretory pathway. The ABC transporter (80) and the Golgi-associated protein p115 (81) have been reported to exert this function.

1.8.4 MIF function

MIF activity was described in the late 60's as T-cell derived factor with the ability to inhibit the migration of macrophages and monocytes, correlating with delayed type hypersensitivity (68,69). Almost 30 years later, MIF was re-discovered as cytokine secreted in a hormone-like manner by pituitary cells during endotoxemia (70). Innate immune cells, especially macrophages, are an

important source of MIF. Macrophages stimulated with LPS rapidly secrete large quantities of preformed MIF. Additionally, organs containing large amounts of MIF are those commonly associated with a high macrophage content like the kidney, liver or brain (82).

Different mechanisms by which cells respond to MIF stimulation have been identified. Extracellular MIF signals through a receptor composed of CD74 (*i.e.* the invariant chain of the MHC class II), the MIF binding receptor, and CD44, the signal transducing molecule of the MIF receptor complex (83,84). MIF also interacts with the chemokine receptors CXCR2 and CXCR4, thereby triggering leukocyte recruitment (85-87). Besides signaling through a membrane receptor, intracellular MIF modulates cell activity by interacting with JAB-1, a co-activator of AP-1 transcription factor (88), and p53 (89).

Once released, MIF sustains inflammatory and immune responses, stimulating the production of inflammatory mediators by macrophages and providing a stimulus for the activation of T and B cells. Notably, MIF has been reported to up-regulate the expression of TLR4 in macrophages, allowing a quick response to Gram-negative bacteria (90). MIF also possesses the characteristic to be induced by physiological concentrations of glucocorticoids and to override the immunosuppressive and anti-inflammatory properties of glucocorticoids (91,92).

MIF is considered as an integral component of the host antimicrobial alarm system and stress response that promotes pro-inflammatory functions of immune cells. Indeed, MIF is required for optimal host defenses against infection, as shown by its capacity to limit the growth of intracellular pathogens (93,94). Yet, excessive amounts of MIF produced during the acute phase of sepsis is deleterious, as shown by the fact that serum MIF levels are increased and correlate with outcome in patients with septic shock (95) and that neutralization of MIF activity improves outcome in animal sepsis models (96). Overall, MIF has been implicated in the pathogenesis of numerous inflammatory and auto-immune diseases, including sepsis, adult respiratory distress syndrome, glomerulonephritis, arthritis, colitis, pancreatitis, systemic lupus erythematosus, autoimmune uveïtis and sarcoidosis (**Table 1.6**) (67,97).

Table 1.6. MIF mechanisms of action and biological activities in pathologic conditions. Adapted from *Lue et al., Microbes and Infection, 2002* (98).

Diseases/ pathologic conditions	Associated MIF activity	Mechanism(s)
Septic Shock	↑ TNF, NO, IL-1, IL-6, IL-8 ↑ LPS signaling	Up-regulation of TL4 Binding of MIF to CD74 leading to ERK1/2 pathway activation
Stress and glucocorticoid functions	Counter-regulation of glucocorticoid action	Counteracts steroid-induced upregulation of cytosolic IκBα Counteracts glucocorticoid-induced expression of MKP-1
Inflammatory lung disorders	↑ TNF, IL-8 ↑ arachidonic acid release	Counter-regulation of glucocorticoid action ↑ arachidonic acid release by MIF
Rheumatoid arthritis	↑ MMP-1/MMP-3 in synovial fibroblasts ↑ PLA-2 / COX-2 acitvity ↑ TNF	↑ PKC, Ap-1 and TK by MIF ↑ MIF expression by 10^{-10}-10^{-12}M glucocorticoids in synoviocytes ↑ PLA-2/COX-2 activity by MIF
Cancer and tumorigenesis	↓ p53 activity ↓ Redox- and stress-induced apoptosis ↑ PI3K/Akt survival pathway ↑ Cell proliferation ↑ Tumor invasion ↑ Tumor cell metastasis and motility	↓ p53 activity by MIF ↑ of ERK1/2 activity By MIF ↑ PI3K/Akt survival pathway by MIF MIF interacts with JAB1/CSN5 that interact with p53 Modulation of JNK activity by MIF

1.8.5 MIF as a link between inflammation and cancer

Recent studies strongly support an important role for MIF in the control of cell growth and tumorigenesis (**Table 1.6**). MIF stimulates cell proliferation, promotes cell survival and tumor-associated neovascularization and inhibits antitumor natural killer (NK) and cytotoxic T-lymphocyte (CTL) responses. Moreover, MIF regulates tumor cell motility and invasion (summarized in (99)). MIF promotes tumorigenesis by suppression of p53 tumor suppressor activity (89) and by sustaining ERK1/2 activation and cell proliferation (100). MIF has also been shown to increase matrix metaloproteinases (MMPs) secretion, thus favouring tumor invasion. Furthermore CD44, a partner of the MIF receptor complex, has been demonstrated to promote the motility of tumor cells and metastasis, especially in breast cancer (101). Finally, human cancer tissues, such as prostate, breast, colon, brain, skin and lung-derived tumors, have been shown to overexpress MIF (99).

1.9 Aim of the study

When we initiated this project, several studies suggested that inhibitors of class I, II and IV HDAC (HDAC1-11) have anti-inflammatory activity. Yet, their influence on innate immune responses was largely uncharacterized. Thus, the overall aim of the study was to investigate the role of HDAC1-11 inhibitors on host innate immune responses against bacterial and fungal infections. The three main objectives were to:

- examine the influence of HDAC1-11 inhibitors on macrophage and dendritic cell functions *in vitro* and to investigate the molecular basis of the inhibition of innate immune responses by these compounds (**chapter 2 and 3**).
- evaluate the impact of treatement with HDAC1-11 inhibitors on the susceptibility to bacterial and fungal sepsis in preclinical models of infection (**chapter 2**).
- determine whether, and if so by which mechanisms, HDAC1-11 inhibitors interfere with the expression of MIF (**chapter 4 and 5**).

Lately, we moved our attention towards the analysis of the influence of inhibitors of class III HDACs (*i.e.* sirtuins) on innate immune responses. This decision was motivated by the fact sirtuins and HDAC1-11 share numerous common substrates. In **chapter 6** of this manuscript, we will first introduce sirtuins and sirtuin inhibitors and then present the preliminary results we obtained *in vitro* and *in vivo*.

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2 HISTONE DEACETYLASE INHIBITORS IMPAIR INNATE IMMUNE RESPONSES TO TOLL-LIKE RECEPTOR AGONISTS AND INFECTION

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***Blood*, January 27, 2011, Volume 117, number 4, Pages 1205-1217**

2.1 Summary

Inhibitors of class I, II and IV HDACs (*i.e.* HDAC1-11) are amongst the most promising anti-cancer drugs. Several studies have suggested that inhibitors of HDAC1-11 (thereafter called HDACi) have anti-inflammatory activity. Yet, their influence on innate immune responses remains largely uncharacterized. In the present study, we investigated the effects of HDACi (trichostatin A, valproate, and suberoylanilide hydroxamic acid) on host innate immune responses against TLR agonists and bacterial and fungal infections.

Strikingly, genome-wide expression studies revealed that trichostatin A acts essentially as negative regulator of basal and microbial product-induced expression of critical immune receptors and antimicrobial products expressed by macrophages. These results were confirmed in a number of settings analysing the production of cytokines or the expression of co-stimulatory molecules by mouse and human macrophages, dendritic cells, splenocytes and whole blood treated with HDACi and stimulated with a broad range of bacterial and fungal products. At the molecular level, we demonstrate that HDACi impair pro-inflammatory cytokine expression without interfering with the activation of the NF-κB and MAP kinases signaling pathways. Most importantly, we identified a new molecular mechanism whereby HDACi stimulate the expression and the recruitment of the transcriptional repressor Mi-2β on the promoter of pro-inflammatory genes targeted by HDACi. The relevance of the above findings was evaluated *in vivo* in experimental models of bacterial and fungal sepsis or toxic shock titrated to cause either mild or severe infections or shock. These experiments revealed that mice treated with valproate are more susceptible to non-severe bacterial and fungal infection, but are protected against toxic and septic shock. Altogether these data confirm the potent anti-inflammatory potential of HDACi *in vivo*.

Overall, these studies ascribe HDAC a pivotal role in controlling the biologic functions of innate immune cells, and identified HDACi as powerfull anti-inflammatory drugs which may increase the susceptibility to infection in immunocompromised patients. Yet, the anti-inflammatory and immunosuppressive properties of HDACi may be beneficial as adjunctive therapy for septic shock.

Histone deacetylase inhibitors impair innate immune responses to Toll-like receptor agonists and to infection

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Regulated by histone acetyltransferases and deacetylases (HDACs), histone acetylation is a key epigenetic mechanism controlling chromatin structure, DNA accessibility, and gene expression. HDAC inhibitors induce growth arrest, differentiation, and apoptosis of tumor cells and are used as anticancer agents. Here we describe the effects of HDAC inhibitors on microbial sensing by macrophages and dendritic cells in vitro and host defenses against infection in vivo. HDAC inhibitors down-regulated the expression of numerous host defense genes, including pattern recognition receptors, kinases, transcription regulators, cytokines, chemokines, growth factors, and costimulatory molecules as assessed by genome-wide microarray analyses or innate immune responses of macrophages and dendritic cells stimulated with Toll-like receptor agonists. HDAC inhibitors induced the expression of Mi-2β and enhanced the DNA-binding activity of the Mi-2/NuRD complex that acts as a transcriptional repressor of macrophage cytokine production. In vivo, HDAC inhibitors increased the susceptibility to bacterial and fungal infections but conferred protection against toxic and septic shock. Thus, these data identify an essential role for HDAC inhibitors in the regulation of the expression of innate immune genes and host defenses against microbial pathogens. (*Blood*. 2011;117(4):1205-1217)

Introduction

The innate immune system plays an essential role in antimicrobial defenses. Detection of microbial pathogens is carried out by sentinel cells of the innate immune system that are located in tissues (macrophages and dendritic cells [DCs]) in close contact with the host's natural environment or that are rapidly recruited to the site of infection (neutrophils). Recognition of invasive pathogens by immune cells relies on their capacity to detect microbial- or pathogen-associated molecular patterns, such as endotoxin, peptidoglycan, lipopeptides, glucans or mannans, flagellin, and nucleic acids. This process involves the coordinated actions of soluble and cellular molecules composing components of the complement system, acute phase proteins, and membrane-associated or intracellular pattern-recognition molecules, including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors, retinoic acid-inducible gene I like receptors, C-type lectin receptors, and scavenger receptors. Ligand-activated receptors trigger the mitogen-activated protein kinase, nuclear factor-κB (NF-κB), and interferon-related factor (IRF) signal transduction pathways that induce the transcription and production of immune genes, including cytokines that are critical for the activation of innate and adaptive immunity.[1,2]

Chromatin structure plays a central role in regulating gene expression. Acetylation of histones is an essential epigenetic mechanism controlling chromatin structure, DNA accessibility for transcription factors, and gene expression. The net state of acetylation of the ϵ amino groups of lysine residues of histones is regulated by the opposing actions of histone acetyltransferases and histone deacetylases (HDACs). Acetylation of histones relaxes the chromatin structure promoting gene transcription, whereas deacetylation of histones compacts the chromatin structure favoring gene silencing. HDACs have been classified into 4 subclasses based on their homology with yeast HDACs, their subcellular localization, and their enzymatic activity.[3] Beside histones, nonhistones proteins (such as α-tubulin, heat shock protein 90, steroid receptors, and regulators of nuclear import) are also modified by reversible acetylation.[4,5] Therefore, histone acetyltransferases and HDACs affect diverse biologic functions, principally cell differentiation, growth, and survival.[6-8]

HDACs are at the center of great interest for 2 major reasons. First, dysregulated HDAC expression or activity has been linked to the pathogenesis of cancer and inflammatory and autoimmune diseases. Second, small-molecule inhibitors of class I, II, and IV HDACs have been shown to exhibit anticancer activity with good safety profiles notably in patients with hematologic malignancies. Thus, these drugs are among the most promising anticancer agents under development.[6,8-10] Here we analyzed the impact of HDAC inhibitors on gene expression profiles in macrophages and DCs in vitro and on the host response to bacteria and fungi in vivo. We found that HDAC inhibitors exerted profound inhibitory effects on the host innate immune antimicrobial defense

Submitted May 10, 2010; accepted October 7, 2010. Prepublished online as *Blood* First Edition paper, October 18, 2010; DOI 10.1182/blood-2010-05-284711.

An Inside *Blood* analysis of this article appears at the front of this issue.

The online version of this article contains a data supplement.

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response, down-regulating the expression of innate immune receptors, interfering with transcriptome remodeling after stimulation with TLR agonists, and inhibiting the expression of key antimicrobial cytokines and accessory molecules in whole blood, macrophages, DCs, and splenocytes. Consistent with these immunosuppressive effects, HDAC inhibitors enhanced the susceptibility of mice to bacterial and fungal infections. Conversely, HDAC inhibitors protected mice from septic shock.

Methods

Ethics statement

All animal procedures were approved by the Office Vétérinaire du Canton de Vaud (authorizations 876.5, 876.6, and 877.5) and performed according to the institution guidelines for animal experiments.

Mice, cells, and reagents

Eight- to 12-week-old female BALB/c mice (Charles River Laboratories) were housed under specific pathogen–free conditions. Bone marrow derived macrophages (BMDMs), thioglycollate-elicited peritoneal macrophages, and RAW 264.7 macrophages (ATCC TIB-71) were cultured as previously described.[11] Bone marrow–derived DCs (BMDCs) were obtained by culturing bone marrow cells in Iscove modified Dulbecco medium containing 10% fetal calf serum (FCS; Sigma-Aldrich), 50μM 2-mercaptoethanol, and granulocyte-macrophage colony-stimulating factor. Splenocytes were cultured in RPMI medium containing 2mM L-glutamine and 10% FCS and 50μM 2-mercaptoethanol. Human myeloid DCs (moDCs) were produced as described previously.[12] Human whole blood assay was performed as described previously.[13]

Cells were exposed to *Salmonella minnesota* ultra pure lipopolysaccharide (LPS; List Biologicals Laboratories), Pam_3CSK_4 (EMC Microcollections), CpG oligonucleotide (CpG ODN; Invivogen), toxic shock syndrome toxin-1 (Toxin Technology), staphylococcal enterotoxin B, concanavalin A (Sigma-Aldrich), or heat-inactivated *Escherichia coli* O18 (*E coli*), *Staphylococcus aureus*, and *Candida albicans*. Trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), and valproic acid (VPA) were from Sigma-Aldrich. The concentrations of TSA and VPA used in vitro did not affect the viability of BMDMs (> 85% cell recovery after 18 hours). Orfiril (Desitin Pharmaceuticals), a commercial injectable solution of sodium valproate, was used for in vivo experiments.

Microarray analysis and quantitative real-time PCR

For each experimental condition, 2 independent samples were processed in parallel. Low RNA input fluorescent linear amplification kit (Agilent Technologies) was used for cDNA synthesis and cRNA amplification. Experimental samples were labeled with cyanine 5-CTP, whereas a control Universal mouse RNA mixture (Stratagene) was labeled using cyanine 3-CTP (PerkinElmer). Labeled cRNA was hybridized onto high-density oligonucleotide microarrays containing approximately 20 000 60-mer (Mouse Development Oligo Microarray Kit, reference G4120A, and Mouse Oligo Microarray Kit, V2, reference G4121B, Agilent Technologies). Slides were scanned using a Microarray Scanner G2565AA system (Agilent Technologies) at a resolution of 5 μm. For data analysis, local background-subtracted signals were calculated using Feature Extraction software (Agilent Technologies, Version A6.1.1). To ensure spot quality, features and their respective background, which were not uniform in pixel fluorescence intensity distribution in both channels, were flagged (nonuniformity outlier flagging algorithm). Data were imported in GeneSpring, Version 7.0 (Agilent Technologies) and then normalized using both per spot (signal channel divided by the corresponding control channel and generation of $\log_{10}$ ratio) and per chip (to the 50th percentile). The microarray dataset has been deposited in the Gene Expression Omnibus database (GEO; National Center for Biotechnology Information; accession numbers GPL7291, GSE22409).

Real-time polymerase chain reaction (RT-PCR) was performed with a 7500 Fast Real-Time PCR System using the Power SYBR Green PCR Master Mix (Applied Biosystems) and primer pairs (supplemental Table 4, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).[14] Samples were tested in triplicates. A standard made of successive dilutions of a reference cDNA was processed in parallel. Gene-specific expression levels were assessed relative to the expression of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *Hprt* and reported in arbitrary units. In selected experiments, standards consisted of serial dilutions of a plasmid containing the target gene.

Cytokine measurements

Tumor necrosis factor (TNF) and interleukin-6 (IL-6) concentrations were measured by bioassay,[15] IL-12p40, and interferon-γ (IFN-γ) by enzyme-linked immunosorbent assay (ELISA; BD Biosciences), and a broad screening of cytokines and chemokines production was performed using the Luminex technology (Luminex Corporation).[14]

Proliferation

Splenocytes (1.5×10^5) were cultured for 48 hours in 96-well culture plates. Proliferation was monitored by measuring ^{3}H-thymidine incorporation over 18 hours.

Flow cytometry

BMDCs were incubated with 2.4G2 monoclonal antibody (mAb) and mAbs (BD Biosciences) specific for mouse major histocompatibility class II (14-4-4S-fluorescein isothiocyanate), CD11c (HL3-phycoerythrin [PE]), and CD40 (3/23-biotin revealed with CyChrome-conjugated streptavidin; BD Biosciences). moDCs were incubated with the lineage Cocktail 1 (fluorescein isothiocyanate–conjugated mAbs specific for CD3, CD14, CD16, CD19, CD20, and CD56) and mAbs specific for human CD11c (B-ly6-PE-Cy5), HLA-DR (L243-APC) and CD40 (5C3-PE), CD80 (L307.4-PE), CD86 (FUN-1-PE), or CC-chemokine receptor 7 (CCR7; 3D12-PE).[12] Data were analyzed using FlowJo Version 8.5.3 software (TreeStar).

Electrophoretic mobility shift assay

A total of 2 μg of nuclear extracts was incubated for 15 minutes at room temperature with a radiolabeled consensus NF-κB probe (Santa Cruz Biotechnology) and analyzed by electrophoretic mobility shift assay.[16]

Western blot analyses

Cell lysates were electrophoresed through polyacrylamide gels and transferred onto nitrocellulose membranes.[14] Membranes were incubated with antibodies specific for phosphorylated (phospho)-extracellular signal-regulated kinase (ERK)1/2, total-ERK1/2, phospho-p38, total-p38, phospho-IRF3, (Cell Signaling Technology), c-jun, Mi2b (Santa Cruz Biotechnology), SNF2β/BRG1 (Millipore), IRF7 (Invitrogen), phospho-signal-transducer and activator of transcription protein (STAT1; BD Biosciences), and α-tubulin (Sigma-Aldrich), and then revealed with secondary horseradish peroxidase-conjugated goat antirabbit IgG and the ECL Western blotting analyses system (GE Healthcare). Acid-soluble proteins were extracted and analyzed by Western blotting using antiacetylated histone H3 and H4 antibodies (Cell Signaling Technology).[13]

Chromatin immunoprecipitation

Chromatin immunoprecipitation analysis was performed using the ChIP assay kit (Millipore) using antiacetylated histone H4 (06-866, Upstate Biotechnology) or Mi2b (sc-11378X, Santa Cruz Biotechnology) rabbit polyclonal antisera and normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) as described previously.[13] Immunoprecipitated DNA was amplified by PCR using primers described in supplemental Table 4.

siRNA silencing in RAW 264.7 mouse macrophages

RAW 264.7 macrophages (6×10^4 cells per well) were seeded in 24-well plates and transfected the next day with 187.5 ng of Mi-2b or Brg1 siRNA

duplexes or negative control (sequences available in supplemental Table 4) and HiPerFect transfection reagent (QIAGEN) according to manufacturer's instruction. After 3 days, cells were stimulated for 4 hours with 0 to 10 ng/mL LPS. Gene expression was analyzed by real-time PCR. Mi-2b and Brg1 mRNA levels were decreased by 60% in cells transfected with the specific siRNAs.

In vivo models

Klebsiella pneumonia *sepsis*. A total of 10 CFU of a clinical isolate of *K pneumoniae* was injected intranasally into mice treated 15 minutes earlier with a single dose of valproate (Orfiril, 200 mg/kg intraperitoneally).[17]

***Systemic candidiasis*.** Mice were challenged through the tail vein with 1.2×10^5 CFU of a clinical isolate of *C albicans*. Valproate treatment (200 mg/kg intraperitoneally) was administrated 15 minutes before *C albicans* challenge and repeated daily during 24 days.

***Pam_3CSK_4-induced shock*.** Mice were injected with D-galactosamine (2 g/kg intraperitoneally) followed immediately after by Pam_3CSK_4 (1.6 mg/kg intraperitoneally).[11] Valproate injections (200 mg/kg intraperitoneally every 12 hours) were started 2 days before D-galactosamine injection and discontinued 48 hours after Pam_3CSK_4 challenge.

***CLP*.** Animals were pretreated intraperitoneally 1 hour before cecal ligation and puncture (CLP) with valproate (200 mg/kg) and injected subcutaneously every 12 hours with gentamicin (10 mg/kg), clindamycin (30 mg/kg), and buprenorphine (0.1 mg/kg).

Doses of valproate were selected based on previous publications and adjusted to the specific conditions of the sepsis models.

Statistical analysis

Comparisons among treatment groups were performed using the Fisher 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. The analyses were performed using Prism software Version 5.03 (GraphPad). All reported *P* values are 2-sided, and values less than .05 were considered to indicate statistical significance. For microarray analyses, statistical significance of differentially expressed genes (2-fold changes) was evaluated by analysis of variance using the Benjamini and Hochberg false discovery rate correction (5%).

Results

Inhibition of HDACs down-regulates the expression of innate immune genes in macrophages

***Transcriptome analyses*.** The transcriptome of BMDMs was examined using the Agilent high-density Mouse Development Oligoarrays, which contained 20 280 unique 60-mer corresponding predominantly to expressed sequence tags and with the Mouse Oligo Microarray Kit (V2) containing 20 156 indexed gene probes. At baseline, TSA (t = 4 hours of incubation), a prototypical broad-spectrum inhibitor of class I, II, and IV HDACs, modified the expression of 1594 macrophage genes (7.7% of the transcriptome), of which 772 (3.6%) were found to be down-regulated and 822 (4.1%) up-regulated. HDAC inhibitors significantly reduced the expression of numerous pattern recognition molecules and immune receptors involved in the sensing of a broad range of microbial products, including bacterial lipopeptides, lipoteichoic acid, peptidoglycan, endotoxin, flagellin, viral nucleic acids, and fungal β-glucan (supplemental Table 1). We then performed time-course analyses of gene expression profiles of BMDMs preincubated for 1 hour with TSA before stimulation for 1, 2, 4, and 20 hours with TLR1/TLR2 (Pam_3CSK_4 lipopeptide) or with TLR4 (LPS) agonists (Figure 1A-B). TSA inhibited the up-regulation of 32% to 58% and 33% to 60% of the genes induced by LPS or Pam_3CSK_4, respectively. Conversely, TSA counter-regulated the down-regulation of 60% to 73% and 51% to 75% of the genes repressed by LPS or Pam_3CSK_4. In contrast, TSA potentiated the effects of LPS and Pam_3CSK_4 on only a small proportion of genes (2%-16%). The genes modulated by TSA included signal transduction, immunoregulation, cytoskeleton and cell structure, metabolism and cell cycle, growth, and apoptosis (complete listing of the genes: Figure 1C-D, supplemental Tables 2-3).

We then focused our analyses on innate immune gene families known to play a critical role in the host antimicrobial defense response. TSA exerted prominent inhibitory effects on LPS- or Pam_3CSK_4-induced genes (Figure 1C-D). Indeed, TSA inhibited LPS- and/or Pam_3CSK_4-induced up-regulation of genes encoding for molecules involved in the sensing of microbial compounds, such as Tlrs, Cd14, Md-2, scavenger receptors (Scarb2), cytosolic microbial sensors (Aim1, Mda-5, Nlrp3, Nod1, Nod2, Eif2ak2/Pkr, Pycard/Asc, Mevf/Pyrin, and RIG-I), c-type lectins (Clecs and Msr1), formyl peptide receptors (Fprs), IgE and IgG Fc receptors, complement and complement receptors (C1qa, C1r, and Cfb/H2-Bf), and adhesion molecules (Icam1, integrins, and Vcam1; Table 1). The effect of TSA on the expression of Tlrs, Cd14, Cd36, and Md-2 was confirmed by real-time PCR (Figure 1E). TSA also inhibited the expression of adaptor molecules (MyD88 and Ticam2), kinases (Iraks, Jaks, Lck, Map3ks, Ripk2, Syk, Tank, Tbk1, and Traf1), phosphatases, and transcription modulators (Atfs, Cebps, Irfs, Junb, Nfkbs, Spic, Stats, and Socs1; Table 1). In addition, TSA down-regulated a wide range of LPS- and/or Pam_3CSK_4-stimulated mediators involved in chemotaxis, inflammation, tissue repair, and antigen processing and presentation. This list of genes included cytokines (Il1a, Il1rn, Il6, Il12a, Il12b, Il15, Il18, Il23a, Il27, Ltb, Tnf, Tnfaip3, Tnfsf4, and Tnfsf9), chemokines (Ccl4, Ccl7-9, Ccl12, Ccl17, Ccl22, Ccl24, Cklfsf3, Cklfsf6, Cklfsf7, Cxcl2, Cxcl5, Cxcl12, Cxcl16, and Cx3cl1), growth factors (Csf2, Edn1, and Tmpo), and their receptors (Il1rl1, Il2rg, Il4ra, Il10rb, Il13ra1, Crlf3, Cxcl12, Cxcl16, Pdfrl, Tnfrsf1a, Tnfrsf14, Ifnar1, Ifnar2, Ifngr2, Csf3r, and Ednrb), cathelicidin antimicrobial peptide (Calmp), matrix metalloproteinases, ubiquitins, proteasome subunits and molecules involved in autophagy (Atg16l), antigen transport (Tap1 and Tap2), and peptide presentation (H-2D, H-2E, and H-2Q; Table 1). Therefore, TSA strongly affected transcriptome remodeling of BMDMs stimulated with microbial products exerting predominantly inhibitory effects, indicating that acetylation of histones or nonhistone proteins is required for optimal transcription of a large number of macrophage genes involved in microbial sensing and host defenses.

***Cytokine production*.** To validate the observations generated from microarray profiling, we quantified the production of cytokines (ie, TNF, IL-6, and IL-12p40) in BMDMs exposed to LPS, Pam_3CSK_4, *E coli*, or *S aureus*. Real-time PCR analyses (Figure 2A), bioassay, and ELISA measurements (Figure 2B-C) confirmed that TSA strongly inhibited TNF, IL-6, and IL-12p40 production in a time- and dose-dependent manner. Yet, TSA did not inhibit LPS-induced Tnf mRNA and protein (Figure 2A-B). Similar results were obtained in thioglycolate-elicited peritoneal macrophages (data not shown).

To confirm the findings obtained with TSA, we tested the effects of 2 other HDAC inhibitors: SAHA, a hydroxamate, and VPA, a short chain fatty acid. Like TSA, SAHA and VPA used at clinically relevant concentrations (4-100nM and 4-100μM) dose-dependently inhibited TNF, IL-6, and IL-12p40 production in BMDMs stimulated with Pam_3CSK_4 (Figure 2D). VPA also markedly reduced (up to 50-fold) the production of 13 of 15 mediators induced by LPS or Pam_3CSK_4 in whole blood (Figure 2 E-F).

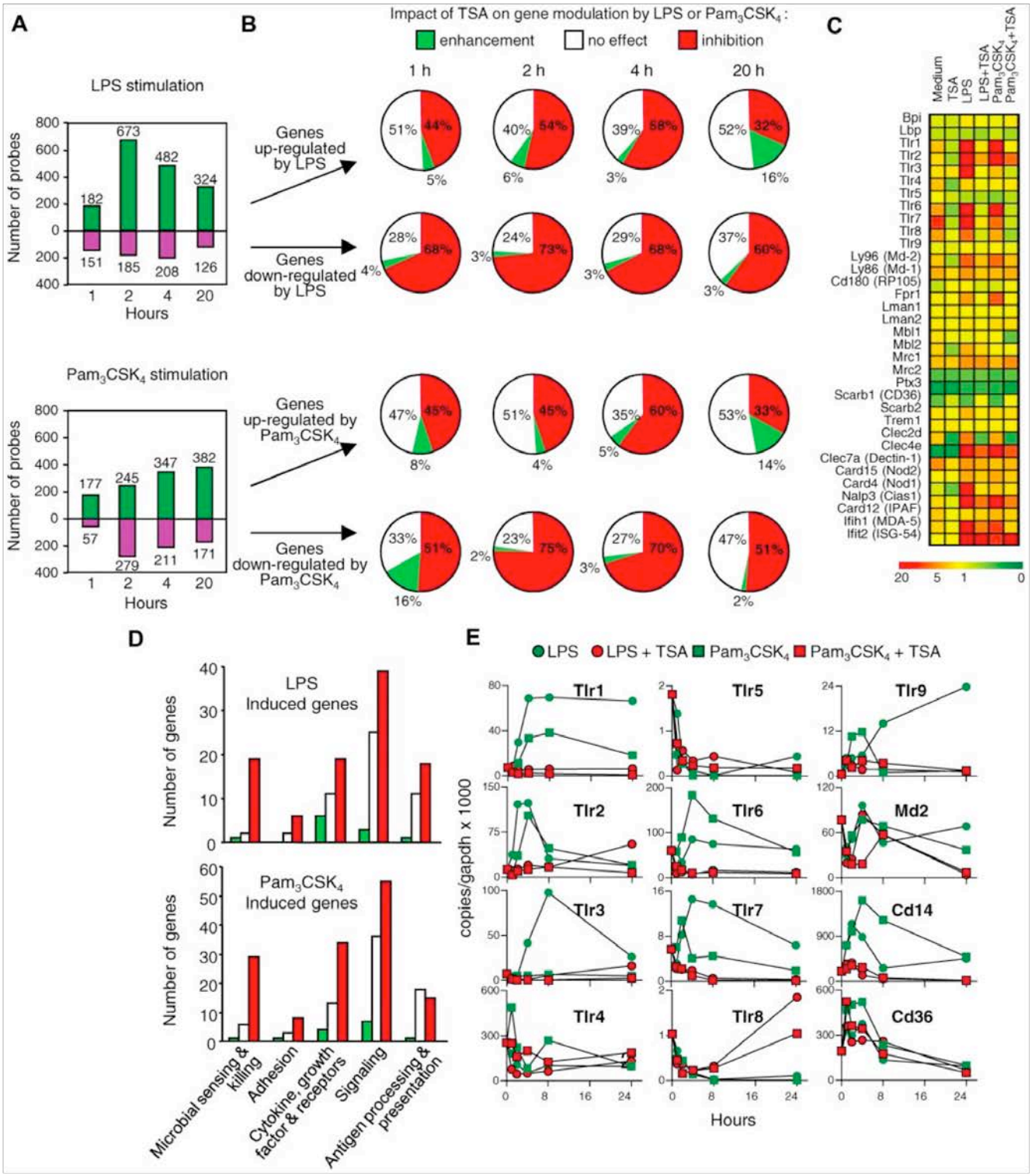


Figure 1. Trichostatin A inhibits the expression of innate immune genes in macrophages. BMDMs were preincubated for 1 hour with or without TSA (100nM) before exposure (C-D, 4 hours) to LPS (100 ng/mL) or Pam_3CSK_4 (100 ng/mL). Transcriptome was analyzed with Agilent Mouse Development Oligoarrays (A-B) or Mouse Oligo Microarray Kit V2 (C-D). (A) Number of genes either up-regulated or down-regulated by LPS and Pam_3CSK_4 without preincubation with TSA (fold change $>$ 2 vs medium). (B) Effect of 1-hour preincubation with TSA on genes (expressed in percentage) either up-regulated or down-regulated by LPS or Pam_3CSK_4 (fold change $>$ 2 vs medium). White represents no change; red, inhibition; and green, increase by TSA compared with stimulation with LPS or Pam_3CSK_4 alone. (C) Heat map of selected pattern recognition molecules (medium, TSA, LPS and Pam_3CSK_4: 4 hours of incubation, LPS + TSA and Pam_3CSK_4 + TSA; 1-hour preincubation with TSA followed by 4-hour incubation with LPS and Pam_3CSK_4). (D) Effect of 1-hour preincubation with TSA (fold changes were calculated vs LPS or Pam_3CSK_4 alone) on a selection of genes up-regulated by LPS or Pam_3CSK_4 and grouped into various categories based on their biologic functions (microbial sensing and killing; adhesion; cytokine, growth factor and receptors; signaling; antigen processing and presentation). White represents no change; red, inhibition; and green, increase by TSA compared with stimulation with LPS or Pam_3CSK_4 alone. (E) Tlr1-9, Md-2, Cd14, and Cd36 mRNA copy number was determined by RT-PCR and expressed relative to that of GAPDH. Data are representative of 2 independent experiments.

Table 1. Selection of genes whose up-regulation by LPS or Pam_3CSK_4 is inhibited by TSA in BMDMs

Adhesion molecule	Innate receptor	Signal transduction	Phosphatase	Transcription regulator	Cytokine, chemokine, and growth factor	Cytokine, chemokine, and growth factor receptor	Antigen processing and presentation	Ubiquitination	Others
Alcam	*Aim1*	*Csnk1a1*	*Dusp1 (Mkp-1)*	*Atf3*	*Ccl4*	*Ccrl2*	*Atg16l*	*Ubc*	*Calmp*
Cd47	*Birc (cIAP2)*	*Irak2*	*Dusp4*	*Atf4*	*Ccl7*	*Crlf3*	*Ctsz*	*Ube1l*	*C1qa*
Icam1	*Card4 (Nod1)*	*Irak3*	*Dusp16*	*Atf5*	*Ccl8*	*Csf3r*	*H2-DMb1*	*Ube2c*	*C1r*
Itga4	*Carc15 (Nod2)*	*Jak1*	*Ptpn6*	*Batf*	*Ccl9*	*Ednrb*	*H2-Ea*	*Ube2d3*	*H2-Bf*
Itga5	*Cd14*	*Jak2*	*Ptpn9*	*Bcl3*	*Ccl12*	*Ifnar1*	*H2-Eb1*	*Ube2e2*	*F10*
Itgal	*Cias1 (Nlrp3)*	*Lck*	*Ptpn12*	*Bcl7c*	*Ccl17*	*Ifnar2*	*H2-M3*	*Ube2j1*	*Mmp9*
Itgax	*Clec2d*	*Map3k7ip2*		*Bcl10*	*Ccl22*	*Ifngr2*	*H2-Q10*	*Ube2j2*	*Mmp14*
Itgb3	*Clec4a2*	*Map3k8 (Tpl2)*		*Cebpb*	*Ccl24*	*Il1rl1*	*H2-Q7*	*Ube2l6*	*Mmp25*
Vcam1	*Clec4d*	*Mapkapk2*		*Cebpd*	*Ccrl2*	*Il2rg*	*Psma3*	*Ube2m*	*Nos2*
	Clec4e	*Myd88*		*Cited2*	*Csf2*	*Il4ra*	*Psma4*	*Ube2r2*	*Pde4b*
	Eif2ak2 (Pkr)	*Pias1*		*Hmgb2*	*Cklfsf3*	*Il10rb*	*Psma5*	*Ube2v1*	*Pld2*
	Fcer1g	*Prkr*		*Ikbke*	*Cksfs6*	*Il13ra1*	*Psma6*	*Ubl3*	*Ptger4*
	Fcgr2b	*Ptk2*		*Irf1*	*Cklfsf7*	*Pdfrl*	*Psmb7*	*Ubtd1*	*Ptges*
	Frp1	*Ripk2*		*Irf2*	*Cxcl2*	*Ptafr*	*Psmb8*	*Ufm1*	*Ptgir*
	Fpr-rs2	*Socs1*		*Irf5*	*Cxcl5*	*Tnfrsf1a*	*Psmb9*	*Usp12*	*Ptgs2*
	Ifih1 (Mda5)	*Stk19*		*Irf7*	*Cxcl12*	*Tnfrsf14*	*Psmb10*	*Usp18*	*Sod2*
	Ly96 (Md-2)	*Syk*		*Junb*	*Cxcl16*		*Psme1*	*Usp24*	*Txn1*
	Mefv (Pyrin)	*Tank*		*Klf6*	*Cx3cl1*		*Psme2b*	*Usp42*	*Tnfrsf5 (Cd40)*
	Msr1	*Tbk1*		*Nfkb1*	*Edn1*		*Tap1*		
	Pycard	*Ticam2*		*Nfkb2*	*Il1a*		*Tap2*		
	Scarb2	*Traf1*		*Nfkbib*	*Il1rn*		*Tapbp*		
	Ddx58 (RIG-I)	*Trim30*		*Nfkbie*	*Il6*		*Tapbpl*		
	Tlr1			*Nfkbiz*	*Il12a*				
	Tlr2			*Pias1*	*Il12b*				
	Tlr3			*Rel*	*Il13ra1*				
	Tlr6			*Rela*	*Il15*				
	Tlr7			*Relb*	*Il18*				
	Tlr9			*Spic*	*Il23a*				
				Stat3	*Il27*				
				Stat5a	*Inhba*				
					Ltb				
					Pbef1				
					Tnf				
					Tmpo				
					Tnfaip3 (A20)				
					Tnfsf4 (OX-40L)				
					Tnfsf9 (4-1BBL)				

HDAC inhibitors down-regulated the production of IL-1ra and IL-10, suggesting that the reduced expression of proinflammatory cytokines was not the result of an increased expression of anti-inflammatory cytokines.

Expression of IFNs and IFN-dependent genes. Together with cytokines and chemokines, type I interferons (IFN-α and IFN-β) are central mediators of innate and adaptive immune responses against viral and bacterial infections.[18,19] In response to LPS stimulation, macrophages produce copious amounts of IFN-β that stimulates the transcription of *Ifna*, *Ccl2*, *Ccl8*, *Ccl12*, *Cxcl10*, and *Nos2* (encoding for iNOS), *Irf7*, and *Irf8*.[20,21] LPS, but not Pam_3CSK_4, induced rapid (1 hour) Ifnb mRNA expression and sustained IFN-β secretion by BMDMs, whereas LPS or Pam_3CSK_4 induced early (2 hours and 4 hours, LPS) or late (8 hours and 24 hours, Pam_3CSK_4) Ifna4 mRNA up-regulation (Figure 3A-C). Interestingly, TSA up-regulated and markedly prolonged (up to 24 hours) IFN-β mRNA and protein expression after LPS exposure (Figure 3A-B), confirming microarray data (supplemental Table 2). In contrast, it completely inhibited the up-regulation of Ifna4 mRNA (Figure 3C). TSA also inhibited the expression of LPS-induced Ccl8, Ccl12, Cxcl10, Nos2, and Irf7 mRNA (Figure 3D) and of numerous other LPS-induced IFN-β-dependent genes (supplemental Figure 1). Yet, TSA did not reduce LPS-induced Ccl2 and Irf8 mRNA and late (24 hours) IFN-β-independent Ifna4 mRNA (Pam_3CSK_4) and Ccl5 mRNA (LPS and Pam_3CSK_4). Thus, the massive accumulation of IFN-β induced by TSA in BMDMs did not overcome its inhibition of IFN-β-dependent gene expression. Of interest, TSA dose-dependently suppressed the proliferation of splenocytes induced by LPS, Pam_3CSK_4, CpG ODN, or *E coli* (Figure 3E). It also inhibited the release of IFN-γ by splenocytes exposed to staphylococcal enterotoxin B, toxic shock syndrome toxin-1, or concanavalin A (Figure 3F).

HDAC inhibitors impair the response of DCs

We then examined the effects of HDAC inhibitors on innate immune responses of mouse BMDCs and human moDCs. TSA inhibited the production of cytokines (IL-6 and IL-12p40) and the up-regulation of CD40 by BMDCs stimulated with LPS, Pam_3CSK_4, CpG ODN, *E coli*, *S aureus*, or *C albicans* (Figure 4A-D). Similarly, TSA and VPA inhibited proinflammatory and anti-inflammatory cytokines (TNF, IL-1α, IL-1β, IL-1ra, IL-6, IL-10, IL-12p40, and IL-12p70), chemokines (IP-10/CXCL10, MIP-1β/CCL4, MCP-1/CCL2, and RANTES/CCL5), IFN-γ, G-CSF, and

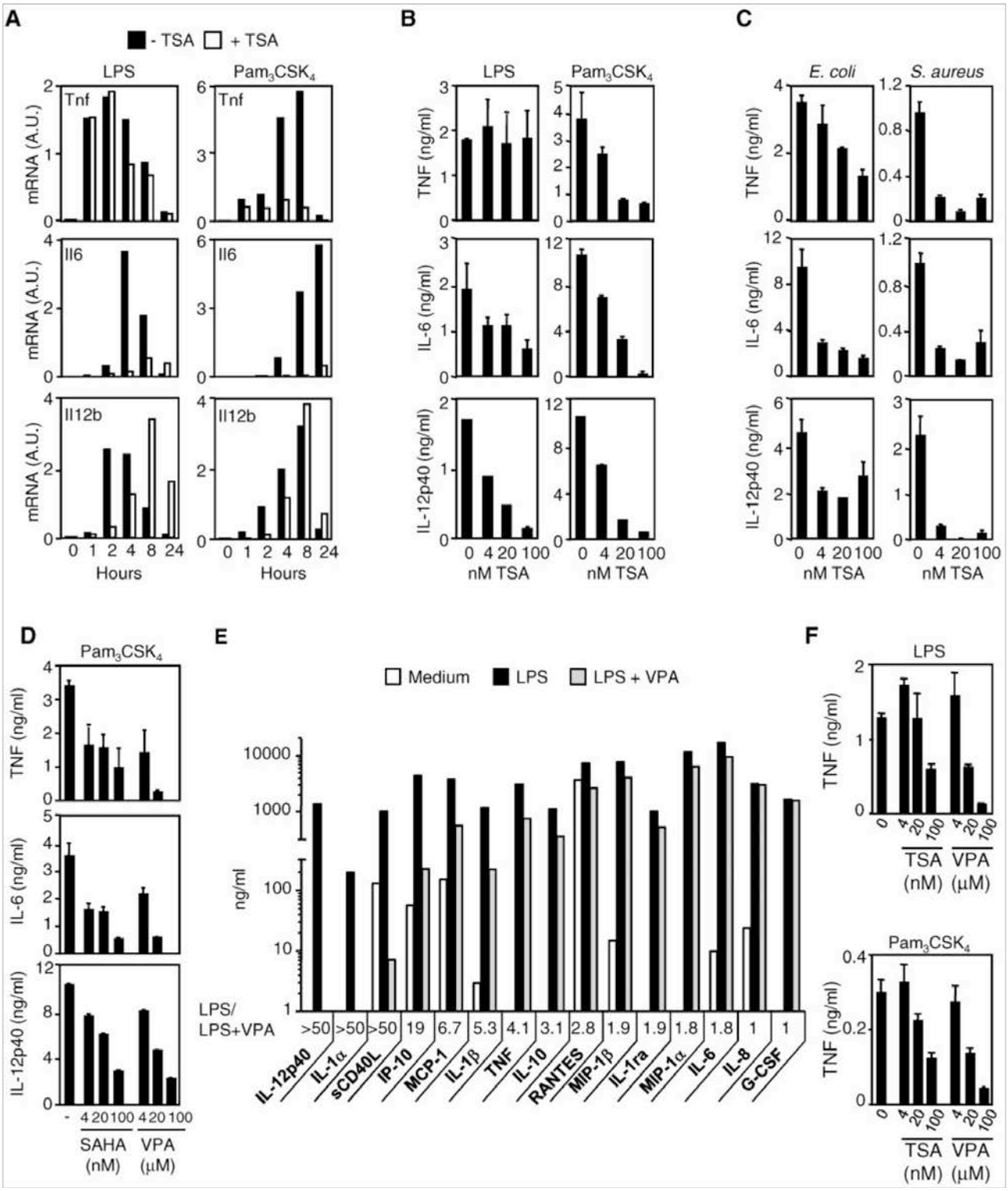


Figure 2. HDAC inhibitors inhibit cytokine release by macrophages exposed to microbial products and bacteria. BMDMs were preincubated for 1 hour with or without TSA (100nM unless specified) before exposure to LPS (100 ng/mL), Pam_3CSK_4 (100 ng/mL), and heat-killed *E coli* or *S aureus* (10^7 CFU/mL). (A-C) TNF, IL-6, and IL-12p40 mRNA (A) and protein (B-C) production by BMDMs. TNF, IL-6, and IL-12p40 mRNA levels were analyzed by RT-PCR, and results are expressed as the ratio of cytokines to GAPDH mRNA levels. Data are representative of 3 independent experiments. Cytokine were quantified in cell culture supernatants collected after 8 hours (TNF) and 18 hours (IL-6 and IL-12p40). Data are mean ± SD of triplicate samples from one experiment representative of 3 independent experiments. A.U. indicates arbitrary units. (D) BMDMs were preincubated for 1 hour with or without SAHA (4, 20, and 100nM) or VPA (4, 20, and 100μM) before exposure to Pam_3CSK_4 (100 ng/mL). TNF, IL-6, and IL-12p40 were quantified in cell culture supernatants collected after 8 hours (TNF) and 18 hours (IL-6 and IL-12p40). Data are mean ± SD of triplicate samples from 1 experiment representative of 3 independent experiments. (E-F) Human whole blood was incubated for 18 hours with VPA (E, 100μM) or TSA together with either LPS (10 ng/mL) or Pam_3CSK_4 (100 ng/mL). Cytokine and chemokine production was assessed by the Luminex technology ("Cytokine measurements"), and LPS/LPS + VPA ratios were calculated (E). TNF was quantified by bioassay. Data are mean ± SD of triplicate samples from one donor and are representative of 2 independent experiments (F).

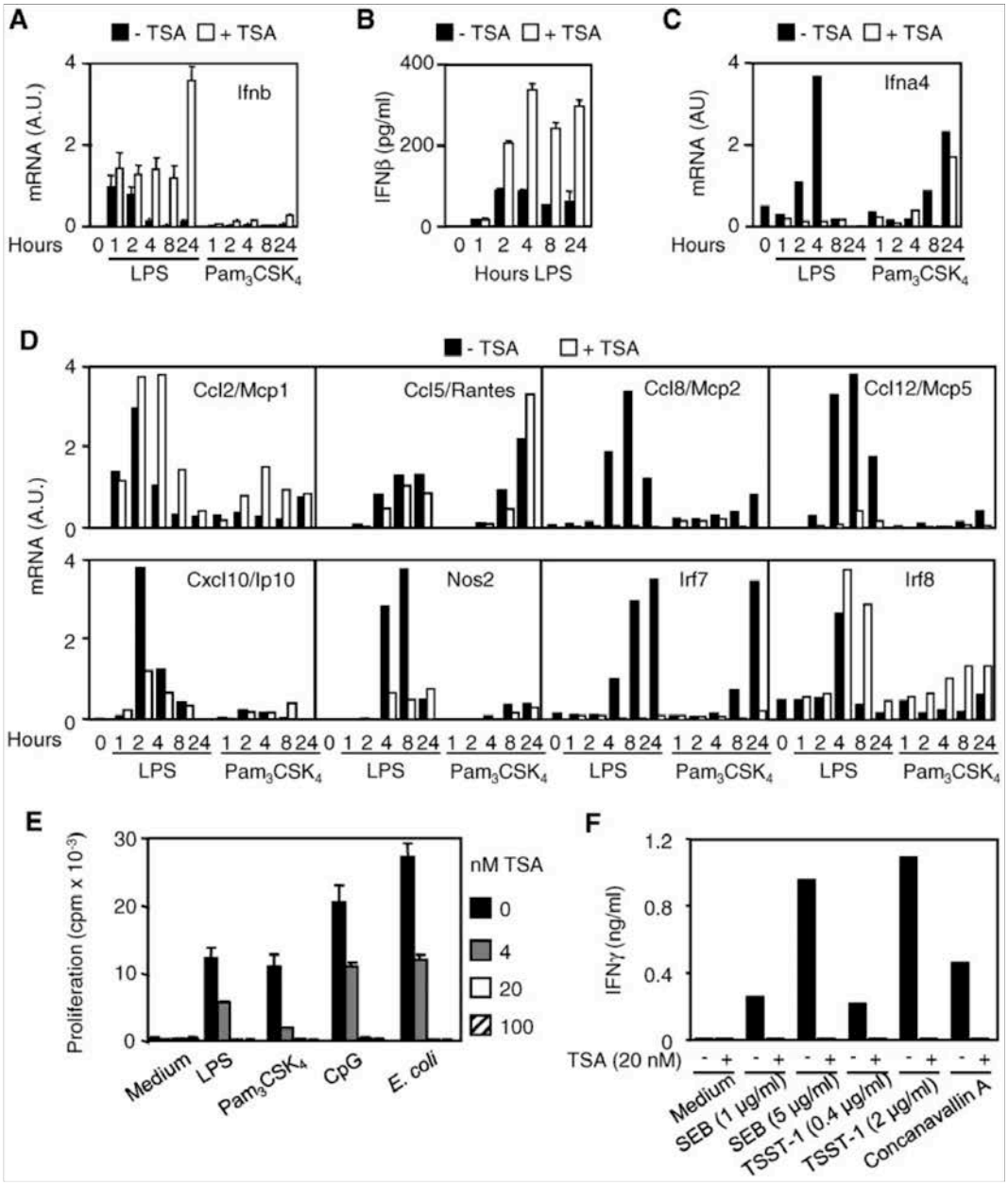


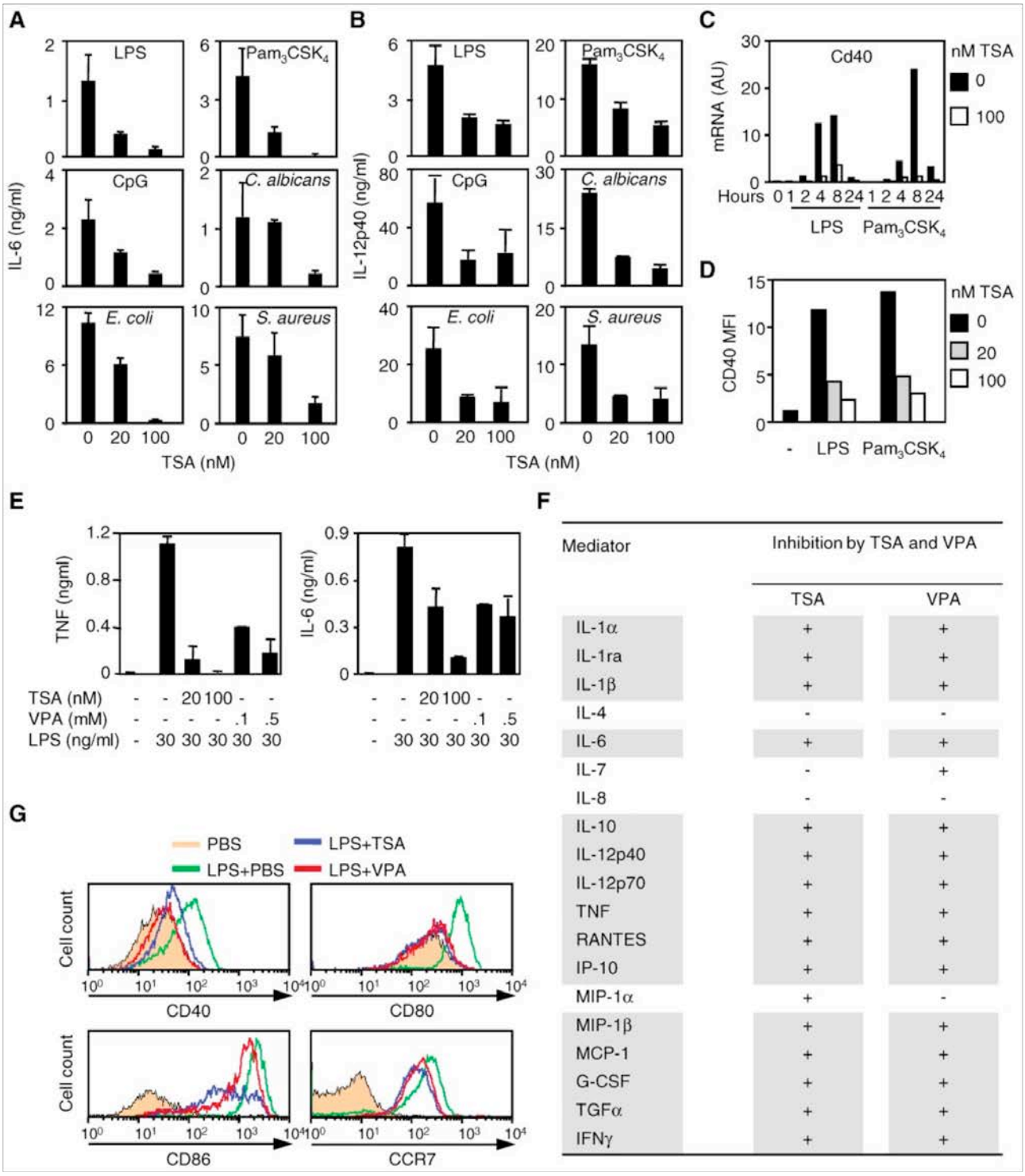
Figure 3. HDAC inhibition enhances the production of IFN-β. (A-D) RNA and cell culture supernatants were collected from BMDMs preincubated for 1 hour with (+) or without (−) TSA (100nM) before exposure to LPS (100 ng/mL) or Pam_3CSK_4 (100 ng/mL). (A-B) IFN-β mRNA and protein expression was quantified by RT-PCR and ELISA. Results are expressed as the ratio of Ifnb mRNA level to that of GAPDH. Data are mean ± SD of triplicate samples from 1 experiment representative of 2 experiments. (C-D) Ifna4 (C), Ccl2, Ccl5, Ccl8, Ccl12, Cxcl10, Nos2, Irf7, and Irf8 (D) mRNA contents were quantified by RT-PCR. Results are expressed as the ratio of mRNA level of the gene of interest to that of GAPDH. Data are representative of 2 independent experiments. A.U. indicates arbitrary units. (E-F) Splenocytes were incubated with TSA and LPS (5 μg/mL), Pam_3CSK_4 (5 μg/mL), CpG ODN (CpG, 0.5μM), *E coli* (5×10^7 CFU/mL), staphylococcal enterotoxin B (SEB, 1-5 μg/mL), toxic shock syndrome toxin-1 (TSST-1, 0.4-2.0 μg/mL), and concanavalin A (5 μg/mL). (E) Proliferation was measured by ^{3}H-thymidine incorporation. Data are mean ± SD of triplicate samples and are representative of 2 independent experiments. (F) IFN-γ production was quantified by ELISA in cell culture supernatants collected after 48 hours. Data are representative of 2 independent experiments.

TGF-α production by moDCs in response to LPS stimulation (Figure 4E-F). TSA and VPA also inhibited the up-regulation induced by LPS of the costimulatory molecules CD40, CD80, and CD86 and of CCR7 (Figure 4G). These results suggested that HDAC inhibitors affect biologic activities of DCs, playing a central role in orchestrating the innate and adaptive responses to infection.

HDAC inhibitors increase the expression and the DNA binding of the transcriptional repressor Mi-2β

We then examined whether HDAC inhibitors affected signal transduction pathways in the macrophage (Figure 5). Neither ERK1/2 or p38 phosphorylation nor NF-κB, c-jun, IRF3, or IRF7 nuclear translocation was inhibited by TSA, VPA, or SAHA in BMDMs, peritoneal macrophages, or RAW 264.7 macrophages (Figure 5A-C; and data not shown). Consistent with the fact that it enhanced LPS-induced IFN-β expression (Figure 3B), TSA markedly prolonged STAT1α/β phosphorylation (Figure 5D). Thus, HDAC inhibitors did not inhibit mitogen-activated protein kinases, NF-κB, IRFs, and STAT1 signal transduction pathways induced by microbial products in macrophages.

Given that histone acetylation is associated with active gene transcription, we then analyzed by chromatin immunoprecipitation



Mediator	Inhibition by TSA and VPA	
	TSA	VPA
IL-1α	+	+
IL-1ra	+	+
IL-1β	+	+
IL-4	-	-
IL-6	+	+
IL-7	-	+
IL-8	-	-
IL-10	+	+
IL-12p40	+	+
IL-12p70	+	+
TNF	+	+
RANTES	+	+
IP-10	+	+
MIP-1α	+	-
MIP-1β	+	+
MCP-1	+	+
G-CSF	+	+
TGFα	+	+
IFNγ	+	+

Figure 4. HDAC inhibitors inhibit the response of DCs to microbial stimulation. (A-C) BMDCs were preincubated for 1 hour with TSA before stimulation for 18 hours or the indicated time with LPS (100 ng/mL), Pam_3CSK_4 (100 ng/mL), CpG oligonucleotide (CpG, 0.7μM), and heat-killed *C albicans*, *E coli*, or *S aureus* (10^7 CFU/mL). (A-B) IL-6 and IL-12p40 production. Data are mean ± SD of triplicate samples and are representative of 4 independent experiments. (C) Cd40 mRNA expression quantified by RT-PCR. Results are expressed as the ratio of Cd40 mRNA level to that of GAPDH. Data are mean ± SD of 1 experiment representative of 3 independent experiments. AU indicates arbitrary units. (D) CD40 mean fluorescence intensity (MFI) determined by flow cytometry. Data are representative of 3 independent experiments. (E-G) Human moDCs were preincubated for 1 hour with TSA (E-F, 100nM) or VPA (E-F, 100μM) before exposure to LPS (30 ng/mL) for 18 hours. (E) TNF and IL-6 production. Data are mean ± SD of triplicate samples and are representative of 2 independent experiments. (F) Effect of TSA and VPA on cytokine and chemokine production by 2 independent preparations of moDCs. Mediators were analyzed using the Luminex technology ("Cytokine measurements"). + indicates inhibition (fold change > 2); and −, no effect. (G) CD40, CD80, CD86, and CCR7 expression analyzed by flow cytometry. Data are representative of 2 independent experiments.

the extent of histone H4 acetylation of the Tnf and Il6 promoters in BMDMs stimulated with LPS or Pam_3CSK_4 (Figure 5E). TSA increased histone H4 acetylation of both promoters, suggesting that there was no correlation between the status of histone acetylation and the observed down-regulatory effect of TSA on gene transcription (Figure 2A).

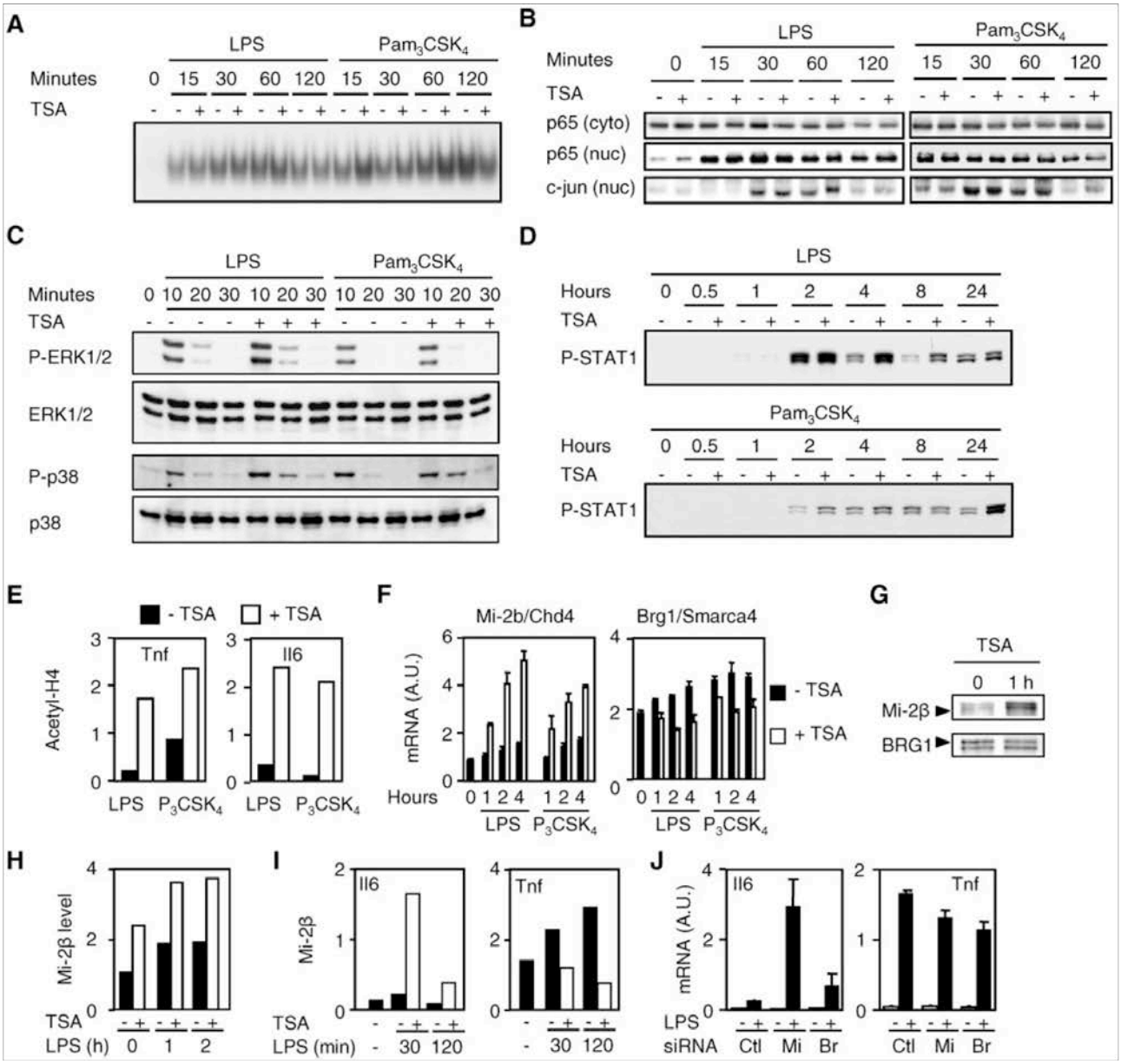


Figure 5. HDAC inhibitors increase Mi-2β expression and recruitment to TSA-sensitive promoter. (A-I) BMDMs were preincubated for 1 hour with (+) or without (−) TSA (100nM unless specified) and exposed to LPS (100 ng/mL) or Pam_3CSK_4 (100 ng/mL) for the indicated time or 1 hour (E). NF-κB DNA binding activity and NF-κB p65, c-jun, phosphorylated (P-), and total ERK1/2 and p38 and P-STAT1 expression were analyzed by electrophoretic mobility shift assay (A) and Western blot (B-D) using nuclear (nuc) and cytosolic (cyto) extracts. The retarded complex detected by electrophoretic mobility shift assay was dose-dependently inhibited by cold wild-type but not mutant NF-κB oligonucleotide, and supershifted using anti-p65 antibody (data not shown). Acetylation of histone H4 (E) and Mi-2β recruitment (I) to Tnf and Il6 promoters were analyzed by chromatin immunoprecipitation. Mi-2β and BRG1 mRNA and protein levels were quantified by real-time PCR (F) and Western blot (G) with densitometric analyses (H). Data are representative of 2 to 5 independent experiments. (J) RAW 264.7 macrophages transfected with control (Ctl), Mi-2β (Mi), or BRG1 (Br) siRNAs. After 3 days, cells were incubated for 4 hours with (+) or without (−) 10 ng/mL of LPS. Il6 and Tnf mRNA levels were analyzed by RT-PCR and results expressed as the ratio of cytokine to GAPDH mRNA levels. Data are representative of triplicate determinations from 1 experiment.

The Mi-2/NuRD and SWI/SNF (also called BAF in mammals) ATP-dependent remodeling complexes play a central role in regulating gene expression.[22] Mi-2β (CHD4) acts as a transcriptional repressor, whereas BRG1 (SMARCA4, the catalytic subunit of the BAF complex) acts as a transcriptional activator of secondary LPS-induced genes in J77.4 macrophages.[23] Given that HDAC inhibitors impaired the expression of secondary (ie, *Il6*) but not primary (ie, *Tnf*) LPS-induced genes in BMDMs, we hypothesized that TSA mediated its effects by affecting the expression of the Mi-2β and BRG1 dyad. Interestingly, TSA markedly upregulated the expression of Mi-2β mRNA in BMDMs exposed to LPS and Pam_3CSK_4 (Figure 5F). In constrast, TSA had a modest inhibitory effect on the expression of BRG1. In line with these findings, TSA increased basal and LPS-induced Mi-2β protein expression (Figure 5G-H). Chromatin immunoprecipitation studies revealed that TSA strongly increased Mi-2β recruitment to the Il6 promoter in BMDMs exposed to LPS, whereas it reduced the binding of Mi-2β to the Tnf promoter (Figure 5I). siRNA-mediated silencing of Mi-2β in RAW 264.7 mouse macrophages greatly enhanced LPS-induced Il6 mRNA expression without a significant effect on Tnf mRNA levels (Figure 5J). Conversely, BRG1 silencing only had a very modest effect on both Il6 and Tnf mRNA expression. Altogether, these data suggest that TSA may inhibit cytokine production via an increased expression of the transcriptional repressor Mi-2β in macrophages.

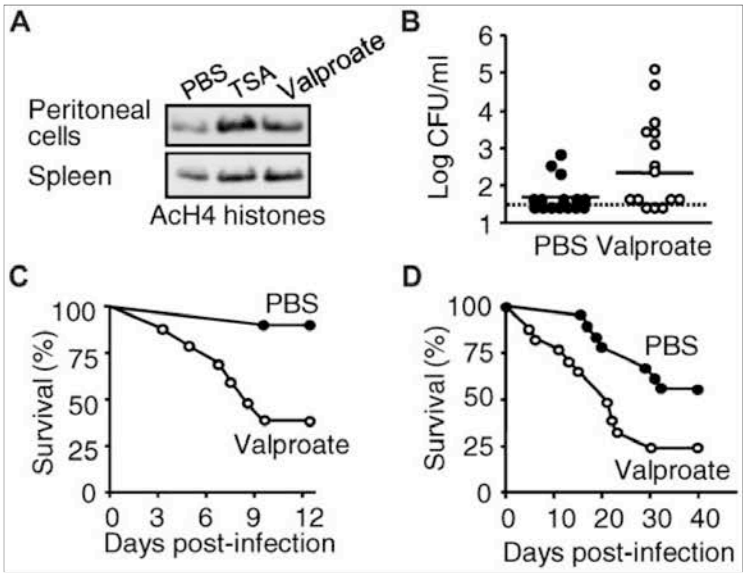


Figure 6. HDAC inhibition increases mortality to nonsevere infection with *K pneumonia* and *C albicans*. BALB/c mice were injected intraperitoneally with valproate (Orfiril, 200 mg/kg) or phosphate-buffered saline (PBS). (A) Peritoneal exudate cells and splenocytes were collected after 1 hour. Histone H4 acetylation (AcH4) was analyzed by Western blotting. (B-C) Mice were infected intranasally with 10 CFU of *K pneumoniae* 15 minutes after valproate. (B) Circulating bacterial counts 3 days after infection and (C) survival (n = 15 mice per treatment groups; $P = .04$ and .0004 for bacterial counts and survival, respectively). The dashed line represents the lower limit of detection. (D) Survival of BALB/c mice injected with 1.2×10^5 CFU of *C albicans* and treated with valproate or PBS daily (n = 18 mice per treatment group; $P = .02$).

HDAC inhibitors impair innate immune responses in vivo

We next investigated the effects of HDAC inhibitors in experimental models of bacterial and fungal sepsis or toxic shock titrated to cause either mild or severe infections or shock. We first verified that valproate enhanced the acetylation of histone H4 in vivo in peritoneal exudate cells and splenocytes (Figure 6A). In an otherwise nonsevere, acute *K pneumoniae* pneumonia model, valproate increased the proportion (53% vs 80%) and magnitude of bloodstream infections (Figure 6B; $P = .04$) and mortality (from 6% to 60%, $P = .0004$; Figure 6C). Consistent with valproate-induced impaired cytokine production by *C albicans*–infected BMDMs and BMDCs (Figure 4; and data not shown), valproate treatment was associated with accelerated (mean time to death: 21.5 days vs > 40 days) and increased mortality (44% vs 75%, $P = .02$) in a model of chronic *Candida* infection (Figure 6D). Thus, inhibition of HDACs impairs host defenses in vivo, increasing the susceptibility to and mortality of bacterial and fungal sepsis.

Severe sepsis and septic shock are characterized by an early overwhelming inflammatory response to microbial invasion, and inhibition of proinflammatory mediators confers protection in sepsis models.[24,25] We therefore tested whether valproate might exert protective effects in models of fulminant toxic shock induced by Pam_3CSK_4 or CLP. Administration of valproate caused a 2- to 3-fold reduction of IL-6 and IL-12p40 circulating levels (Figure 7A) and a notable increase in survival (0%-64%, $P < .001$) in the Pam_3CSK_4 toxic shock model (Figure 7B). Similarly, valproate treatment increased survival from 17% to 42% ($P = .04$) in the CLP model (Figure 7C).

Discussion

These studies identify an essential role for acetylation of histones and nonhistone proteins in the regulation of inflammatory and innate immune gene expression, and in host defensive responses against microbes. Genome-wide microarray analyses revealed a critical role for HDACs in the expression of host defense genes, including pattern-recognition receptors, adaptor molecules, kinases, transcription regulators, complement factors, cytokines, chemokines, and growth factors. HDAC inhibitors exert both immunosuppressive (a predominant effect) and immunostimulatory activities. Located at the forefront of the host defenses against microbial invasion, macrophages and DCs are an important source

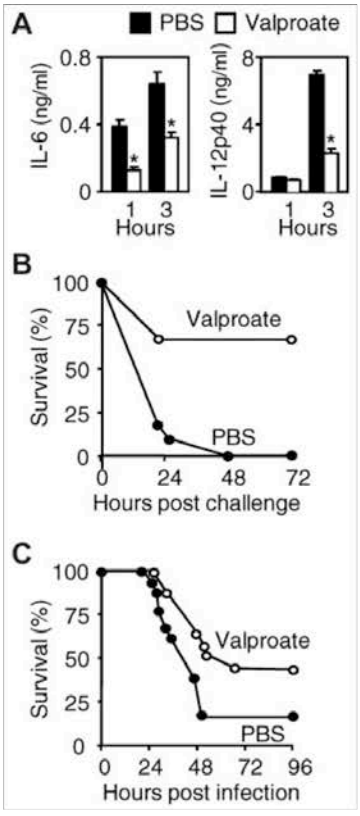


Figure 7. HDAC inhibition protects from lethal toxic shock and severe sepsis. (A-B) BALB/c mice were injected intraperitoneally with valproate (Orfiril, 200 mg/kg started 2 days before and discontinued 2 days after induction of shock) or PBS. Animals were sensitized with D-galactosamine and injected intraperitoneally with Pam_3CSK_4. (A) Plasma levels of IL-6 and IL-12p40 were determined 1 hour and 3 hours after challenge with Pam_3CSK_4. Data are mean ± SD of 8 mice per treatment group. *$P < .001$ for valproate versus PBS. (B) Survival of BALB/c mice subjected to Pam_3CSK_4-induced shock (n = 11 mice per treatment group; $P = .001$). (C) Survival of BALB/c mice subjected to CLP and treated with either valproate or PBS (every 12 hours, starting 30 minutes after surgery; n = 16-18 mice per treatment group). $P = .04$.

of cytokines. Inhibition of HDACs caused a marked reduction of cytokine and chemokine production induced by both cell types after exposure to TLR agonists. Of note, HDAC inhibitors also suppressed the release of IFN-α and IFN-γ by macrophages and splenocytes. Likewise, TSA inhibited IFN-α–stimulated gene activation and late IFN-β induction by Sendai virus and double-stranded RNA, and prevented IFN-α–mediated inhibition of the cytopathic effects of vesicular stomatitis virus.[26,27] Unexpectedly, we observed that HDAC inhibitors sustained LPS-induced IFN-β production in BMDMs. Nevertheless, the expression of numerous IFN-β/STAT1-dependent genes was strongly inhibited by TSA and VPA, indicating that the increased production of IFN-β did not overcome the potent inhibitory effects of HDAC inhibitors.

Phagocytes and professional antigen-presenting cells help bridge innate and adaptive immunity. Macrophages and DCs are key producers of IL-12 and IL-23 promoting the generation of protective Th1 and Th17 responses against intracellular and extracellular pathogens.[28,29] HDAC inhibitors were found to be potent inhibitors of IL-12 and IL-23 production by macrophages and DCs as well as CCR7, which is critical for the migration of DCs to secondary lymphoid organs (present data).[30-32] TSA inhibited the expression of several genes involved in antigen processing and presentation. Moreover, HDAC inhibitors have been reported to inhibit DC-stimulated allogeneic T-cell proliferation and Th1-cell activation[32-34] and to block the differentiation of IL-17–producing T cells.[35] Thus, inhibition of HDACs impacts on several key macrophages and DCs functions likely to affect the production of critical cytokines and the mounting of protective Th1 and Th17 immune responses. Of note, TSA did not inhibit TNF production by BMDMs and peritoneal macrophages stimulated with LPS. In contrast, TSA exerted potent inhibitory effects on whole blood, DCs, and BMDMs stimulated with microbial products other than LPS (Pam_3CSK_4, *E coli*, and *S aureus*). Similar results were obtained using SAHA and VPA. These observations suggest that HDAC inhibitors differentially affect gene expression according to the cell type or the stimulus studied. This could account for the discrepant effects of HDAC inhibitors on TNF production reported in the literature.[30-33,36] Indeed, delaying TSA treatment until 1 hour after the addition of Pam_3CSK_4 to BMDMs, or inhibiting protein synthesis at the time of preincubation with TSA, abrogated TSA-mediated inhibition of Pam_3CSK_4-induced TNF production. Thus, inhibition of stimulus-induced Tnf mRNA expression by TSA requires de novo protein synthesis, which cannot overcome a rapid induction of gene expression in BMDMs stimulated with LPS.

HDAC inhibitors have been reported to interfere with the activation of the mitogen-activated protein kinases, IRFs, STAT1, AP-1, or NF-κB signal transduction pathways. However, these findings have been inconsistent and controversial.[26,30,31,36,37] In BMDMs, we did not observe any impact of 3 HDAC inhibitors (TSA, VPA, and SAHA) on ERK1/2 or p38 phosphorylation or on NF-κB, c-jun, IRF3, or IRF7 nuclear translocation induced by LPS or Pam_3CSK_4. Yet, we found that TSA markedly inhibited the recruitment of NF-κB p65 and of RNA polymerase to the Il6 promoter (data not shown). This finding is consistent with the notion that acetylation of the NF-κB subunits themselves or of molecules involved in the NF-κB signal transduction pathway controls the extent, potency, and duration of NF-κB–mediated transcriptional activity.[38]

Transcriptional repression mediated by HDAC inhibitors may also rely on acetylation-dependent recruitment of transcriptional corepressors or changes in chromatin architecture.[38] In line with this hypothesis, we have previously shown that TSA inhibits the expression of the proinflammatory cytokine macrophage migration inhibitory factor via a local deacetylation of chromatin impairing the recruitment of the basal transcriptional machinery to the macrophage migration inhibitory factor promoter.[13,39] In the present study, we provide data suggesting that HDAC inhibitors induce the expression of Mi-2β and the activity of the Mi-2/NuRD complex, which acts as a transcriptional repressor of secondary LPS-induced cytokines, such as IL-6. Although little is known about the in vivo function of Mi-2β, mice deficient in metastasis-associated protein 2, a component of the Mi-2/NuRD complex, develop a lupus-like syndrome characterized by the hypersecretion of cytokines.[40]

A main finding of our study is the fact that HDAC inhibitors impair the host natural defenses against microbial pathogens. Administration of valproate increased the susceptibility of mice to bacterial and fungal infections converting a nonsevere bacterial pneumonia into a highly lethal infection and markedly increasing the mortality of invasive candidiasis. Moreover, we have also observed that HDAC inhibitors reduced the expression of phagocytic receptors and phagocytosis and killing of bacteria by macrophages (M.M., J.L., T.C., T.R., manuscript in preparation). These results clearly demonstrate that the inhibitory effects of HDAC inhibitors on innate immune cells in vitro translate into robust immunosuppressive effects in vivo that negatively impact on the susceptibility to and outcome of infections. Several arguments led us to believe that these observations may have clinical implications. A vast amount of data indicate that interfering with critical mediators of innate or adaptive immunity (eg, TNF or IL-1) increases the risk of infections.[41] Indeed, treatment of patients with TNF antagonists has been associated with an overall increased risk of bacterial (tuberculosis, nontuberculous mycobacteriosis, listeriosis, and salmonellosis) and fungal (candidiasis) infections.[42,43] HDAC inhibitors are currently used for the treatment of hematologic malignancies and solid tumors often in combination with other cancer or immune suppressive therapies increasing the risk of infection. In phase 1 and 2 clinical trials, patients treated with valproate, SAHA, MS-275, and ITF2357 have developed severe infections, even without neutropenia, that could be the result of the immune-suppressive activities of these HDAC inhibitors.[44-49] These data suggest that monitoring of infections may be warranted in clinical trials of HDAC inhibitors.

Taken advantage of their broad anti-inflammatory and immunomodulatory properties targeting several key mediators implicated in the pathogenesis of septic shock (such as TLRs, MyD88, signal transducing molecules, and proinflammatory cytokines), we reasoned that HDAC inhibitors may prove to be beneficial as adjunctive therapy for septic shock. Consistent with this assumption, valproate exhibited remarkable protective effects in a toxic shock model induced by Pam_3CSK_4 and in the CLP peritoneal sepsis model. In a recent fascinating article, Xu et al detected histones in the circulation of septic patients and baboons and showed that these proteins played a pathogenic role in sepsis.[50] Purified histones H3 and H4 were found to be toxic for endothelial cells, to cause microvascular thrombosis, and to be lethal when injected intravenously into mice. Notably, antihistone H4 antibodies rescued mice from lethal shock induced by LPS, TNF, or CLP. Given that histones appear to act as noxious danger-associated endogenous molecular patterns, one may wonder whether the acetylation status of extracellular histones correlates with toxicity. If so, deacetylation of histones released by necrotic and apoptic cells could exert detoxifying and cytoprotective effects.

Taken together, the present data identify protein acetylation as a key mechanism regulating the expression of important innate

immune genes critically implicated in the sensing of and host responses to microbial pathogens. Inhibition of HDACs impairs essential biologic functions of innate immune cells, reducing their capacity to induce a proinflammatory response, to engulf and kill pathogens, and to mount an adaptive response increasing the susceptibility to infection. On the other hand, the broad anti-inflammatory and immune-suppressive properties of HDAC inhibitors were found to be beneficial as adjunctive therapy for septic shock. Thus, these results suggest that the use of HDAC inhibitors as anticancer agents may increase the risk of infection and sepsis, whereas they may offer new therapeutic options for the management of patients with septic shock.

Acknowledgments

This work was supported by research funding from the Swiss National Science Foundation (310000-114073/1 and 310030-132744/1, T.R.; 310030-118266, T.C.; 3100A0-112370/1, J.S.; and 3100A0-116075, P.F.), by the Swiss Society for Infectiology (Merck Sharp & Dohme–Chibret AG Award; T.R.), and by the Leenaards Foundation (T.R., J.S.).

Authorship

Contribution: T.R. conceived and supervised the studies, performed experiments, and wrote the paper; J.L., G.G., X.C.D., M.M., A.-L.C., M.K.R., and I.M. carried out in vitro experiments; D.L.R. performed in vivo experiments; T.K., P.F., and J.S. performed microarray analyses; and T.C. discussed the study results and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Supplemental Tables S1, S2 and S3 are large datasets that cannot be inserted in this document for a matter of space. However they are available online at:

http://bloodjournal.hematologylibrary.org/content/suppl/2010/10/07/blood-2010-05-284711.DC1/Document1.pdf

Table S4. Oligonucleotides used in this study

	Forward (5'->3')	**Reverse (5'->3')**
For RT-PCR		
Actg1	CGCAAAGACCTGTATGCCAAT	GGGCTGTGATCTCCTTCTGC
Brg1	AAGGATGTGATGCTGCTGTG	CTTTCCTCGCCTTCACTGTC
Ccl2	GGATCAGAGATACTCATGAT	GAGAAGATTACCTGAGTACA
Ccl5	CTGCCGCGGGTACCATGAAG	TACAGGGTCAGAATCAAG
Ccl8	CGAGGGATTGAGAGGACGCT	ATGAGAAAACACGCAGCCCA
Ccl12	TTGGCTGGACCAGATGCG	GGGACACTGGCTGCTTGTGA
Cd14	CCCGACCCTCCAAGTTTTAG	GCTTCAGCCCAGTGAAAGAC
Cd36	TCCCTCACTGGAGGAAACTG	TGTGATATCTGGCCTTGCTG
Cd40	AGGTTTAAAGTCCCGGATGC	CCTTTGGTTTCTTGACCACCT
Cxcl10	GGATGGCTGTCCTAGCTCTGTAC	TGGGCATGGCACATGGT
Gapdh	CTCATGACCACAGTCCATGC	CACATTGGGGGTAGGAACAC
Hprt	GTTGGATACAGGCCAGACTTTGTTG	GATTCAACTTGCGCTCATCTTAGGC
Ifnb	GCATTTGAAAGGTCAAAGGAA	CCCTTTATAAGAAGTGTCCAGCA
Ifna4	CCTGTGTGATGCAGGAACC	TCACCTCCCAGGCACTGA
Il6	AGCAGTAGCAGTTCCCCTGA	AGTCCCTTTGGTCCAGTGTG
Il12b	GGAAGCACGGCAGCAGAATA	AACTTGAGGGAGAAGTAGGAATGG
iNos	CTCTGACAGCCCAGAGTTCC	GAAAGGGAGAGAGGGGAGG
Irf7	CTGGAGCCATGGGTATGCA	AAGCACAAGCCGAGACTGCT
Irf8	GATCAAGGAACCTTCTGTGG	GAAGCTGATGACCATCTGGG
Md2	CAACTCCTCCGATGCAATTA	GGCACAGAACTTCCTTACGC
Mi2b	CAGCAAACAGCGTTTCATGT	AGCCAGCAGCCAGTAATCAT
Tlr1	CAATGTGGAAACAACGTGGA	TGTAACTTTGGGGGAAGCTG
Tlr2	AAGAGGAAGCCCAAGAAAGC	CGATGGAATCGATGATGTTG
Tlr3	CACAGGCTGAGCAGTTTGAA	TTTCGGCTTCTTTTGATGCT
Tlr4	ACCTGGCTGGTTTACACGTC	CTGCCAGAGACATTGCAGAA
Tlr5	AAGTTCCGGGGAATCTGTTT	GCATAGCCTGAGCCTGTTTC
Tlr6	CAGAACTCACCAGAGGTCCAA	CGAGTATAGCGCCTCCTTTG
Tlr7	AATCCACAGGCTCACCCATA	CAGGTACCAAGGGATGTCCT
Tlr8	GACATGGCCCCTAATTTCCT	GACCCAGAAGTCCTCATGGA
Tlr9	ACTGAGCACCCCTGCTTCTA	AGATTAGTCAGCGGCAGGAA
Tnf	CCAGGCGGTGCCTATGTCT	GGCCATTTGGGAACTTCTCAT
For ChIP		
Tnf	CAACTTTCCAAACCCTCTGC	CTGGCTAGTCCCTTGCTGTC
Il6	AGGGCTAGCCTCAAGGATGA	AACCCACAATGCTGGCTCT
Il12b	TCTGCCTCCTTCCTTTTTCC	AGCTGCCTGGTCTGATGTG
For siRNA		
Control	AGGUAGUGUAAUCGCCUUGdTdT	
Mi2b	GAAACCUCGAGACCCUAAAdTdT	
Brg1	GGUAGAGUAUGUCAUCAAAdTdT	

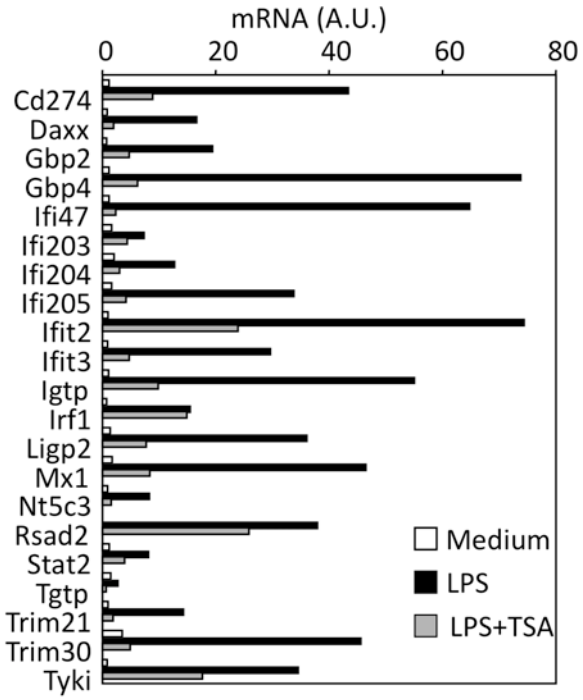

Figure S1. Trichostatin A inhibits the expression of LPS-induced IFN-β-dependent genes in macrophages. Bone marrow derived macrophages were pre-incubated for 1 h with or without TSA (100 nM) prior to exposure for 4 h to LPS (100 ng/mL). The expression of LPS-induced (fold-change > 2 versus medium) IFN-β-dependent genes (Thomas et al, J. Biol. Chem. 2006; 281:31119-31130) was analyzed with Agilent Mouse Oligo Microarray Kit (V2).

3 HISTONE DEACETYLASE INHIBITORS IMPAIR ANTIBACTERIAL DEFENCES OF MACROPHAGES

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***The Journal of Infectious Diseases*, 2011, in press**

3.1 Abstract

Histone deacetylases (HDACs) control gene expression by deacetylating histones and non-histone proteins. HDAC inhibitors (HDACi) are powerful anticancer drugs that exert anti-inflammatory and immunomodulatory activities. We recently reported a proof of concept study demonstrating that HDACi increase susceptibility to bacterial infections *in vivo*. Yet, still little is known about the effects of HDACi on host antimicrobial innate immune defences. Here we show that HDACi belonging to different chemical classes inhibit at multiple levels the response of macrophages to bacterial infection. HDACi reduce the phagocytosis and the killing of *Escherichia coli* and *Staphylococcus aureus* by macrophages. In line with these findings, HDACi decrease the expression of phagocytic receptors and inhibit bacteria-induced production of reactive oxygen and nitrogen species by macrophages. Consistently, HDACi impair the expression of NADPH oxidase subunits and inducible nitric oxide synthase. These data indicate that HDACi have a strong impact on critical antimicrobial defence mechanisms in macrophages.

3.2 Introduction

The innate immune system plays a crucial role in host defences against invasive microorganisms. Professional phagocytes are key sentinel cells of the innate immune system. Pathogen recognition relies on the capacity of phagocytes to sense microbial molecular motifs (e.g. lipopolysaccharide, peptidoglycan, lipopeptides, mannans, glucans, flagellin and nucleic acids) via pattern-recognition receptors comprising Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I) like receptors (RLRs), C-type lectin receptors (CLRs) and scavenger receptors (1). The engagement of phagocytic receptors, either through a direct interaction with microbial motifs or through the recognition of opsonized infectious agents, stimulates the engulfment and the delivery of the pathogen to the phagosome. Phagosome maturation by fission and fusion with endosomes and lysosomes generates the phagolysosome which provides a powerful microbicidal microenvironment usually resulting in efficient microbial killing (2, 3). The release of pro-inflammatory cytokines during the course of an infection stimulates the production of powerful phagocyte activating molecules like interferon γ (IFNγ).

Reversible acetylation of the ε amino groups of lysine residues from histones and non-histone proteins (such as α-tubulin, steroid receptors, HSP90 and regulators of nuclear import and transcription) is controlled by histone acetyltransferases and histone deacetylases (HDACs). Generally, acetylated histones are associated with active gene transcription, whereas deacetylated histones are associated with transcription repression (4-6). The eighteen mammal HDACs have been classified into class I (HDAC1-3 and 8), class IIa (HDAC4, 5, 7 and 9), class IIb (HDAC6 and 10), class III (SIRT1-7) and class IV (HDAC11) HDACs (4, 7). Small molecule inhibitors of class I, II and IV HDACs were originally identified for their potential to induce cellular differentiation, growth arrest and apoptosis of transformed cells. HDACi targeting class I and II HDACs have been reported to counteract cancer development by reducing tumor angiogenesis, metastasis and invasion and antitumor immunity (4-6).

Beside their anti-cancer properties, HDACi exert immunomodulatory activities that have been exploited for the treatment of inflammatory and auto-immune disease (8). Recently, we reported that HDACi interfere with the response of innate immune cells stimulated with TLR agonists and increase the mortality of mice to microbial sepsis (9). Yet, whether HDACi impair the phagocytosis and the killing of bacteria by phagocytes remains unknown. To more deeply characterize the influence of HDACi on innate immune responses, we investigated whether HDACi have an impact on key antibacterial defence mechanisms of macrophages. We report that HDACi reduce the expression of phagocytic and opsonophagocytic receptors and inhibit the phagocytosis of *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*), two of the most common infectious agents, by macrophages. Moreover, HDACi impair the generation of reactive oxygen and nitrogen species by macrophages infected with bacteria, resulting in a marked reduction of bacterial killing.

3.3 Material and Methods

3.3.1 Cells and reagents

Animal procedures were approved by the Office Vétérinaire du Canton de Vaud (authorizations n° 876.6) and performed according to institution guidelines for animal experiments. Eight to ten-week-old female BALB/c mice were purchased from Charles River Laboratories (L'Arbresle, France). Mouse bone marrow-derived macrophages (BMDMs) and thioglycollate-elicited macrophages were obtained as previously described (10, 11). RAW 264.7 macrophages were cultured in RPMI 1640 medium containing 2 mM glutamine and 10% FCS (12). *E. coli* O18:K1:H7 (*E. coli*) and *S. aureus* AW7 (*S. aureus*) are clinical isolates obtained from septic patients hospitalized at the Centre Hospitalier Universitaire Vaudois (Lausanne, Switzerland). Trichostatin A (TSA) and valproic acid (VPA) were purchased from Sigma-Aldrich (St Louis, MO), *Salmonella minnesota* Ultra Pure LPS from List Biologicals Laboratories (Campbell, CA) and IFNγ from R&D Systems (Abingdon, UK). The concentrations of TSA (dissolved in ethanol) and VPA (dissolved in PBS) used in this study were selected based on previous publications (13-18) and did not affect the viability (Trypan blue staining and MTT Cell Proliferation and Viability Assay) of BMDMs (> 85 % cell recovery after 18 h of culture with 20-40 nM TSA and 1-2 mM VPA with or without bacteria. n = 6-9 determinations. P > 0.5 for all conditions). Ethanol and PBS vehicle controls were performed in each experiment. For simplicity only one set of data is presented in each figure.

3.3.2 Assay for bacterial uptake and bacterial killing

E. coli and *S. aureus* were grown overnight at 37°C in tryptic soy broth (BD Biosciences, Erembodegem, Belgium), washed in PBS and adjusted to 10^7 CFU/ml in RPMI medium containing 10% FCS. BMDMs (4 x 10^5 cells in 24-well cell-culture plates, Costar, Cambridge, MA) were treated with TSA or VPA for 18 h. Medium was changed and cells were incubated for 1 h with bacteria at a multiplicity of infection of 20 bacteria per macrophage. Non-adherent bacteria were removed by washing with PBS. Extracellular bacteria were killed by a 30-min exposure to either 100 mg/ml of gentamicin (*E. coli*) or to 10 mg/ml of lysostaphin (*S. aureus*). BMDMs were washed and lysed. Serial dilutions of cell lysates were plated on agar plates and colonies enumerated to calculate the number of

phagocytosed bacteria. In parallel wells, BMDMs were treated as above except that, after 30 min of incubation with antibiotics, cells were washed and incubated for a further 24 h in culture medium containing 20 mg/ml gentamicin or 10 mg/ml lysostaphin. Bacteria were enumerated and results expressed as percent changes in bacterial counts using the following formula: (count after 24 h / count after 1 h) x 100. Of note, neither TSA nor VPA at the concentrations used in the above assays were toxic for bacteria.

3.3.3 RNA analysis by quantitative real-time polymerase chain reaction

RNA was isolated using the RNeasy kit (Qiagen, Hombrechtikon, Switzerland). Reverse transcription was carried out using the ImProm II RT System kit (Promega, Dübendorf, Switzerland). Quantitative real-time PCR was performed with a 7500 Fast Real-Time PCR System using the Power SYBR Green PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland) and primer pairs (**Supplementary Table 1**) as previously described (19). Samples were tested in triplicates. For each measurement, a standard made of successive dilutions of a reference cDNA was processed in parallel. The relative expression levels of NADPH oxidase subunits and iNOS were reported to the relative expression of Gapdh and expressed in arbitrary units (A.U.). The expression of phagocytic receptors and TLRs was calculated with the Comparative Ct Method (ΔΔCt method). The expression of the target gene was first normalized to the endogenous control (Gapdh) and then to that of a calibrator (i.e. data obtained from cells cultured with vehicle set at 1). Importantly, the Ct values of Gapdh were not affected by TSA or VPA (19.24±0.13, 19.10±0.03, 19.30±0.48, 19.21±0.06 and 19.10±0.15 in BMDMs cultured for 18 hours with medium, 20 nM TSA, 40 nM TSA, 1 mM VPA and 2 mM VPA, respectively. n = 6 determinations. $P > 0.05$ for all conditions compared to cells cultured in medium). In selected experiments, results were validated using *Hprt* as an endogenous control.

3.3.4 Flow cytometric analysis

BMDMs cultured for 18 h with TSA (40 nM) and VPA (2 mM) were incubated 30 min at 4°C in PBS containing 5% FCS, 5 mM EDTA, 2.4G2 mAb and mAbs specific for macrophage scavenger receptor 1 (Msr1/CD204), CD11c, CD14 and MHC-II (20). Acquisition and analysis were performed using a FACSCalibur^{TM} (BD Biosciences) and FlowJo 8.5.3 software (FlowJow, Ashland, OR).

3.3.5 Analysis of oxidative burst using the dichlorofluorescein diacetate fluorescence assay

BMDMs (4 x 10^5 cells in 24-well cell-culture plates) were cultured as previously described (21) and incubated for 18 h with TSA and VPA. Dichlorofluorescein diacetate (DCFDA) (20 mM, Sigma-Aldrich) was added to the cultures followed 15 min later by bacteria (5 x 10^8 CFU/ml). After 30 min, cell fluorescence was measured by flow cytometry.

3.3.6 Western blot analysis

Cell-lysates were electrophoresed through polyacrylamide gels and transferred onto nitrocellulose membranes as previously described (12). Membranes were incubated with antibodies directed against iNOS (BD Biosciences), $p47^{phox}$ (Santa Cruz, Santa Cruz, CA) and tubulin (Sigma). After washing, membranes were incubated with HRP-conjugated secondary antibody (Pierce). Signals were revealed using the ECL Western blotting Analysis System (GE Healthcare).

3.3.7 Nitrite/nitrate measurements

BMDMs (10^5 cells in 96-well cell-culture plates) were pre-incubated for 1 h with TSA and VPA and stimulated with LPS (100 ng/ml), IFNγ (100 U/ml), *E. coli* and *S. aureus* (10^8 CFU/ml). Cell culture supernatants were collected after 24 h. The concentrations of nitrite/nitrate were measured using the Griess reagent.

3.3.8 Statistics

Statistical analyses were performed using PRISM (GraphPad Software, La Jolla, CA). Comparisons between the different groups were performed by analysis of variance using ANOVA and appropriate post-hoc analyses. *P* values are two-sided and values of less than 0.05 were considered to indicate statistical significance.

3.4 Results

3.4.1 HDACi inhibit bacterial phagocytosis by macrophages

We recently reported that HDACi impair host defenses to bacterial infection *in vivo* (9). Whether HDACi impact on the phagocytosis and the killing of bacteria is currently unknown. To fill in this gap, we first analyzed the phagocytosis of Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria by bone marrow-derived macrophages (BMDMs) pretreated with two chemically unrelated HDACi: trichostatin A (TSA), a hydroxamate widely used as a prototypical broad spectrum HDACi, and valproic acid (VPA), a clinically relevant short fatty acid. The dose and duration of treatment with TSA and VPA were in the range of those used in cancer preclinical studies or measured in patients enrolled in cancer clinical trials (VPA) (see for example (13-18)). Phagocytosis was evaluated after 1 h of contact between bacteria and macrophages. As shown in **Figure 3.1**, HDACi dose dependently reduced the number of *E. coli* (2 to 4-fold; $P < 0.05$) and *S. aureus* (1.5 to 2-fold; $P < 0.05$) phagocytosed by BMDMs.

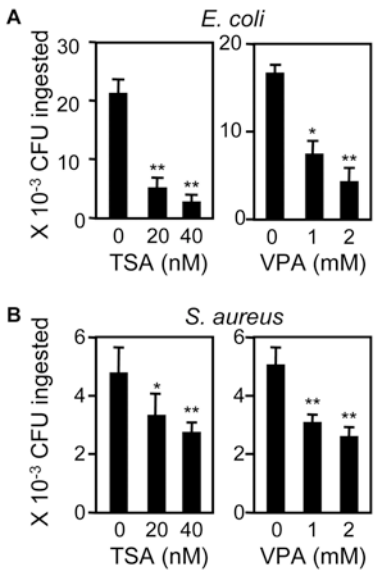


Figure 3.1. Histone deacetylase inhibitors impair the phagocytosis of *E. coli* (A) and *S. aureus* (B). BMDMs were incubated for 18 h with increasing concentrations of TSA and VPA before the addition of 10^7 CFU of *E. coli* or 1.5 x 10^7 CFU of *S. aureus*. The number of bacteria ingested by BMDMs was determined 1 h later. Data are means ± SD of quadruplicate samples from one experiment representative of two to three experiments. *, $0.05 < P < 0.005$; **, $P < 0.005$.

3.4.2 HDACi impair the expression of phagocytic receptors

Macrophages express phagocytic scavenger receptors, including macrophage scavenger receptor 1 (Msr1/SR-AI/CD204), CD14 and CD36 and C-type lectins such as Dectin-1 (encoded by

Cle7a) which mediate the recognition of microbial ligands expressed at the surface of pathogens and initiate phagocytosis. Macrophages also express opsonic phagocytic receptors of the integrin family (integrin$_{\alpha X}$/Itgax/CD11c, integrin$_{ß2}$/Itgb2/CD18, integrin$_{\alpha 5}$/Itga5/CD49e) that facilitate the uptake of microorganisms coated with opsonins like the mannose binding lectin, complement sub-components, growth arrest specific 6, ficolins and pentraxins (2, 22). TSA and VPA reduced 2-10-fold Msr1, CD14, Dectin-1 and Itgax mRNA levels in BMDMs (**Figure 3.2A**). TSA inhibited Itgb2 expression more efficiently than VPA (2.4-fold with 40 nM TSA versus 1.4-fold with 2 mM VPA), whereas HDACi did not affect CD36, Itga5 and Itga6 expression. Flow cytometry analyses confirmed that TSA and VPA inhibited the expression of Msr1, CD11c and CD14 by BMDMs (**Figure 3.2B**). As a control of non-specific broad inhibitory effects of HDACi, MHC-II expression was not affected by HDACi. Altogether, these data suggest that reduced expression of phagocytic receptors may contribute to impair the phagocytosis of *E. coli* and *S. aureus* in macrophages treated with HDACi.

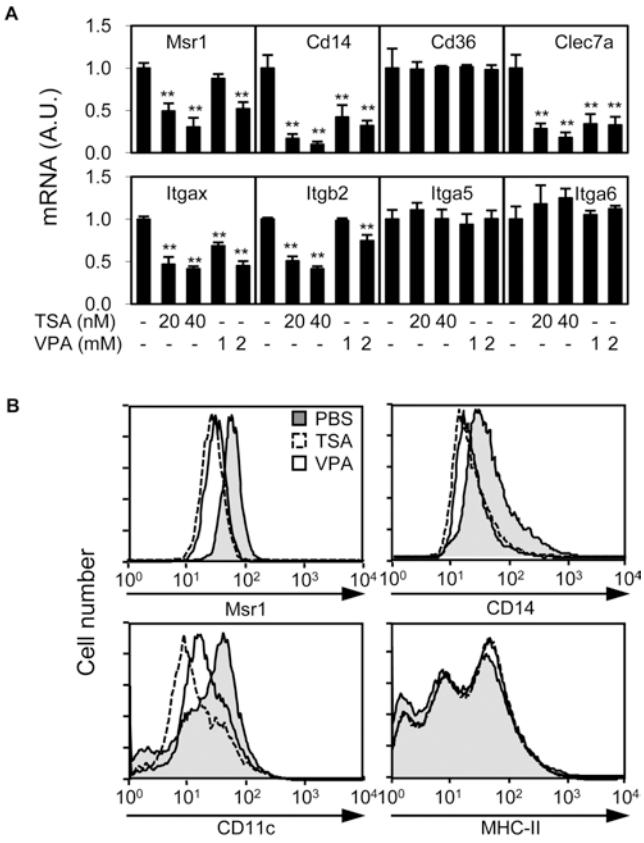
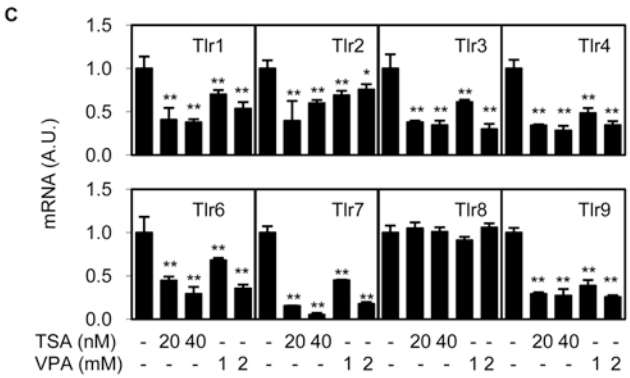


Figure 3.2. Histone deacetylase inhibitors inhibit the expression of phagocytic and Toll-like receptors. (A) Real-time PCR analysis of Msr1, CD14, CD36, Clec7a, Itgax, Itgb2, Itga5 and Itga6 mRNA expression in BMDMs incubated for 8 h with increasing concentrations of TSA and VPA. Data are means ± SD of triplicate samples from one experiment and are representative of two independent experiments. (B) Flow cytometry analysis of Msr1, CD14, CD11c and MHC-II expression by BMDMs incubated for 18 h with medium (grey area), TSA (dashed line) and VPA (solid line). Results are representative of two independent experiments. (C) Real-time PCR analysis of TLRs in BMDMs incubated for 8 h with increasing concentrations of TSA and VPA. Data are means ± SD of triplicate samples from one experiment and are representative of two independent experiments. A.U.: arbitrary units. *, $0.05 < P < 0.005$; **, $P < 0.005$.

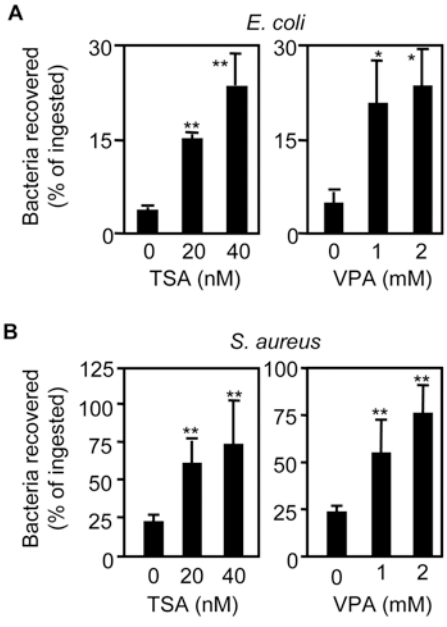
Figure 3.2 (continued). Histone deacetylase inhibitors inhibit the expression of phagocytic and Toll-like receptors.



3.4.3 HDACi inhibit bacterial killing

TLRs play a crucial role in the sensing of invasive microorganisms and in transmitting signals involved in the maturation of phagosomes (23). Interestingly, we observed that HDACi strongly reduced baseline expression of TLR1-7 and TLR9 in BMDMs (**Figure 3.2C**). Pathogen delivery to phagolysosomes usually results in effective microbial killing (2, 24). In agreement, less than 5% of *E. coli* and 25% of *S. aureus* phagocytosed by BMDMs were recovered 24 h later in macrophages ($P < 0.001$) (**Figure 3.3**). TSA and VPA reduced 5-fold and 3-fold *E. coli* and *S. aureus* killing respectively (*i.e.* increasing bacteria recovery to 25% and 75% of the ingested inoculum; $P < 0.05$). Thus, HDACi inhibit both the phagocytosis and the killing of bacteria by macrophages, in agreement with the observation that HDACi increased the susceptibility of mice to microbial infection (9).

Figure 3.3. Histone deacetylase inhibitors impair the killing of *E. coli* and *S. aureus*. BMDMs were cultured for 18 h with increasing concentrations of TSA and VPA before the addition of 10^7 CFU of *E. coli* or 1.5 x 10^7 CFU of *S. aureus*. The number of bacteria recovered from macrophages after 24 h was divided by to the number of bacteria recovered after 1h and expressed in percentage using the formula (i.e. (count after 24 h / count after 1 h) x 100). Data are means ± SD of quadruplicate samples from one experiment representative of two to three experiments. *, $0.05 < P < 0.005$; **, $P < 0.005$.



3.4.4 HDACi interfere with the generation of reactive oxygen species

In response to microbial challenge, macrophages produce highly toxic reactive oxygen species (ROS), which contribute to pathogen destruction (25). The generation of ROS in BMDMs was analyzed by flow cytometry using the cell permeable non-fluorescent dye DCFDA that is transformed upon oxidation into the highly fluorescent DCF. *E. coli* and *S. aureus* strongly increased DCF fluorescence in BMDMs, which was inhibited 2- to 3-fold by TSA or VPA (**Figure 3.4A** and **3.4B**). HDACi also inhibited the production of ROS in BMDMs stimulated with phorbol myristate acetate (> 10-fold reduction, data not shown) indicating that HDACi inhibit the oxidative burst induced by microbial and non-microbial stimuli.

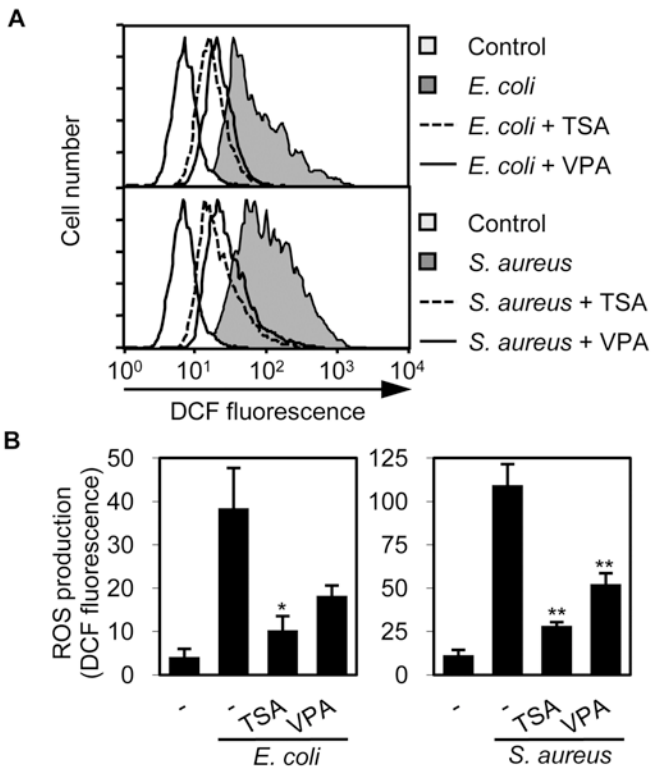


Figure 3.4. Histone deacetylase inhibitors interfere with the generation of reactive oxygen species. BMDMs were incubated with TSA (40 nM) and VPA (2 mM) and exposed to *E. coli* or *S. aureus* as described in *Material and Methods*. (A) The generation of ROS was quantified by flow cytometry by measuring dichlorofluorescein (DCF) diacetate oxidation into fluorescent DCF. (B) Data are means ± SD of two independent determinations. $P = 0.03$ and 0.08 for *E. coli* and *S. aureus* versus control. *, $P = 0.05$; *, $0.05 < P < 0.005$ versus *E. coli* and *S. aureus* treated cells.

In macrophages, ROS are generated during the respiratory burst through the action of the phagocytic NADPH oxidase, an enzymatic complex composed of two membrane associated subunits, $gp91^{phox}$/NOX2 and $p22^{phox}$, three cytosolic subunits, $p47^{phox}$, $p40^{phox}$ and $p67^{phox}$, and the Rac2

regulatory subunit (26, 27). Cytokines, particularly IFNγ, and microbial products released during the course of an infection prime and amplify macrophage respiratory burst through the induction of NADPH oxidase subunits (21). Real-time PCR and Western blot analyses revealed that TSA and VPA dose-dependently inhibited baseline expression of NADPH oxidase subunits and potently inhibited the up-regulation of the catalytic gp91^{phox} and regulatory p47^{phox} subunits in LPS+IFNγ-stimulated macrophages (**Figure 3.5**). Altogether, these data provide compiling evidence that HDACi inhibit ROS production in macrophages.

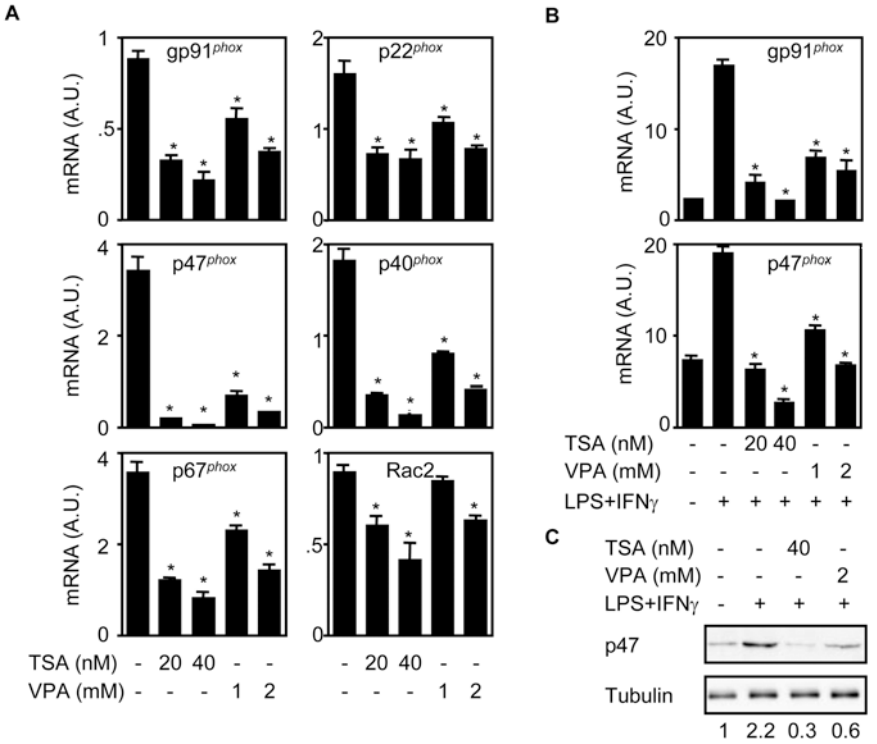


Figure 3.5. Histone deacetylase inhibitors inhibit NADPH oxidase subunits expression. (A) Real-time PCR analysis of gp91, p22, p47, p67, p40 and Rac2 mRNA expression in BMDMs cultured for 8 h with TSA and VPA. Results are expressed as the ratio of the gene of interest to that of Gapdh. Real-time PCR (B) and Western blot (C) analyses of gp91 and p47 expression in BMDMs cultured for 1 h with TSA and VPA and then stimulated for 6 h with LPS+IFNγ (100 ng/ml and 100 U/ml). Data are means ± SD of triplicate samples from one experiment and are representative of two independent experiments (A, B). *, $P < 0.005$ versus control (A) and LPS+IFNγ (B). A.U.: arbitrary units.

3.4.5 HDACi inhibit nitric oxide production and *iNos* gene expression

Nitric oxide (NO) is produced during the nitrosative burst by iNOS and represents an important antimicrobial effector mechanism (25, 27). TSA and VPA dose-dependently inhibited the production of NO by BMDMs stimulated with LPS+IFNγ (50-80% reduction; $P < 0.05$), in line with

previous work showing that HDACi inhibit cytokine-induced NO release (28, 29). More relevant for microbial infection, TSA and to a lesser extend VPA inhibited NO production induced by *E. coli* and *S. aureus* (50-60% reduction using 20 and 40 nM TSA and 30-35% reduction using 2 mM VPA; $P < 0.05$) (**Figure 3.6A**). In agreement with these findings, real-time PCR and Western blot analyses demonstrated that TSA and VPA inhibited iNOS mRNA and protein expression in BMDMs (**Figure 6B and 6C**). Similar results were obtained using thioglycollate-elicited peritoneal and RAW 264.7 macrophages (data not shown). Taken together, these results suggest that HDACi impair NO production by BMDMs in response to bacterial challenge by interfering with iNOS expression.

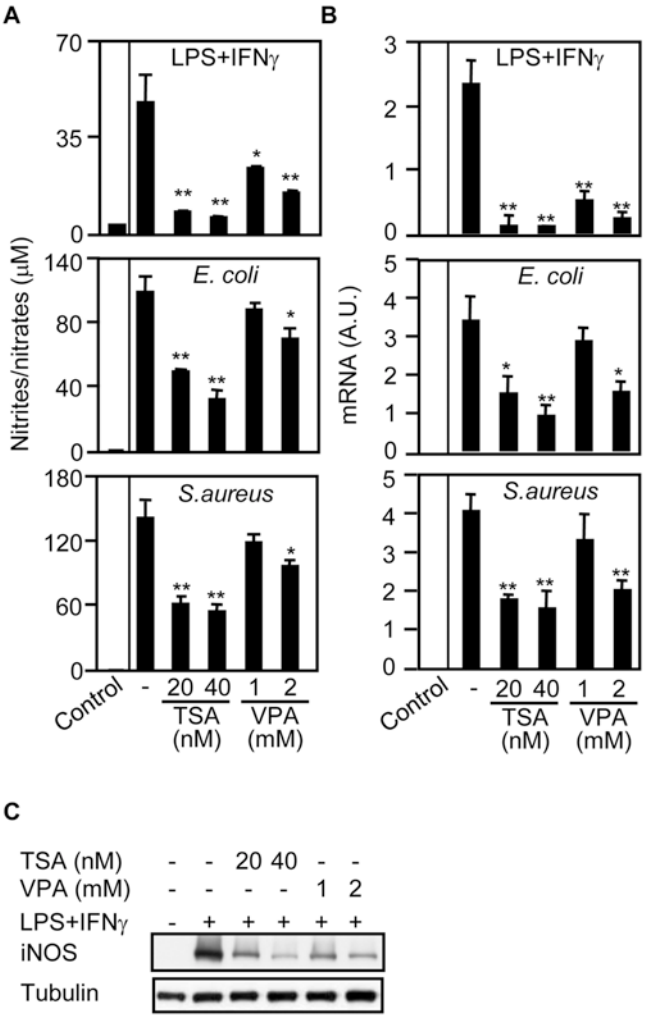


Figure 3.6. Histone deacetylase inhibitors impair nitric oxide production and *iNos* gene expression. BMDMs were cultured for 1 h with TSA and VPA and then stimulated with LPS+IFNγ (100 ng/ml and 100 U/ml), *E. coli* and *S. aureus* (10^8 CFU/ml) for 24 h (A) or 8 h (B-C). (A) Nitrite/nitrate concentration in cell culture supernatants was measured using the Griess reagent. Data are means ± SD of triplicate samples from one experiment and are representative of four independent experiments. Real-time PCR (B) and Western blot (C) analyses of iNOS expression. Results are expressed as the ratio of iNos mRNA levels to that of Gapdh. Data are means ± SD of triplicate samples from one experiment and are representative of three independent experiments. A.U.: arbitrary units. *, $0.05 < P < 0.005$; **, $P < 0.005$.

3.5 Discussion

In the present study, we report for the first time that HDACi inhibit the phagocytosis and the killing of bacteria, the expression of phagocytic receptors and the generation of oxidative and nitrosative bursts induced by bacteria in macrophages. These data extend our previous work demonstrating that HDACi interfere with cytokine production by macrophages and impair host defenses to bacterial infection (9).

The inhibition of *E. coli* and *S. aureus* phagocytosis by HDACi was associated with a reduced expression of phagocytic receptors, among which Msr1 (scavenger receptor A1). Mrs1 binds a wide range of microbial ligands and mediates non-opsonic phagocytosis of *E. coli* and *S. aureus* (30). Moreover, *Msr1*$^{-/-}$ mice are more susceptible than wild-type mice to *S. aureus* infection (31). Thus, HDACi-mediated inhibition of Msr1 expression may well contribute to impair bacterial phagocytosis, although HDACi may target other phagocytic receptors such as the mannose receptor, MARCO or CD14. Of note, HDACi decreased Dectin-1 expression in BMDMs. Considering that Dectin-1 is a major receptor involved in the recognition of ß-glucan, we speculate that HDACi may affect the phagocytosis of yeast. In line with this hypothesis, we observed that VPA increases mortality of mice infected with *Candida albicans* (9).

The αx and ß2 integrin subunits contribute to the structure of complement receptor (CR) 3 and CR4 which mediate the recognition of opsonized microorganisms by phagocytes. ß2 integrins play an important role in anti-microbial defences as suggested by the observation that patient with leukocyte adhesion deficiency type I (LADI) syndrome (i.e. patients deficient in functional ß2 integrin) have defects in phagocytosis and are prone to bacterial infections (32). Altogether, inhibition of the expression of αx and ß2 integrins and scavenger and lectin receptors by HDACi support the contention that HDACi interfere with bacterial opsonic and non-opsonic phagocytosis.

HDACi powerfully inhibited the killing of *E. coli* and *S. aureus* by macrophages. This observation is congruent with the fact that VPA treatment increased the proportion and the magnitude of bloodstream infections in mice infected with *Klebsiella pneumoniae* (9). Reactive oxygen and

nitrogen species are among the most deleterious components produced by phagocytes and implicated in the destruction of microorganisms (2, 24). Deficiency in members of the NADPH oxidase complex or in iNOS, which control the generation of superoxide ($O_2^{-\circ}$) and nitric oxide (NO°) radicals, impair the killing of *E. coli* and *S. aureus* by innate immune cells and compromise mouse survival (33-36). Moreover, germ-line mutation in one of the components of NADPH oxidase complex results in chronic granulomatous disease characterized by recurrent bacterial and fungal infections and reduced life expectancy (25). Inhibition of NADPH oxidase subunit and iNOS expression and of reactive oxygen and nitrogen species generation by HDACi likely represents an effective mechanism by which these drugs impair the killing of bacteria.

In agreement with the notion that phagocytosis is coupled with a proinflammatory cytokine response and with the observation that HDACi inhibit TLR expression, HDACi strongly impaired the secretion of cytokines and chemokines (TNF, IL-6, IL-12p40 and MIP-2α, data not shown) by macrophages infected with *E. coli* and *S. aureus*. These data expand on recent studies showing that HDACi inhibit cytokine production induced by cytokines and purified microbial products in innate immune cells (14, 15, 28, 37, 38). Considering that proinflammatory mediators released during the course of an infection coordinate the development of innate and adaptive immunity, one may anticipate that HDACi interfere with the generation of pathogen-specific adaptive immune response.

HDACi have been reported to interfere with signaling pathways controlling the expression of genes particularly relevant for the present study. Indeed, HDACi down-regulate the expression of PU.1 transcription factor (37, 39), which regulates constitutive expression of HDACi-target genes encoding for integrins, scavenger receptors, TLR4, CD14 and p40, p47 and p67 NADPH oxidase sub-units (20, 40). Moreover, HDACi have been reported to interfere with the activation of mitogen activated protein kinases (MAPKs), NF-κB and AP-1 which control inflammatory and antimicrobial host responses (15, 41). Albeit less well characterized, HDACi also impair gene expression through chromatin modifications or aceytlation-dependent recruitment of transcriptional repressors. For example, TSA inhibits the expression of the proinflammatory cytokine macrophage migration inhibitory factor (MIF) through a local deacetylation of MIF-promoter associated histones impairing

the recruitment of the basal transcriptional machinery (42, 43). Finally, we have recently shown that TSA inhibits macrophage response to LPS stimulation by inducing the expression of Mi-2β and the activity of the Mi-2/NuRD transcriptional repressor complex (9).

HDACi have been used to treat inflammatory diseases in mouse models (8). Abundant preclinical and clinical studies indicate that interfering with critical mediators of innate or adaptive immunity increases the risk of infections. Thus, one may question whether HDACi might affect natural host defenses in patients, as could be anticipated from the powerful immunomodulatory and anti-inflammatory activities of HDACi *in vivo* (8) and the increased susceptibility to bacterial and fungal infections of mice treated with HDACi (9). Patients treated with HDACi (SAHA, MS-275, valproate and ITF2357) in phase I and II clinical trials have developed episodes of severe infection even in the absence of treatment-induced neutropenia (44-49), suggesting the need of monitoring the immune status and susceptibility to infection of patients treated with HDACi, especially immunosuppressed cancer patients (4-6, 50).

In summary, the present study demonstrates that HDACi impair the capacity of macrophages to ingest and destroy Gram-positive and Gram-negative bacteria. The fact that HDACi impede the expression of phagocytic receptors, the generation of oxygen and nitrogen reactive species and the release of proinflammatory cytokines provide a plausible mechanism whereby HDACi negatively impact on critical antimicrobial functions of innate immune cells and increase the susceptibility of mice to bacterial and fungal infection (9).

Conflict of interest. All the authors declare no conflict of interest.

Funding. This work was supported by the Swiss National Science Foundation [grant 310000_114073 and 310030_132744 to T.R. and 310000_118266 to T.C.], a MSD award from the Swiss Society for Infectious Diseases and the Leenaards Foundation to TR.

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Supplementary Table 1. Oligonucleotides used for real-time PCR.

Gene	Forward (5'->3')	Reverse (5'->3')
Cd14	CCCGACCCTCCAAGTTTTAG	GCTTCAGCCCAGTGAAAGAC
Cd36	TCCCTCACTGGAGGAAACTG	TGTGATATCTGGCCTTGCTG
Gapdh	CTCATGACCACAGTCCATGC	CACATTGGGGGTAGGAACAC
Hprt	GTTGGATACAGGCCAGACTTTGTTG	GATTCAACTTGCGCTCATCTTAGGC
iNos (Nos2)	CTCTGACAGCCCAGAGTTCC	GAAAGGGAGAGAGGGGAGG
Itgax (Cd11c)	CTCCTGAGTGAGGCTGAAATCA	TTATACATCTCCAGCACTGTCTTCGT
Itgb2 (Cd18)	ACAATCTTGCCGCAGAGC	AAGTTGGGGCCACCTTTACT
Itga5 (Cd49e)	CTCGGCTTCTTCAAACGTTC	AAGAAGAGCTTCTCCCCAGC
Msr1 (Cd204)	AGTGTAGGCGGATCAACCC	TCACTTCATTCAGCCATATTGG
$p22^{phox}$ (Cyba)	AGGGGTCCACCATGGAGCGA	GCTCAATGGGAGTCCACTGC
$p40^{phox}$ (Ncf4)	CCGCCGCTATCGCCAGTTCTAC	CCGGCAGGCTCAGGAGGTTCT
$p47^{phox}$ (Ncf1)	CTATCTGGAGCCCCTTGACA	ACAGGGACATCTCGTCCTCTT
$p67^{phox}$ (Ncf2)	CCAGAAGACCTGGAATTTGTG	AAATGCCAACTTTCCCTTTACA
$gp91^{phox}$ (Cybb)	CCAACTGGGATAACGAGTTCA	GAGAGTTTCAGCCAAGGCTTC
Rac2	GACACCATCGAGAAGCTGAAG	GTGAGTGCAGAACATTCCAAGT
Tlr1	CAATGTGGAAACAACGTGGA	TGTAACTTTGGGGGAAGCTG
Tlr2	AAGAGGAAGCCCAAGAAAGC	CGATGGAATCGATGATGTTG
Tlr3	CACAGGCTGAGCAGTTTGAA	TTTCGGCTTCTTTTGATGCT
Tlr4	ACCTGGCTGGTTTACACGTC	CTGCCAGAGACATTGCAGAA
Tlr6	CAGAACTCACCAGAGGTCCAA	CGAGTATAGCGCCTCCTTTG
Tlr7	AATCCACAGGCTCACCCATA	CAGGTACCAAGGGATGTCCT
Tlr8	GACATGGCCCCTAATTTCCT	GACCCAGAAGTCCTCATGGA
Tlr9	ACTGAGCACCCCTGCTTCTA	AGATTAGTCAGCGGCAGGAA

4 HISTONE DEACETYLASE INHIBITORS REPRESS MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF) EXPRESSION BY TARGETING *MIF* GENE TRANSCRIPTION THROUGH A LOCAL CHROMATIN DEACETYLATION

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***Biochimica et Biophysica Acta*, November 2009, Volume 1793, Issue 11, pages 1749-1758**

4.1 Summary

Macrophage migration inhibitory factor (MIF) is an important player of inflammation and tumorigenesis. Moreover, MIF levels correlate with tumor aggressiveness and metastatic potential. Given that inhibitors of class I, II and IV HDACs (HDACi) are potent antitumor agents with anti-inflammatory activity, we tested the hypothesis that MIF represents a target of HDACi. Confirming our hypothesis, PCR analysis of *MIF* gene expression revealed that HDACi of various chemical classes (trichostatin A, valproate and suberoylanilide hydroxamic acid) strongly inhibit *MIF* gene expression in a broad range of cell lines including myeloid cells, epithelial cells, keratinocytes and melanoma. Moreover MIF protein expression was deacreased in human whole blood and in the circulation of mice treated with trichostatin A. Nuclear run on coupled to transient transfection with MIF promoter reporter constructs and transduction with MIF expressing adenovirus revealed that trichostatin A inhibits endogenous, but not episomal, *MIF* gene transcription. These results highlighted the importance of the endogenous chromatin landscape in HDACi-mediated MIF inhibition. Interestingly, chromatin immunoprecipitation (ChIP) studies showed that trichostatin A induced a local and specific deacetylation of MIF promoter-associated H3 and H4 histones. Curiously however, the local deacetylation of histones did not affect chromatin accessibility as revealed by chromatin accessibility by real-time PCR (CHART-PCR). Nonetheless, the deacetylation of the MIF promoter was associated with an impaired recruitment of RNA polymerase II and Sp1 and CREB, two transcription factors eseential to drive basal *MIF* gene transcription. Overal, this study describes a new molecular mechanism by which HDACi inhibit *MIF* gene expression, and suggests that inhibition of MIF expression by HDACi may contribute to the antitumorigenic effects of this class of drugs.

Biochimica et Biophysica Acta 1793 (2009) 1749–1758

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Histone deacetylase inhibitors repress macrophage migration inhibitory factor (MIF) expression by targeting *MIF* gene transcription through a local chromatin deacetylation

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ARTICLE INFO

Article history:
Received 8 June 2009
Received in revised form 30 August 2009
Accepted 3 September 2009
Available online 10 September 2009

Keywords:
Macrophage migration inhibitory factor
Histone deacetylase inhibitor
Trichostatin A
Chromatin
Transcription factor

ABSTRACT

The cytokine macrophage migration inhibitory factor plays a central role in inflammation, cell proliferation and tumorigenesis. Moreover, macrophage migration inhibitory factor levels correlate with tumor aggressiveness and metastatic potential. Histone deacetylase inhibitors are potent antitumor agents recently introduced in the clinic. Therefore, we hypothesized that macrophage migration inhibitory factor would represent a target of histone deacetylase inhibitors. Confirming our hypothesis, we report that histone deacetylase inhibitors of various chemical classes strongly inhibited macrophage migration inhibitory factor expression in a broad range of cell lines, in primary cells and *in vivo*. Nuclear run on, transient transfection with macrophage migration inhibitory factor promoter reporter constructs and transduction with macrophage migration inhibitory factor expressing adenovirus demonstrated that trichostatin A (a prototypical histone deacetylase inhibitor) inhibited endogenous, but not episomal, *MIF* gene transcription. Interestingly, trichostatin A induced a local and specific deacetylation of macrophage migration inhibitory factor promoter-associated H3 and H4 histones which did not affect chromatin accessibility but was associated with an impaired recruitment of RNA polymerase II and Sp1 and CREB transcription factors required for basal *MIF* gene transcription. Altogether, this study describes a new molecular mechanism by which histone deacetylase inhibitors inhibit *MIF* gene expression, and suggests that macrophage migration inhibitory factor inhibition by histone deacetylase inhibitors may contribute to the antitumorigenic effects of histone deacetylase inhibitors.

1. Introduction

Macrophage migration inhibitory factor (MIF), one of the first cytokine activity identified [1,2], is a proinflammatory mediator that plays a central role in the development of inflammation and innate and adaptive immune responses [3]. MIF has been implicated in the pathogenesis of acute and chronic infectious, inflammatory and autoimmune diseases. In addition, recent studies strongly support an important role for MIF in the control of cell growth and tumorigenesis at multiple levels. MIF stimulates cell proliferation through a sustained activation of the ERK1/2 MAPKs [4] and the induction of cyclin D1 (CCND1) [5] and promotes cell survival by inhibiting the p53 and retinoblastoma/E2F tumor suppressor pathways [6–8] and by activating the phosphoinositide-3-kinase (PI3K)/Akt survival pathway [9]. Moreover, MIF promotes tumor-associated neovascularization [10] and inhibits antitumor natural killer (NK) and cytotoxic T-lymphocyte (CTL) responses [11–14]. MIF also plays a role in the control of the response to DNA damage [15] and regulates tumor cell motility and invasion in a Rac1-dependent cell manner [16]. Finally, human cancer tissues, such as prostate, breast, colon, brain, skin and lung-derived tumors, have been shown to overexpress MIF [17,18]. Altogether, an accumulating body of evidence indicates that MIF have a crucial role in the development of inflammatory diseases and neoplasia.

The structure of chromatin is influenced by covalent modifications of amino-terminal tails of histones, particularly acetylation at lysine residues [19–21]. Acetylation is regulated by the opposing actions of histone acteyl transferases and histone deacetylases (HDACs). Increased histone acetylation has been linked to gene overexpression [22]. Transcription coactivators like p300/CBP and PCAF display acetyltransferase activity whereas transcription co-repressor complexes contain HDAC activity [23,24]. The current view is that histone acetylation induces chromatin scaffolding to assume a more relaxed, open structure increasing the accessibility of regulatory factors to DNA and favoring active gene transcription. Yet, several observations have challenged this traditional view. First, genome-wide expression

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doi:10.1016/j.bbamcr.2009.09.007

studies have revealed that HDAC inhibitors (HDIs) affect the expression of a small subset of cellular genes with similar proportions of genes down-regulated and up-regulated [25–28]. Second, an increasing number of non-histone proteins among which transcription regulators have been shown to be modified by acetylation [21].

An abundant literature indicates that deregulation of acetylation contributes to abnormal gene expression observed in many forms of cancer. Therefore, HDACs are considered among the most promising targets for intervention expected to reverse abnormal epigenetic status in cancer cells [19–21]. In agreement, HDIs have shown encouraging therapeutical results in preclinical studies [29] and the broad spectrum HDI suberoylanilide hydroxamic acid (SAHA) has been approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma [30]. The powerful and specific anticancer activities of HDIs result, to a great extent, from their capacity to induce differentiation, growth arrest and/or apoptosis of transformed cells. HDIs may also affect cancer development by modifying tumor angiogenesis, metastasis and invasion and antitumor immunity [19–21].

Considering that MIF is overexpressed in malignant cells and the role played by MIF in tumorigenesis, we hypothesized that MIF could be a target of HDIs. Here we report that HDIs strongly inhibit MIF expression in various cell lines, primary cells and *in vivo*. Moreover, we describe a new molecular mechanism by which TSA, a broad spectrum HDI, inhibits *MIF* expression through a local and specific deacetylation of histones associated with the proximal MIF promoter. Altogether this study expands our comprehension of the mechanisms by which HDIs inhibit gene expression and suggests that MIF is a target of the antitumorigenic effects of HDIs.

2. Materials and methods

2.1. Cells, mice and reagents

Human HeLa cervix epithelial cells and HaCat keratinocytes and mouse B16-10 melanoma were cultured in DMEM containing 4.5 g/l glucose and 10% glutamax (Invitrogen). Human HL-60 promyelocytic, U-937 promonocytic and THP-1 monocytic cell lines, KG1a promyeloblasts and A549 airway epithelial cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine (Invitrogen). All media contained 10% heat-inactivated fetal calf serum (Seromed).

Animal procedures were approved by the Office Vétérinaire du Canton de Vaud (authorizations no. 876.5 and 876.6) and performed according to institution guidelines for animal experiments. Eight to ten-week-old female BALB/c mice were purchased from Charles River Laboratories (L'Arbresle, France). Mouse bone-marrow-derived macrophages were obtained as previously described [31]. Mice (3 animals per group) were injected intraperitoneally with TSA (10 mg/kg) or an equal volume of diluent every 24 h. After 2 days, blood was collected. MIF in plasma (8 μl) was analyzed by Western blotting.

TSA and valproic acid (VPA) and mithramycin A were purchased from Sigma-Aldrich and SAHA from ALEXIS Biochemicals. Recombinant MIF and anti-human MIF antibodies isolated from the sera of MIF-immunized New Zealand White rabbits were obtained as previously described [32]. HeLa cells were transduced with the empty control and MIF-expressing adenoviruses [16,33] at a multiplicity of infection of 100. The medium was replaced by fresh medium 1 h after viral infection, and incubation prolonged for 18 h before the addition of TSA.

2.2. mRNA analysis

Total RNA was isolated using the RNeasy kit (Qiagen). MIF and $p21^{WAF1/CIP1}$ (p21 or cyclin-dependent kinase inhibitor 1A, CDKN1A) mRNA steady state levels were assessed by Northern blotting using PCR amplified DNA probes (primers are listed in Table 1). Quantification of specific signals was performed using an Instant Imager 2024 (Packard). Total RNA was reverse transcribed using the ImProm-II Reverse Transcription System (Promega). MIF and hypoxanthine phosphoribosyltransferase 1 (HPRT) mRNA expression was analyzed by PCR. Quantitative PCR was performed with a 7500 Fast Real-Time PCR System using the Power SYBR Green PCR Master Mix (Applied Biosystems) as previously described [34]. MIF mRNA expression was expressed relative to HPRT mRNA expression in arbitrary units.

2.3. Western blot and co-immunoprecipitation analyses

Cells were lysed in Mammalian Protein Extraction Reagent (Pierce). To detect histones, acid-soluble proteins were extracted as previously described [35]. Cell-lysates and acid-insoluble proteins were electrophoresed through polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were incubated with antihuman MIF, α-tubulin (T 5168, Sigma-Aldrich) and acetylated histones H3 and H4 antibodies (06-755 and 05-858, Millipore), washed and incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies. Signals were revealed using the ECL Western blotting analysis system (GE Healthcare).

For co-immunoprecipitation studies, total cell extracts were obtained by incubating 10^7 HeLa cells in a lysis buffer containing 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate, 4 mM Na3VO4 and 1 μg/ml leupeptin. 200 μg of proteins were pre-absorbed with Protein G-Sepharose ™ 4FF beads (Amersham). Supernatants were incubated overnight at 4 °C with acetylated-Lysine antibody (9441S, Cell Signaling Technology) and then with Protein G-Sepharose™ 4FF beads for an additional 3 h. The beads were washed and heated 3 min at 100 °C in electrophoresis sample buffer. Samples were analyzed by SDS-PAGE and Western blotting using anti-Sp1 (sc-59x, Santa Cruz Biotechnology) and HRP-conjugated immunopure rabbit antigoat IgG antibody (31402, Pierce).

MIF protein decay was measured in HeLa cells pre-incubated for 1 h with or without TSA (1 μM) and then cultured with or without cycloheximide (CHI, 10 μg/ml, Sigma-Aldrich). Protein extracts were collected at intervals and analyzed by Western blotting as described above.

Table 1
Oligonucleotides used in this study.

	Oligonucleotide	5′ -> 3′ sequence
Probe synthesis	MIF S	CACGCTCGCAGTCTCTC
	MIF AS	GAGGCTCAAAGAACAGC
	p21 (CDKN1A) S	GACACCACTGGAGGGTGACT
	p21 (CDKN1A) AS	GGATTAGGGCTTCCTCTTGG
Nuclear run-on	MIF NRO S	CCGGACAGGGTCTACATCAA
	MIF NRO AS	AATTCTCCCCACCAGAAGGT
	ACTB NRO S	TAAGGAGAAGCTGTGCTAGG
	ACTB NRO AS	TTGCTGATCCACATCTGCTG
EMSA	$Sp1^P$ wt	GCCTCGCGGGGGCGGGGCCTGGCG
	$Sp1^P$ mt	GCCTCGCGGGTAGAGGGCCTGGCG
	CRE wt	GCGGTGGCGTCACAAAAGGCGG
	CRE mt	GCCGGCGGTGGATACACAAAAGG
PCR	MIF S	GGTCTCCTGGTCCTTCTGC
	MIF AS	TGCACCGCGATGTACTGG
	Ad-MIF S	GCCAGAGGGGTTTCTGTC
	Ad-MIF AS	GTTCGTGCCGCTAAAAGTCA
	HPRT S	GAACGTCTTGCTCGAGATGTG
	HPRT AS	CCAGCAGGTCAGCAAAGAATT
ChIP and CHART	MIF S3	TCCCAGCATCCTATCCTCTT
	MIF S8	CGGTGACTTCCCCACTCG
	MIF AS1	GGCACGTTGGTGTTGTTTACGAT
	MIF AS3	TGGGGATGCGGCGGTGAACC

Base substitutions are underlined.
S: sense; AS: antisense; wt: wild-type; mt: mutant.

2.4. Whole blood MIF secretion assay

Whole blood (200 μl) from healthy donors was diluted one in five in RPMI medium with or without TSA and incubated 20 h at 37 °C in a humidified incubator containing 5% CO_2. Samples were centrifuged at 3000×*g* for 3 min. MIF levels in supernatants were measured by ELISA as previously described [36].

2.5. Nuclear run-on

Nuclei isolation and *in vitro* transcription using biotin-16-UTP (Roche) were performed as described [37]. RNA was isolated using TRIzol® (Invitrogen), extracted with chloroform, precipitated with isopropanol and resuspended in 50 μl sterile water. Total RNA (10 μl) was conserved to monitor RNA input. Purification of biotinylated RNA transcripts was performed using the μMACS Streptavidin Kit according to the manufacturer's instructions (Miltenyi Biotec). Input RNA and biotinylated RNA were used for cDNA synthesis with random hexamer primers. The level of MIF and β-actin cDNA in the preparations was determined by PCR using specific oligonucleotides (Table 1). The PCR conditions were as follows: 95 °C 15 min, followed by 40 cycles consisting of 95 °C 30 s, 56 °C 30 s and 72 °C 1 min. PCR products were analyzed by electrophoresis through agarose gels.

2.6. Transient transfection

The MIF promoter driven luciferase vectors have been described in details [38]. HeLa cells (5×10^4) were platted in 24-well culture plates. The following day, cells were transiently transfected using Fugene™ 6 transfection reagent (Roche Diagnostics) and 100 ng of luciferase reporter vectors [39]. Six hours later, transfected cells were exposed to the drugs. After 18 h, cells were harvested. Luciferase activity was measured using the Dual-Luciferase™ Reporter Assay System (Promega).

2.7. Chromatin immunoprecipitation (ChIP)

Cells were treated with 1% formaldehyde for 30 min at 37 °C, harvested, washed twice with cold PBS containing 1 mM PMSF and 0.9 μg/ml aprotinin, lysed in 50 mM Tris–HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS, 1 mM PMSF and 0.9 μg/ml aprotinin (200 μl per 10^6 cells) and sonicated using a Soniprep 150 (MSE) sonicator (5 × 30 s, amplitude 14). Cleared lysates (200 μl) were diluted 10-fold in 1% Triton X-100, 150 mM NaCl, 20 mM Tris–HCl pH8.0, 2 mM EDTA pH 8.0, 1 mM PMSF and 0.9 μg/ml aprotinin. Twenty microliters were kept for controls (input DNA). The rest of the preparation was incubated overnight at 4 °C with 2.5 μg of control antibody (sc-2027, Santa Cruz Biotechnology) or anti-acetylated histones H3 and H4 (06-599 and 06-866, Upstate), anti-phosphorylated CREB (9198, Cell Signaling Technology), anti-Sp1 (07-645, Upstate) and anti-RNA polymerase II (sc-9001x, Santa Cruz Biotechnology) antibody. One hundred microliters of Protein A Sepharose slurry (GE healthcare) were added to the lysates and the mixtures were incubated 2 h at 4 °C. Beads were extensively washed and immune complexes were eluted in 450 μl 1% SDS and 100 mM $NaHCO_3$. Input DNA and immunoprecipitated DNA were reverse-crosslinked, purified by phenol:chloroform:isoamyl alcohol extraction, precipitated with ethanol and resuspended in 50 μl of water. MIF promoter sequence was detected by PCR using the S8 and AS1 primers (Table 1). The PCR conditions were as for nuclear run-on. p21 promoter sequence was detected as previously described [40]. Signals were quantified using an Eagle eye video imaging system (Stratagene).

2.8. HDAC activity assay

HDAC activity in 50 μg of nuclear proteins was measured using the HDAC assay kit (Millipore and BioVision) according to the manufacturer's recommendations.

2.9. Chromatin accessibility by real-time PCR (CHART-PCR)

Cells were incubated 10 min on ice in 15 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 0.1 mM EGTA, 50 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 0.2% NP40 and 5% sucrose. The suspension was layered on a 10% sucrose cushion. After centrifugation, the nuclei were recovered in 15 mM NaCl, 10 mM Tris pH 7.4, 50 mM KCl, 0.15 mM spermine, 0.5 mM spermidine and 8.5% sucrose. Nuclei from 10^6 cells were diluted in the recommended 1× restriction enzyme dilution buffer and 1 U/μl of *Dra*I or *Not*I. The reaction mixture was incubated 1 h at 37 °C. A non-digested control was included in the assay as well as a purified DNA positive control. Genomic DNA was isolated using the DNeasy Tissue kit (Qiagen), eluted in water, digested to completion with *Xma*I and used in real-time PCR. Quantitative PCR was performed with a Light Cycler (Roche) using the SYBR®-Green PCR assay (Qiagen) and primer pairs S3/AS3 and S8/AS1 (Table 1) overlapping the region of DNA targeted by restriction enzymes (Fig. 6). PCR conditions were as for nuclear run-on. Results of restriction digest were normalized to the intact region and expressed as percentage of non-digested DNA.

3. Results

3.1. HDIs down-regulates MIF expression

We used TSA, a broad spectrum prototypical HDI [19], to examine whether HDIs affect MIF expression. Analyses of MIF mRNA steady state levels in HeLa epithelial cells revealed that TSA decreased MIF expression in a time- and dose-dependent manner (Fig. 1A and B). Of note, other HDIs such as SAHA and valproic acid (VPA) inhibited MIF mRNA expression in HeLa cells (Fig. 1C). TSA strongly reduced MIF mRNA expression also in HaCat keratinocytes, HL-60, KG1a and U-937 leukemic cell lines, THP-1 monocytic cells, A549 airway epithelial cells and B16 melanoma (Fig. 1D and data not shown). Conversely, TSA was shown to reactivate the expression of *p21* (*CDKN1A*) in all cell lines (Fig. 1A and D), indicating that the effect on MIF mRNA expression was specific. In line with a strong inhibition of MIF mRNA expression, TSA decreased intracellular MIF protein content in U-937, HaCat and A549 cells in a time-dependent manner (Fig. 1E and data not shown). TSA also strongly decreased MIF mRNA level in mouse bone-marrow-derived macrophages (Fig. 1F) and MIF release in human whole blood (Fig. 1G). Strengthening these *in vitro* observations, injection of TSA in mice (10 mg/kg intraperitoneally q24 h for 2 days) reduced MIF blood levels two-fold (Fig. 1H). Overall, these data demonstrated that HDIs inhibit MIF mRNA and protein expression in cells lines, primary cells and *in vivo*.

3.2. TSA decreases MIF gene transcription

Nuclear run-on assays were used to determine whether TSA inhibited MIF mRNA expression via a transcriptional mechanism. Nuclei of U-937 cells cultured with or without TSA were isolated and subjected to *in vitro* transcription using biotinylated UTP. Neo-synthesized biotinylated messengers were isolated, reversed transcribed and amplified by PCR using MIF and β-actin specific oligonuleotides. The level of nascent MIF, but not β-actin, mRNA was substantially reduced upon treatment with TSA (Fig. 2). These data definitively indicated that TSA decreased MIF mRNA expression by inhibiting the transcription of the *MIF* gene.

3.3. TSA targets endogenous MIF gene expression, but not ectopically expressed MIF

To test whether TSA impaired *MIF* transcription by interfering with MIF promoter activity, we first thought to analyze the transcriptional activity of MIF promoter (−858/+129, −308/+129,

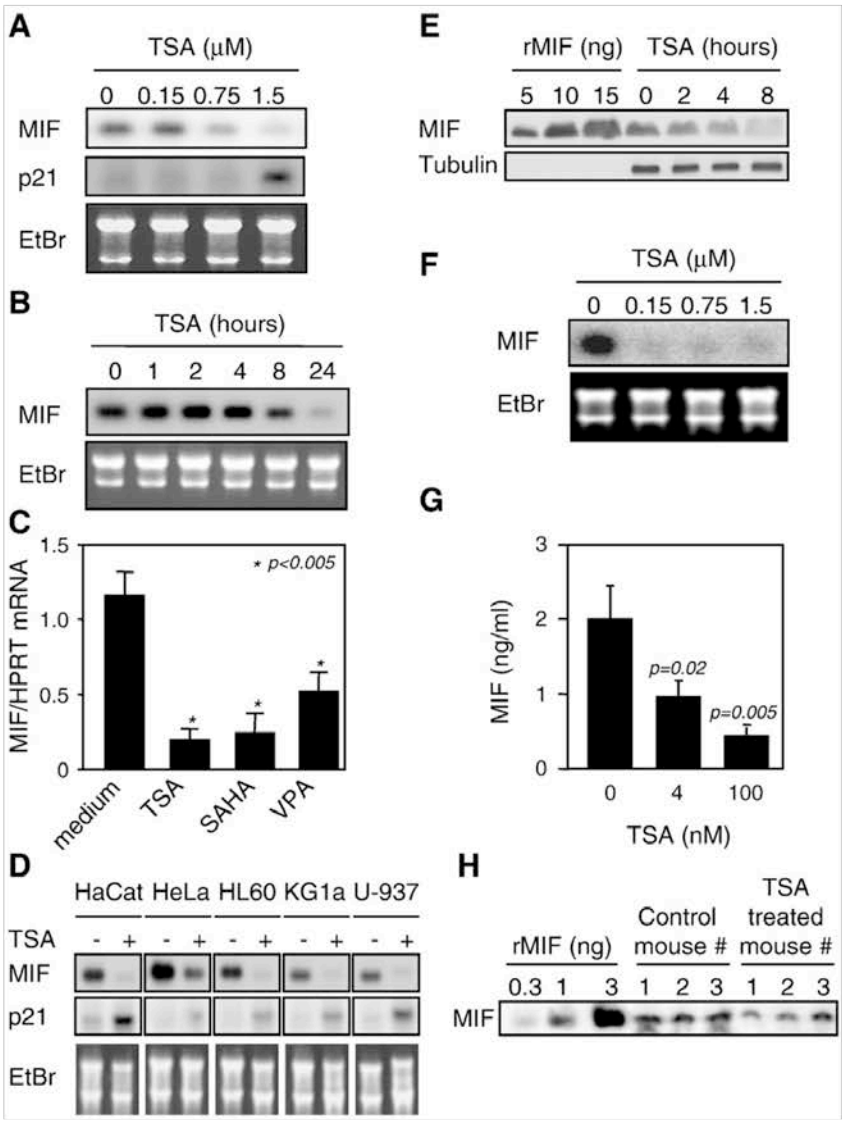

Fig. 1. TSA down-regulates MIF expression. (A, B) Northern blot analysis of MIF and p21 (CDKN1A) mRNA expression in HeLa cells cultured for 18 h with increasing concentrations of TSA (A) or cultured for increasing time with 1 μM TSA (B). Uniform RNA loading was verified by ethidium bromide (EtBr) staining of 28S and 18S RNA. (C) MIF mRNA expression in HeLa cells cultured 18 h with TSA (1 μM), SAHA (1 μM) and valproic acid (VPA, 1 mM) analyzed by real time-PCR. Results are expressed as the ratio of MIF to HPRT mRNA levels. Data are means ± S.D. of triplicate samples from one experiment ($p<0.005$ for HDIs versus medium by two-tailed paired Student's *t*-test). (D) MIF and p21 mRNA expression in HaCat, HeLa, HL60, KG1a and U-937 cells cultured for 18 h with or without TSA (1 μM). (E) Western blot analysis of intracellular MIF in U-937 cells treated for 0, 2, 4 and 8 h with TSA (1 μM). Recombinant MIF (rMIF) was used as a standard. (F) MIF mRNA expression in mouse bone-marrow-derived macrophages cultured for 24 h with increasing concentrations of TSA. Results are representative of at least two independent experiments (A-F). (G) Whole blood was incubated for 24 h with TSA. MIF secretion was measured by ELISA. Data are means ± S.D. of triplicate samples from one experiment ($p=0.02$ and 0.005 for 4 and 100 nM TSA compared to medium by two-tailed paired Student's *t*-test). (H) BALB/c mice ($n=3$) were injected with TSA (10 mg/kg i.p. q24h) or diluent (control) for 2 days. Blood was collected and a fixed volume of plasma was used to analyze MIF expression by Western blotting.

−157/+129, −81/+129 and +44/+129) luciferase reporter vectors in HeLa cells. In agreement with high MIF mRNA expression in resting HeLa cells, all but one construct (+44/+129) drove strong luciferase activity (Fig. 3A). In contrast to expectations, TSA treatment strongly increased luciferase activity driven by all MIF promoter reporter constructs active at baseline. The proximal region of the MIF promoter contains several DNA-binding sites among which are one c-Myb, two Sp1 ($Sp1^p$ and $Sp1^d$) and two cAMP response element (CRE^p and CRE^d) sites (Fig. 3B). We previously reported that Sp1 and CREB bind to the $Sp1^p$ and CRE^p sites to positively regulate MIF promoter activity [38]. To analyze their involvement in TSA-induced transcriptional activity of MIF promoter, the c-Myb, Sp1 and CRE sites were disrupted in −157/+129 construct (Fig. 3B). Mutation of the cMyb and CRE sites did not impact on TSA-induced MIF promoter activity. In contrast, disruption of the $Sp1^p$ site and, to a lesser extent, of the $Sp1^d$ site reduced MIF promoter induction by TSA. Furthermore, mithramycin A, an antibiotic that interferes with Sp1 binding to DNA, abrogated TSA-mediated increased activity of wild-type but not the $Sp1^p$ mutant MIF promoter (Fig. 3C), confirming that TSA increased episomal MIF promoter activity through Sp1 DNA binding. Overall, the effect of TSA on ectopically expressed MIF promoters did not recapitulate the inhibitory effect observed on endogenous *MIF* gene,

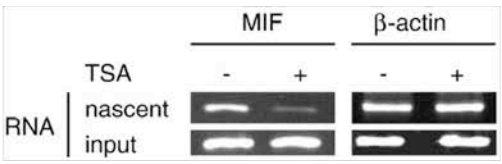

Fig. 2. TSA inhibits *MIF* gene transcription. Nuclei were isolated from U-937 cells treated for 8 h with or without TSA (1 μM) and subjected to *in vitro* transcription analysis. MIF and β-actin mRNA levels in elongated and initiated transcripts (nascent) and in total nuclear transcripts (input) were amplified by RT-PCR (see Materials and methods). PCR products were analyzed by gel electrophoresis.

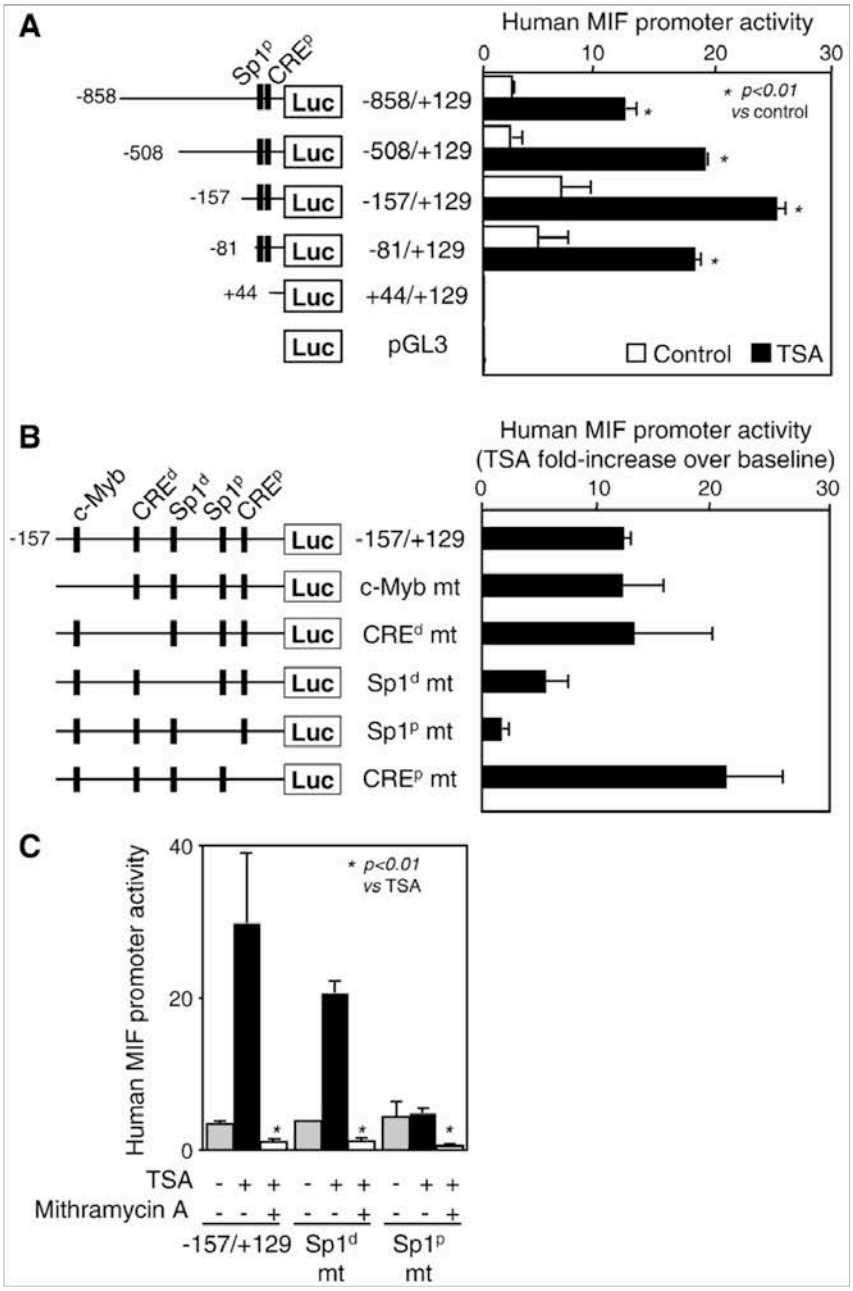

Fig. 3. TSA increases episomal MIF promoter activity. (A–C) HeLa cells were transfected with an empty pGL3 vector, human MIF promoter deletion constructs (−858/+129, −508/+129, −157/+129, −81/+129 and +44/+129) or the −157/+129 promoter construct with mutations (mt) in c-Myb, CRE^{d}, Sp1^{d}, Sp1^{p} and CRE^{p} DNA-binding sites. Of note, the Sp1 and CRE DNA-binding sites that control constitutive transcriptional activity of the *MIF* gene are deleted from construct +44/+129. After 6 h, cells were treated with or without TSA (1 μM) and mithramycin A (100 nM). Luciferase activity was determined 18 h latter (right panel). Data are means ± S.D. of six determinations from one experiment representative of two experiments. $p<0.01$ for medium versus TSA by two-tailed paired Student's *t*-test for −858/+129, −308/+129, −157/+129 and −81/+129 and for Sp1^{p} mt versus the −157/+129.

which probably relies on the fundamental differences that exist between episomal reporter constructs and endogenous chromosomal genes.

To further demonstrate that TSA specifically inhibited endogenous MIF expression, we transduced HeLa cells with an adenoviral vector encoding *MIF* under the control of the CMV promoter. A PCR discriminating between endogenous MIF mRNA and adenoviral-derived MIF mRNA was used to evaluate the effect of TSA. As previously observed (Fig. 1), TSA dose-dependently inhibited the endogenous MIF mRNA steady state level (Fig. 4A). In contrast, TSA did not affect adenoviral-derived MIF mRNA expression. In agreement with these observations, TSA reduced MIF protein expression two-fold in cells transduced with the control adenovirus, whereas it slightly increased MIF protein level in cells transduced with the MIF-encoding adenovirus (Fig. 4B). Altogether, these data argued in favor of a direct inhibitory effect of TSA on endogenous *MIF* gene transcription.

3.4. TSA deacetylates the proximal MIF promoter

Because the association of trans-regulatory elements with promoters occurs in the context of chromatin, we tested whether TSA affected the acetylation status of histones H3 and H4 associated with the proximal MIF promoter using chromatin immunoprecipitation (ChIP) (Fig. 5A and B). Interestingly, TSA markedly reduced (two- to three-fold) the level of acetylated histones H3 and H4 associated with the MIF promoter in both U-937 and HeLa cells exposed to TSA. The

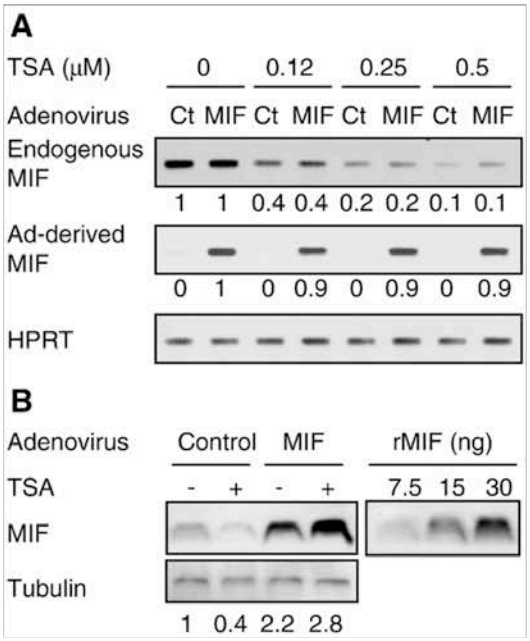

Fig. 4. TSA does not inhibit ectopically expressed MIF. HeLa cells were transduced with an empty control (Ct) or MIF-expressing adenovirus (Ad) and treated 18 h later with TSA (0.5 μM unless specified). RNA and proteins were recovered after 18 h. (A) Endogenous MIF, adenovirus (Ad)-derived MIF and HPRT mRNA expression levels were assessed by PCR. (B) MIF and tubulin expression was analyzed by Western blotting. Recombinant MIF (rMIF) was processed in parallel to estimate MIF content in HeLa cells. Results are representative of two independent experiments.

effect was specific of MIF promoter-associated histones since TSA increased the acetylation of histones H3 and H4 related to the p21 promoter (Fig. 5C). Moreover, as positive controls, we confirmed that TSA dose-dependently inhibited HDAC activity (Fig. 5D) at concentrations shown to inhibit MIF mRNA expression (Fig. 1) and raised global acetylation of histones H3 and H4 (5.2- and 8.3-fold for histones H3 and H4, Fig. 5E) in TSA-treated cells. Therefore, deacetylation of the histones H3 and H4 associated with the MIF promoter was an MIF-specific HDI effect.

To determine whether TSA-induced inhibition of MIF mRNA expression and MIF promoter histone acetylation required newly synthesized factors, U-937 cells were pretreated for 1 h with cycloheximide before exposure to TSA. Cycloheximide given alone gradually decreased MIF mRNA levels over time, indicating that MIF mRNA expression required protein synthesis. TSA alone decreased MIF mRNA levels similarly to CHI. The combination of cycloheximide plus TSA increased MIF mRNA levels at an early time point (1.6-fold increase after 2 h of treatment) but decreased MIF mRNA levels after 8 and 16 h as observed with cycloheximide or TSA (Fig. 6A). Cycloheximide did not modify the acetylation of MIF promoter-associated histones H3 and H4, whereas it counter-acted TSA-mediated inhibition of histone acetylation (Fig. 6B). Thus, TSA decreased MIF promoter histone acetylation and MIF mRNA expression through a molecular mechanism requiring de novo protein synthesis.

3.5. TSA does not modify chromatin accessibility to the proximal MIF promoter

The proximal region of the MIF promoter contains several DNA-binding sites among which are one Sp1 (at −42 bp) and one cAMP response element (CRE, at −20 bp) sites that we previously reported to bind Sp1 and CREB to positively regulate MIF promoter activity [38]. We therefore developed a chromatin accessibility by real-time PCR (CHART-PCR) assay to test whether hypoacetylation of MIF-associated histones rendered the MIF promoter less accessible to Sp1 and CREB. Nuclei were isolated and incubated with *Dra*I and *Not*I that recognize sequences present in the distal and proximal regions of the MIF promoter (at nucleotide −528 and +41, respectively, Fig. 7A). Genomic DNA was subjected to real-time PCR amplification using primer pairs surrounding the *Dra*I and *Not*I restriction sites. The amount of material amplified was inversely proportional to the

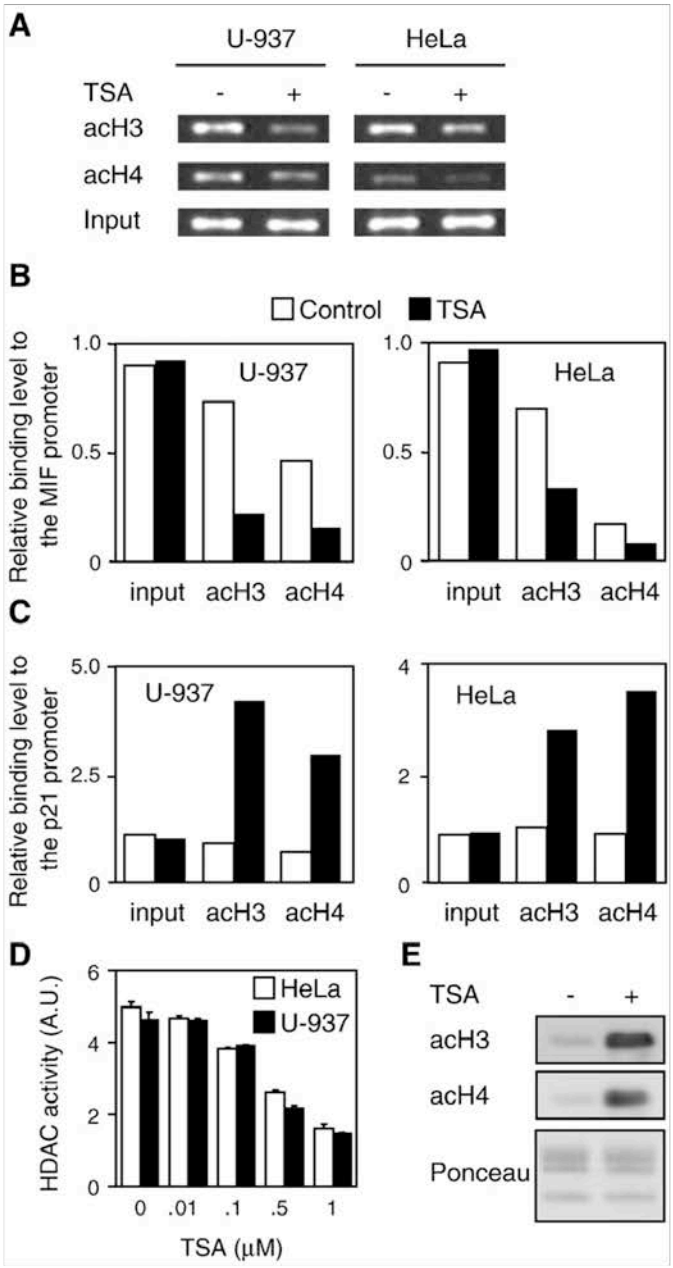

Fig. 5. TSA deacetylates the histones associated with the proximal region of the MIF promoter. (A–C) U-937 and HeLa cells were treated for 10 h with or without TSA (1 μM) and analyzed by ChIP assay using anti-acetylated (ac) H3 and anti-acH4 antibodies. Input and immunoprecipitated DNA were subjected to PCR amplification using primers specific of the promoter region of the *MIF* (A, B) and *p21* (C) genes. PCR were analyzed by gel electrophoresis (A) and signals quantified by densitometry (B, C). Results are representative of two independent experiments. (D) HDAC activity in nuclear extracts from HeLa and U-937 cells treated for 10 h with increasing concentrations of TSA (0–1 μM). Data are means ± S.D. of duplicate samples from one experiment. (E) Histones were extracted from U-937 cells treated for 10 h with or without TSA (1 μM) and subjected to Western blot analysis using anti-acH3 and anti-acH4 antibodies. The gel was colored with Ponceau Red to visualize total histones. Results are representative of three independent experiments.

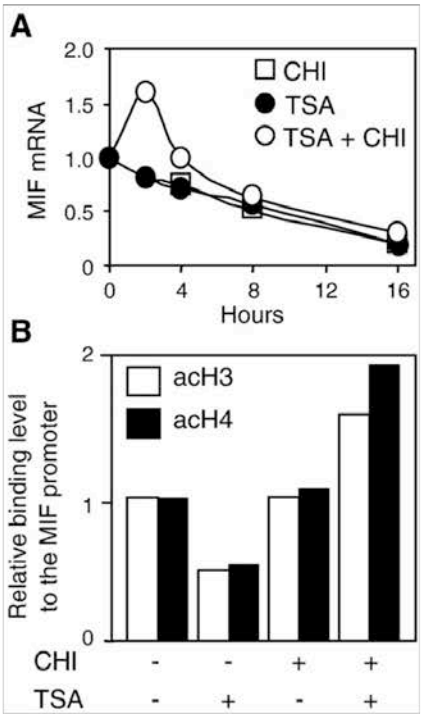

Fig. 6. TSA decreases MIF promoter histone acetylation through a molecular mechanism requiring de novo protein synthesis. Quantification of MIF mRNA expression determined by Northern blotting (A) and ChIP assay of acH3 and acH4 histones associated with the proximal MIF promoter (B) in U-937 cells cultured for 1 h with or without cycloheximide (CHI, 10 μg/ml) and then incubated for 10 h with or without TSA (1 μM). Results are representative of two independent experiments.

restriction digestion of DNA, and therefore to the accessibility of the chromatin. In agreement with the localization of important transcriptional regulatory elements in the vicinity of the *MIF* transcriptional start site, the proximal region of the MIF promoter (examined by *Not*I digestion) was highly accessible in both U-937 and HeLa cells. Conversely, the distal region (examined by *Dra*I digestion) was much less accessible (Fig. 7B and C). When cells were incubated with TSA, the accessibility to the proximal region of the MIF promoter slightly increased in U-937 cells, whereas it was not modified in HeLa cells (right panels in Fig. 7B and C). Finally, the accessibility to the distal region of the MIF promoter was not affected by TSA (left panels in Fig. 7B and C). Thus, remodeling of chromatin accessibility by TSA was not a major mechanism whereby TSA inhibited *MIF* gene transcription.

3.6. TSA decreases the recruitment of regulatory proteins to the proximal MIF promoter

The hypoacetylation of the MIF proximal promoter induced by TSA could potentially affect the binding of transcription regulatory factors essential for basal *MIF* expression such as Sp1 and CREB [38] and thereby prevent efficient RNA polymerase II recruitment. To address this question, we analyzed by ChIP the level of Sp1, CREB and RNA polymerase II interacting with the proximal MIF promoter (Fig. 8A and B). Whereas Sp1, CREB and RNA polymerase II were shown to be bound to the MIF promoter in resting U-937 cells, TSA strongly reduced their binding to DNA, which provided a molecular mechanism by which TSA inhibited *MIF* gene transcription. Of note, Western blot analyses demonstrated that TSA did not reduce the quantity of nuclear Sp1 and CREB (Fig. 8C and D). Moreover, co-immunoprecipitation analyses confirmed that TSA increased Sp1 acetylation (Fig. 8D), which has been associated with Sp1 transcriptional activity [41]. Altogether, these results ruled out the possibility that TSA decreased Sp1 and CREB binding to the endogenous MIF promoter by affecting their expression level or Sp1 acetylation status.

4. Discussion

We report that HDIs down-regulate the expression of the proinflammatory and protumorigenic cytokine MIF through a new molecular mechanism involving a local deacetylation of MIF promoter-associated histones which does not modify chromatin accessibility but impairs the recruitment of the basal transcriptional machinery to the proximal MIF promoter. Although the relative contribution of specific HDACs in these process has not been determined, the observation that chemically unrelated HDIs inhibited MIF expression argues for an important contribution of HDAC in regulating MIF expression.

Histone acetylation has been traditionally seen as a positive marker for transcription. It is generally admitted that elevated histone acetylation level induces a more open, transcriptionally more active chromatin state. This notion is, however, hardly reconcilable with the fact that HDIs, albeit strongly raising global histone acetylation, induce transcriptional changes of only 2–10% of the transcriptome. Interestingly, comparison of the transcriptome of different cancer cell lines treated with HDIs has identified common subsets of genes being

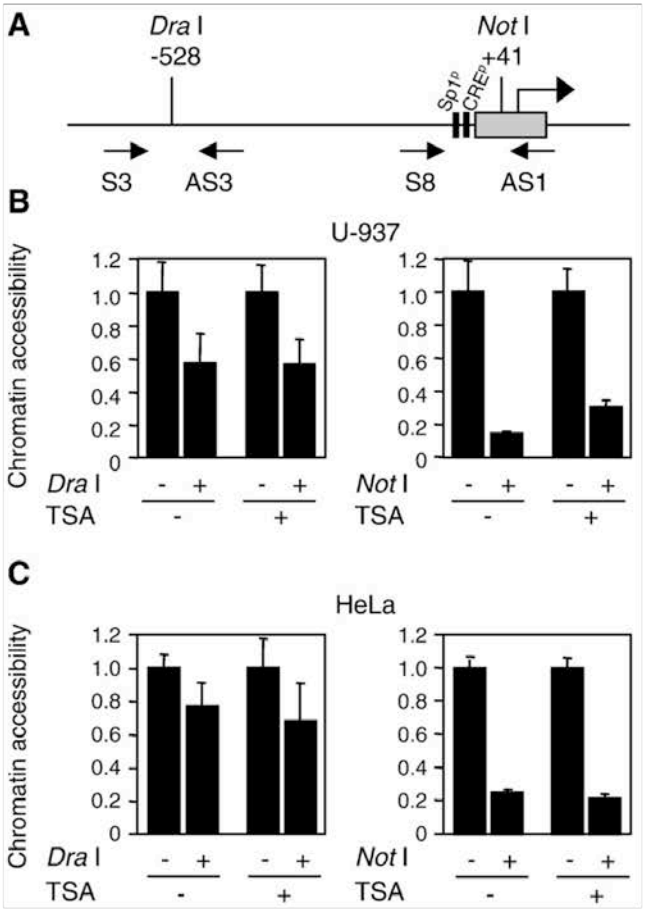

Fig. 7. TSA does not impair the accessibility to the chromatin associated with the MIF promoter. (A) Schematic representation of the MIF promoter showing MIF exon I (grey box), the translational start site (upper arrow), the Sp1 and CRE DNA-binding sites that regulate basal MIF promoter transcriptional activity, the *Dra*I and *Not*I restriction sites (nucleotide localization is relative to the transcriptional start site) and the relative localization of S3/AS3 and S8/AS1 primer pairs used to amplify by real-time PCR the DNA region surrounding the *Dra*I and *Not*I sites. (B, C) Nuclei isolated from U-937 cells (B) and HeLa cells (C) treated for 10 h with or without TSA (1 μM) were subjected to digestion with *Dra*I and *Not*I and analyzed by chromatin accessibility by real-time PCR. A non-digested control as well as a purified DNA positive control was included in the assays (data not shown). Data from restriction digests were normalized to those obtained without digestion set at 1. Data are means ± S.D. of three independent determinations. Similar results were obtained using cells incubated for 4, 10 and 24 h with TSA (data not shown).

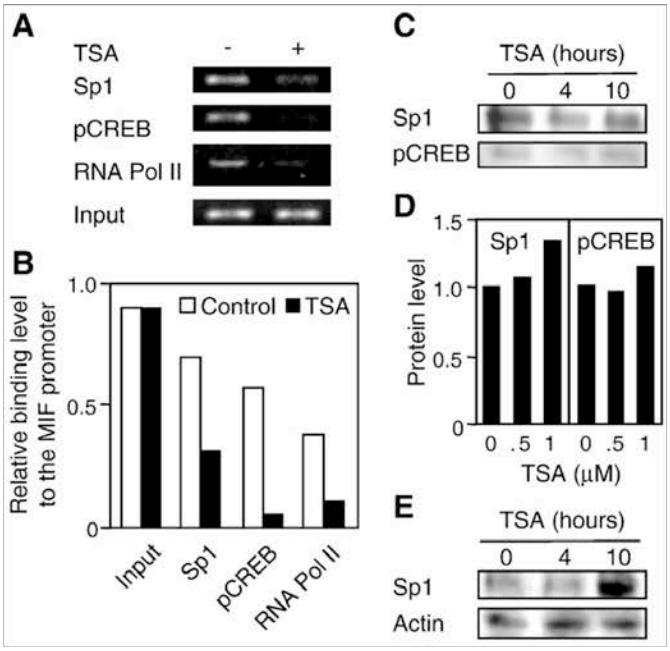

Fig. 8. TSA decreases the binding of Sp1, CREB and RNA polymerase II to the proximal region of the MIF promoter. HeLa cells were treated for 10 h with or without TSA (1 μM) and analyzed by ChIP assay using anti-Sp1, anti-phospho-CREB (pCREB) and anti-RNA polymerase II (RNA Pol II) antibodies. (A) The immunoprecipitated DNA was subjected to PCR amplification using primers specific for the proximal region of the MIF promoter. (B) Densitometric quantification of the gels shown in panel A. (C–E) Western blot analysis of Sp1 and phosphorylated CREB (pCREB) nuclear content (C, D) and co-immunoprecipitation study of acetylated Sp1 expression (D) in HeLa cells treated with TSA (1 μM in C, E) for the indicated time (C, E) or 10 h (D). Similar results were obtained in two independent experiments.

either activated or repressed, primarily genes encoding for proteins involved in cell cycle regulation, apoptosis, signaling and regulation of transcription [25–28]. The basis for this selectivity remains largely unknown and probably involves multiple components as HDIs modify the acetylation status of an increasing list of non-histone substrates including transcription factors and signal transduction mediators.

We found that MIF expression was strongly repressed by HDIs in a broad range of cell lines, including myeloid cells, epithelial cells, keratinocytes and melanoma. Considering that MIF is overexpressed in multiple kinds of tumors and that MIF expression levels have been associated with tumor aggressiveness and metastatic potential [12,42,43], MIF may represent a common target of the anticancer effects of HDIs.

While numerous studies have dissected the molecular mechanisms by which HDIs relieve gene expression, very little is known about how these drugs repress gene expression, particularly constitutive expression. Whereas TSA inhibited *MIF* gene transcription, it vigorously activated ectopically expressed MIF promoters concomitantly with an increased Sp1 acetylation. This effect was lost when the proximal Sp1 DNA binding site was mutated in MIF promoter reporter constructs, which is in fact in agreement with the observation that Sp1 acetylation increases its transcriptional activity [41]. Yet, of importance, these observations definitely illustrate the fundamental differences that exist between episomal reporter constructs and endogenous chromosomal genes and the risk of drawing conclusions from the analysis of transfected reporter gene exposed to chromatin remodeling agents [44].

In the context of HDAC inhibition, Sp1 regulatory elements, which we previously demonstrated to regulate MIF promoter activity [38], may have a dual role on ectopically expressed promoters. Indeed, Sp1 sites both increased (inhibitor of Cdk4, HMG-CoA synthase, CDKN1A and EC-SOD) [45–48] and inhibited (BCL2 and TGFβ type II receptor) [49,50] promoter activity upon TSA treatment. The dual role of Sp1 sites might reflect differences in promoter specific sequences allowing the recruitment of additional transcriptional co-regulators (activators or repressors), acetylation-dependent modifications of Sp1 activity, changes in chromatin architecture or even the cell type considered.

Considering that TSA strongly increased overall histone acetylation, it was unanticipated to detect a local deacetylation of histones associated with the proximal MIF promoter in cells exposed to TSA. MIF is one of the very rare examples of promoter deacetylation by HDIs, with that of *high-mobility-group A2* (*HMGA2*) in NIH-3T3 cells [51] and of *BCL2* in human lymphoma [49]. The local deacetylation of these promoters, while in apparent contradiction with the pro-acetylation properties of HDIs, is actually concordant with the fact that HDIs repressed *MIF*, *HMGA2* and *BCL2* gene expression. Genome-wide ChIP experiments (ChiP on Chip) combined with transcriptome analyses will be required to have a comprehensive view of the reciprocal influence of HDIs on histone acetylation and gene expression.

Transcriptome analyses have revealed that inhibition of protein synthesis by CHI prevents most of the effects of HDIs on gene expression [52]. In line with this observation, CHI prevented histone deacetylation by TSA. Co-treatment with TSA and CHI up-regulated MIF mRNA expression early on, whereas TSA-mediated repression of MIF mRNA expression required several hours. These data suggest that inhibition of MIF mRNA synthesis by TSA is not a direct effect but presumably belongs to a secondary response determined by *de novo* synthesis of transcriptional repressors that remain to be identified. Of note, we observed that TSA reduced MIF protein half-life from more than 18 h in HeLa cells cultured with CHI with or without pre-incubation for 1 h with TSA, to 4.5 h in cells cultured with TSA alone. Therefore, TSA not only affected *MIF* transcription, but also stimulated MIF protein degradation through a process requiring protein synthesis.

Considering that TSA reduced MIF promoter histone acetylation, we speculated that this DNA region would be less accessible to Sp1 and CREB which positively regulate constitutive *MIF* gene expression [38]. Whereas our ChIP experiments confirmed that TSA reduced the binding of Sp1, CREB and RNA polymerase II to the MIF promoter, analysis of chromatin accessibility by real-time PCR clearly demonstrated that the accessibility to the proximal region of the MIF promoter was not affected by TSA. This unique situation challenges the concept that hypoacetylated chromatin forms a compact structure [24]. Since TSA did not impact on Sp1 and CREB expression levels, further work will be required to test whether TSA displaces the binding of Sp1 and CREB from the MIF promoter through a direct modification of these proteins affecting their DNA binding capacity or through the recruitment of a transcriptional repressor competing with, or disrupting, Sp1 and CREB DNA binding.

MIF promotes cell proliferation and survival through activation of the PI3K/Akt survival pathway and through inhibition of the p53/CDKN1A and retinoblastoma/E2F tumor suppressor pathways [6–8]. Conversely, HDIs have been reported to induce tumor cell apoptosis by inhibiting Akt signaling which is constitutively activated in many types of cancer [53–55]. Moreover, HDIs most invariably increase tumor expression of CDKN1A in either a p53-dependent or a p53-independent manner. Therefore, it is tempting to speculate that inhibition of MIF expression by HDIs is a significant contributor of the pro-apoptotic effects of HDIs.

The inflammatory cells and mediators in the tumor microenvironment participate in neoplastic processes, orchestrating survival, proliferation and migration of malignant cells, promoting angiogenesis and altering antitumor adaptive immune responses and response to cancer therapy [56,57]. Besides its effect on cell proliferation and survival, MIF has been reported to promote tumor-associated angiogenesis [10] and to inhibit antitumor NK and CTL responses [11–14]. Following these observations and considering the well-established central role played by MIF in acute and chronic

inflammatory responses [3], inhibition of MIF-mediated inflammation by HDIs may represent an important facet by which HDIs interfere with tumorigenesis.

Recent studies converge to attribute MIF an important role in tumor development. Transplantation of neuroblastoma, ovarian cancer and melanoma cell lines engineered to express reduced levels of MIF resulted in delayed or reduced tumor establishment, progression and metastasis and improved animal survival [14,58–60]. Similarly, the development of chemically induced fibrosarcoma and muscle invasion of bladder cancer was impaired in $MIF^{-/-}$ compared to wild-type mice [61,62]. Considering that TSA reduced MIF blood levels in mice (Fig. 1H), it will be of interest to analyze whether the anticancer activity of HDIs is associated with decreased MIF expression *in vivo*.

In conclusion, we report that HDIs inhibit *MIF* gene expression through a local deacetylation of MIF promoter-associated histones that affects the recruitment of the basal transcription machinery. MIF has a unique spectrum of biological activities, which positions MIF as a central mediator of inflammation and innate immunity and as an important contributor to cell proliferation, malignant transformation and angiogenesis. Bringing together the observations that MIF expression levels correlate with tumor aggressiveness and metastatic potential, that altered expression of HDACs contributes to tumor development and that HDIs powerfully inhibit MIF expression, we propose that MIF could represent a candidate for potential targeted or adjunctive anticancer therapy.

Acknowledgements

This work was supported by grants from the Swiss National Science Foundation (310000-114073/1), the Leenaards Foundation and Merck Sharp & Dohme-Chibret AG to TR and grants from the Swiss National Science Foundation (3100-066972.01), the Bristol-Myers Squibb Foundation, the Leenaards Foundation and the Santos-Suarez Foundation for Medical Research to TC.

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5 EPIGENETIC CONTROL OF MIF EXPRESSION

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In *MIF Handbook*, Edited by Richard Bucala, World Scientific Publishing Co. Pte. Lte., New Jersey, 2011. Chapter II.1

This commissioned manuscript summarizes our current knowledge about the epigenetic mechanisms involved in the control of *MIF* gene expression.

5.1 Abstract

Covalent modifications of DNA by methylation and of histones by acetylation are two main mechanisms by which epigenetics regulates gene expression under physiological and pathological situations. Inhibitors of DNA methyltransferases and histone deacetylases (HDACs) are potent anti-cancer drugs, displaying anti-inflammatory and immunomodulatory activities. MIF is a proinflammatory cytokine involved in the pathogenesis of inflammatory, autoimmune and infectious diseases. Lately, MIF has been shown to promote tumorigenesis, suggesting that epigenetic mechanisms participate in the control of MIF expression. Of note, the *MIF* gene lies in a CpG island, a DNA context prone to regulation by methylation. Yet, the *MIF* promoter is hypomethylated in primary and tumor cells, and demethylating agents do not affect *MIF* expression. In contrast, HDAC inhibitors impair MIF mRNA and protein expression *in vitro* and *in vivo*. At the molecular level, HDAC inhibitors decrease the recruitment of the basal transcriptional machinery to the *MIF* promoter and thereby inhibit *MIF* transcription. These data indicate that HDACs are important regulators of *MIF* expression. Therefore, inhibition of MIF expression may contribute to the anticancer and anti-inflammatory activities of HDAC inhibitors.

5.2 Epigenetics

All cells from a given organism contain essentially the same DNA information. Developmental specification relies on qualitative and quantitative differences in gene expression, which for obvious reasons of parsimony, is primarily controlled at the level of transcription. In recent years, epigenetics, defined as all meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence (1), has profoundly transformed our vision of how gene expression is regulated.

Epigenetics comprises three main and inter-related mechanisms: DNA methylation, small interfering RNAs and post-transcriptional modifications of histones (2). Specific panels of epigenetic modifications shape the transcriptional program in a cell-specific manner. As such, the epigenome plays a central role in conserving cell characteristics by maintaining specific patterns of gene expression during somatic cell division (3-6). Yet, the epigenome is dynamic and flexible and accommodates transcriptional changes during development. Most importantly, epigenetic modifications have been directly linked to the dysregulated gene expression characterizing numerous human diseases (7). Reflecting the great interest of biomedical research in epigenetics, ambitious projects and initiatives (NIH Roadmap Epigenomics Program, ENCODE project, AHEAD project and the Epigenomics NCBI browser) have been developed to provide highly comprehensive epigenomic maps in human stem cells and in healthy and diseased tissues (8, 9). In this chapter, we will focus our attention on DNA methylation and histone acetylation as possible mechanisms involved in the control of *MIF* gene expression, as no MIF-specific micro-RNA (miRNA) has been identified thus far.

5.3 DNA Methylation

DNA methylation is probably the most studied epigenetic modification in mammals. The reversible covalent modification of 5’-methyl cytosine residues mainly occurs in the context of CpG dinucleotides. DNA methylation is catalyzed by DNA methyltransferases (DNMTs). Around 3% of cytosines are methylated in the human genome. Repetitive genomic DNA sequences are heavily methylated whereas CpG rich regions of the genome, also known as CpG islands, are commonly unmethylated in normal cells. The CpG islands are not randomly distributed. About half of these are localized in the promoter region of genes that have a widely expressed, such as *MIF*.

DNA methylation is usually associated with repressed gene expression. The methyl groups added by DNMTs protrude from cytosines and affect gene transcription through two main mechanisms. First, they inhibit the binding of transcription factors that positively regulate transcription. Second, they recruit methyl-CpG-binding proteins and other types of proteins that are involved in histone modification, chromatin compaction and gene silencing (10, 11).

DNA hypomethylation was one of the very first epigenetic alteration reported in human cancer (12). Interestingly, dysregulated DNA methylation have also been reported in neurological disorders and autoimmune diseases (7). Yet, very little is known about the mechanisms involved in DNA demethylation (10,11). Although tumor cells have globally 20-60% less 5-methyl-cytosine methylation than normal cells, they also contain subsets of hypermethylated genes. Indeed, transcriptional silencing of tumor suppressor genes resulting from the hypermethylation of CpG island promoters is a common hallmark of tumor cells (13, 14). DNA demethylating agents have been developed to revert aberrant gene silencing in cancers. The nucleoside analog 5-Azacytidine (5-Aza-CR; azacitidine, Vidaza®) and its derivative 5-Aza-2-deoxycytidine (5-Aza-CdR, decitabine, Dacogen®) have been approved for the treatment of all subtypes of myelodysplastic syndrome (15-17).

5.4 Post-transcriptional modifications by histone deacetylases

The nucleosome, the basic repeating unit of chromatin, is composed of a 147 bp section of DNA wrapped around an histone octamer composed of two copies of each of the four core histones H2A, H2B, H3 and H4. Histones are subjected to post-transcriptional covalent modifications at amino-terminal tails through acetylation, ubiquitination and sumoylation of lysine, methylation of arginine and lysine, and phosphorylation of serine and threonine. Histone acetylation usually associates with specific histone methylation marks (7). Each modification affects the structure and the function of the chromatin. The open structure of transcriptionaly active euchromatin is enriched in acetylated and trimethylated H3K4, H3K36 and H3K79 histones, whereas the transcriptionaly inactive heterochromatin is characterized by hypoacetylated and highly methylated H3K9, H3K27 and H4K20 histones.

Histone acetylation is controlled by the antagonistic action of two enzyme families: histone acetyl transferases (HATs) and histone deacetylases (HDACs). HATs catalyze the transfer of an acetyl group from acetyl-coenzyme A to an amino-group of lysine residues of histones. Conversely, HDACs catalyze the hydrolysis of acetamides resulting in histone deacetylation. The name HDAC was coined because histones were the first substrates identified for lysine deacetylases (18). However, HDACs deacetylate numerous non-histones proteins such as tubulin, heat shock proteins, steroid receptors and nuclear import and transcription regulators (19, 20). HDACs are classified into two main sub-families: HDAC1-11 and the sirtuins (21). In the following sections, we will use HDACs as a generic term referring to HDAC1-11.

Aberrant gene expression due to inactivation of HATs or overexpression of HDACs is common in cancer cells. Moreover, dysregulated recruitment of HDACs to promoters is associated with transcriptional repression, notably that of cell-cycle modifiers and tumor suppressor genes, thereby contributing to oncogenesis (14, 22-24). These observations strengthened the development of pharmacological inhibitors of HDACs as novel cancer therapeutics. Indeed, HDAC inhibitors counteract cancer development by blocking DNA synthesis and inducing growth arrest, differentiation and apoptosis of tumor cells. They also reduce tumor angiogenesis, metastasis and invasion. Suberoylanilide hydroxamic acid (SAHA, vorinostat, Zolinza®) and depsipeptide (romidepsin, Istodax®) have been approved for the treatment of cutaneous T-cell lymphoma (17) and numerous additional HDAC inhibitors are now being tested in clinical trials.

Specific patterns of histone modifications by methylation and acetylation correlate with the expression of inflammatory and immune genes. HDAC inhibitors have been recently reported to exert potent anti-inflammatory and immunomodulatory activities *in vitro* and *in vivo*. HDAC inhibitors improved outcome in models of inflammatory and auto-immune diseases such as sepsis, rheumatoid arthritis, lupus, autoimmune encephalitis, multiple sclerosis, graft versus host disease, asthma and colitis (25-31). Therefore, HDAC inhibitors are attractive therapies not only for the treatment of oncologic disorders, but also possibly for immune-related diseases (32).

Numerous experimental, pre-clinical and clinical observations have ascribed a central role for MIF in the pathogenesis of inflammatory, autoimmune and neoplastic diseases (33-35). Considering that these diseases are characterized by dysregulation of epigenetic marks, we hypothesized that epigenetic mechanisms may participate in the control of *MIF* expression. Since epigenetic modifications profoundly affect gene transcription, we will first summarize our current knowledge about the various DNA binding sites and trans-acting transcription factors controlling *MIF* gene expression.

5.5 *MIF* gene structure, expression and transcriptional regulation

5.5.1 *MIF* gene structure and expression

The first sequence of a human MIF cDNA and of the *MIF* gene were reported by Weiser *et al.* in 1989 (36) and by Paralkar and Wistow in 1994 (37), respectively. A single *MIF* gene spans nucleotide positions 24236191 to 24237414 on chromosome 22 (22q11.2). This region of chromosome 22 is in syntenic conservation with part of mouse chromosome 10 containing the *Mif* gene (38). Also located on chromosome 22 (22q11.23), *D-dopachrome tautomerase* is the only potential human *MIF* paralog (39). The *MIF* gene is composed of three exons of 108, 173 and 67 bp interspaced by two introns of 189 and 95 bp (Figure 1). A single RNA initiation start site located 97 bp upstream of the methionine codon is used to transcribed a 0.8 kb mRNA (37). The 345 bp open-reading frame of MIF mRNA encodes for a 115 amino acid nonglycosylated protein of 12.5 kD.

Sequence analyses of the *MIF* gene revealed that it does not contain a TATA box but numerous CpG dinucleotides forming a CpG island. A CpG island is defined as a sequence of at least 200 bp with a G+C content of 50% or more and an observed to expected CpG dinucleotide ratio greater than 0.6. The *MIF* CpG island spans approximately 1.2 kb, starting 300 bp upstream of the transcriptional start site (Figure 2). In agreement with the fact that broadly expressed genes are typically lying in CpG islands, MIF is constitutively expressed as a single mRNA species of 0.8 kb in virtually all organs and cell types (summarized in (34)).

MIF gene expression increases through the action of cytokines (tumor necrosis factor, interferon-γ, interleukin(IL)-1, IL-2) (40-44), mitogens (40-44), microbial products (40, 41, 45-47)),

glucose (48), low-density lipoproteins (49, 50), UV-B (51), hypoxia (52, 53) and hormones (glucocorticoids, corticotropin-releasing factor [CRF], human chorionic gonadotropin, angiotensin) (54, 55). Of great interest, MIF is commonly over-expressed in prostate, breast, colon, brain, skin and lung cancers (56-63). Altogether, the expression patterns of MIF, its well-characterized pro-inflammatory, pro-proliferative, pro-survival and pro-angiogenic biological activities point towards a crucial role of MIF in the pathogenesis of infectious, inflammatory, auto-immune neoplastic diseases (33-35).

5.5.2 *MIF* gene transcriptional regulation

Despite the involvement of MIF in the pathogenesis of numerous diseases, few studies have analyzed the molecular mechanisms underlying the transcriptional regulation of *MIF*. The *MIF* promoter region contains putative DNA binding sites for transcription factors such as activator protein-1 (AP-1), nuclear factor (NF)-κB, E-twenty six (Ets), GATA, cAMP-responsive element (CRE) binding protein (CREB), specificity protein 1 (Sp1), hypoxia-inducible factor (HIF) and a glucocorticoid receptor (GR). The first insights about *MIF* gene transcriptional regulation were obtained by the analysis of the activity of mouse *Mif* promoter reporter constructs in rat anterior pituitary cells. A CRE site in the vicinity of the transcriptional start site of the mouse *Mif* gene (located at position -48/41) was shown to mediate forskolin- and CRF-induced *Mif* promoter activation (64). Subsequently, Baugh *et al.* reported that hypoxia and HIF-1α activate human *MIF* promoter activity through a HIF responsive element (HRE located at position +25). Conversely, CREB over-expression decreased *MIF* promoter activity under hypoxic conditions, whereas disruption of a proximal (-20/-11) CRE site increased HIF-1α-mediated *MIF* promoter activity (52). The functional role of the HRE site in response to hypoxia was recently confirmed (65). We have shown that CREB and Sp1 interact with proximal CRE (-20/-11) and Sp1 (-42/-34) sites in the human *MIF* promoter to positively regulate constitutive promoter transcriptional activity in human monocytic (THP-1), epithelial (HeLa and A549) and keratinocytic (HaCat) cell lines and in peripheral blood mononuclear cells (PBMCs). The CRE and Sp1 sites also cooperate to mediate microbial product-induced *MIF* gene expression in monocytic cells (66). The CRE site has also been reported to relay glucocorticoid-induced *MIF* gene

expression in CEM-C7 T cells (54). Altogether, these studies indicate that DNA regulatory elements surrounding the transcriptional start site play a central role in controlling *MIF* gene transcription. Yet, several lines of evidence suggest that more distant regulatory elements also may have a functional role (**Figure 5.1**). For example, disruption of a distal consensus GR element (GRE at -742) completely abolished glucocorticoid-inducible *MIF* promoter activity (54). Moreover, NF-kB recruitment to putative kB sites at -2538 or -1389 trans-activates *MIF* promoter in response to IL-1b and TNF in endometrial cells (42, 43).

The *MIF* gene contains two major functional polymorphisms, a five to eight CATT tetranucleotide repeat at -794 (67) and a G/C single nucleotide polymorphism at -173 (68) (**Figure 5.1**). These polymorphisms have been reported to modulate *MIF* promoter activity, to correlate with MIF expression levels, and to be associated with the susceptibility to or the outcome of infectious, inflammatory, autoimmune and neoplastic diseases as discussed in other chapters of this book. The exact mechanisms whereby these polymorphisms affect *MIF* gene transcriptional activity remain poorly understood. The -173*C SNP creates a putative AP-4 DNA binding site (68). HMG box-containing protein 1 (HBP1), a known negative regulator of tumorigenesis, has recently been proposed to inhibit *MIF* gene transcription in prostate cancer cells by interacting with a sequence (-811/-792) covering five CATT repeats (69).

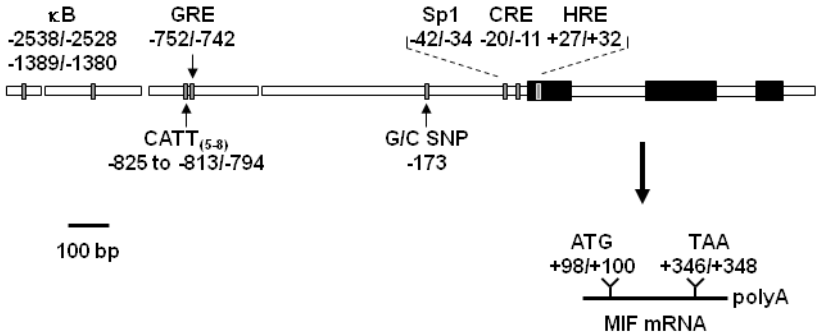

Figure 5.1. Structure of the *MIF* gene and MIF mRNA. The three *MIF* exons are represented by black boxes. The κB, glucocorticoid response element (GRE), specificity protein 1 (Sp1), cAMP-responsive element (CRE) and hypoxia-inducible factor response element (HRE) that have been functionally characterized are depicted by grey boxes. Their localization is relative to the transcriptional start site set at +1. Vertical arrows indicate the positions of the $CATT_{5-8}$ tetranucleotide microsatellite and the -173*G/C single nucleotide polymorphism (SNP). The translational start codon (ATG at +98/+100) and stop codon (TAA at 346+/+348) are pinpointed in the MIF mRNA.

5.6 Epigenetic control of MIF expression

5.6.1 The MIF promoter is not methylated

The human *MIF* promoter contains numerous CpG sites typically found in the proximal promoter of housekeeping genes (**Figure 5.2**). Two CpG sites are part of the proximal Sp1 and CRE binding sequences implicated in basal *MIF* promoter activity (66). To test whether CpG methylation affects *MIF* gene expression in THP-1, HeLa, A549 and HaCat cell lines, we sequenced the proximal *MIF* promoter region using sodium bisulfite-treated genomic DNA, in which 5-methyl-cytosines are protected from bisulfite-induced conversion to uracils. These analyses focused on 34 CpG sites confined in a region extending from position -300 to +1. Only two methylated cytosines located at -211 and -121 were detected in one out of five sequences in HaCat keratinocytes and THP-1 monocytic cells. Thus, the proximal *MIF* promoter is essentially not methylated in cell lines of different origins (70). Given that hypomethylation of the *MIF* promoter could account for higher MIF mRNA expression in tumor cells compared to normal cells, we analyzed CpG methylation in primary cells by bisulfite DNA sequencing. Two methylated cytosines located at -65 and -28 were detected among 12 sequences (408 CpG sites analyzed) obtained from PBMCs isolated from three healthy subjects. Altogether, these data indicate that methylation of CpG sites within the proximal *MIF* promoter is a very rare event and does not account for increased MIF expression in tumor cell lines.

Figure 5.2. The *MIF* gene is located in a cytosine guanine dinucleotide (CpG) island. *In silico* analysis of CpG sites (vertical lines) within the human *MIF* gene. *MIF* exons are depicted by grey boxes.

5.6.2 Inhibition of DNA methyl transferases (DNMTs) does not affect *MIF* gene expression

CpG island shores that refer to regions of lower CpG density close to CpG islands are subjected to methylation (71). Moreover, methylation of CpG sites located in coding sequences or distant from transcriptional start sites have been reported to affect gene expression (72, 73). We thus

explored whether methylation of CpG sites outside the proximal *MIF* promoter influences *MIF* gene expression. As illustrated in **Figure 5.3**, treatment of epithelial (A549 and HeLa), keratinocytic (HaCat) and myeloid (HL-60, KG1a, U-937, THP-1) cell lines with 5-Aza-CdR does not alter MIF mRNA expression. Similar results were obtained with bone-marrow derived macrophages (66). This groundwork argues against a role for DNA methylation as an epigenetic mechanism affecting *MIF* gene expression in tumor cell lines.

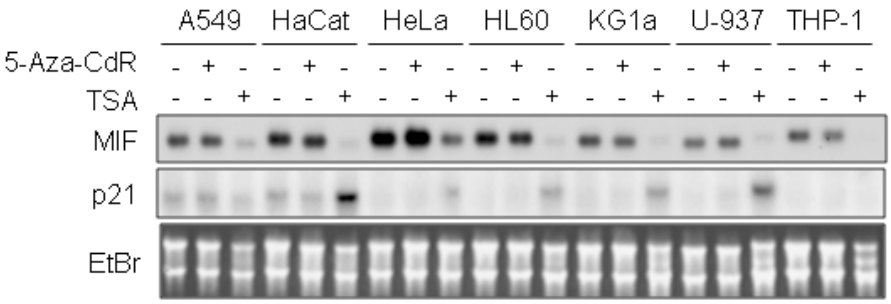

Figure 5.3. Inhibition of histone deacetylases (HDACs), but not of DNA methyl transferases (DNMTs), inhibits MIF mRNA expression. A549, HaCat, HeLa, HL60, KG1a, U937 and THP1 cells were cultured for 18 hours with or without 5-aza-2'-deoxycytidine (5-Aza-CdR at 5 µM), an inhibitor of DNA methyl transferases or trichostatin A (TSA at 1 µM), an inhibitor of histone deacetylases (HDACs). MIF and p21 (CDKN1A) mRNA expression was analyzed by northern blotting. EtBr: ethidium bromide.

5.6.3 Inhibition of histone deacetylases (HDACs) impairs *MIF* gene expression

Dysregulated HDAC activity contributes to abnormal gene expression in tumors. Considering that MIF is overexpressed in tumor cells and that MIF levels correlate with tumor aggressiveness and metastatic potential (59, 74, 75), HDACs were attractive candidate molecules regulating *MIF* gene expression.

Trichostatin A (TSA), a natural hydroxamic acid that has inspired the design of many synthetic HDAC inhibitors including SAHA (76), is widely used to assess the role of HDACs *in vitro* and *in vivo*. Valproic acid (VPA) is a chemically unrelated HDAC inhibitor used to treat epileptic seizures and bipolar disorders. TSA, SAHA and VPA powerfully inhibited MIF mRNA expression in a time- and dose-dependent manner in a panel of tumor cell lines (A549, HeLa, HaCat, HL-60, KG1a, U-937 and THP-1), in B16 melanoma and in primary macrophages (**Figure 5.3** and (70)). The inhibitory effect of TSA on *MIF* expression was specific as TSA reactivated the expression of the *CDKN1A* gene (encoding for the p21/WAF cell-cycle inhibitor). TSA reduced MIF protein expression

in cell lines, whole blood and in the circulation of mice injected with TSA (70). Altogether, these data suggest that HDACs are important regulators of *MIF* gene expression.

Combinatorial epigenetic therapies associating DNMT inhibitors and HDAC inhibitors have been shown to exert additive and synergistic clinical effects in patients with hematologic malignancies (77-79). In HeLa, HL60 and THP-1 cells, the association of 5-Aza-CdR and TSA does not amplify TSA-mediated inhibition of MIF mRNA expression (Roger et al., unpublished data), in line with the observation that the MIF promoter is hypomethylated in tumor cells.

MIF is overexpressed in multiple types of tumors and it promotes malignancies by increasing survival, proliferation and migration of tumor cells (80-82), by promoting angiogenesis (74, 83, 84) and by altering antitumor adaptive immune responses (75, 85-87). This led us to speculate that MIF might be a common target of the anticancer activity of HDAC inhibitors. Similarly, considering that MIF is a central mediator of the pathogenesis of sepsis (45, 47, 55, 88), arthritis (89), colitis (90) and lupus (91), we propose that the benefit afforded by HDAC inhibitors in inflammatory and autoimmune diseases may be related to the inhibition of MIF expression.

5.6.4 Mechanisms by which HDAC inhibitors inhibit *MIF* gene expression

Studies initiated to unravel the molecular mechanisms by which HDAC inhibition affects *MIF* gene expression revealed several unique features. Nuclear run-on assays demonstrated that TSA inhibits *MIF* gene transcription. Surprisingly however, chromatin immunoprecipitation analyses showed that TSA deacetylates histones H3 and H4 associated with the proximal *MIF* promoter (70). This observation was unexpected given that HDAC inhibitors increased overall histone acetylation. Yet, genome-wide expression studies have established that HDAC inhibition impacts on a minority of the transcriptome (2-10%) with similar proportions of genes up-regulated and down-regulated. Moreover, local hypoacetylation following HDAC inhibition have been previously observed within the *High-mobility-group A2* and *BCL2* genes (92, 93).

Because TSA reduced the acetylation of MIF promoter-associated histones, we hypothesized that the MIF promoter was less accessible to the transcription machinery. Indeed, chromatin immunoprecipitation experiments confirmed that TSA impairs the binding of Sp1, CREB and RNA

polymerase II to the proximal MIF promoter. Yet, challenging the concept that hypoacetylated chromatin forms a compact structure less accessible to transcriptional regulators, accessibility studies revealed that the proximal region of the *MIF* promoter was accessible in cells cultured either with or without TSA (70). Blocking protein synthesis with cycloheximide increased early on MIF mRNA expression without modifying the acetylation of histones associated with the *MIF* promoter in cells treated with TSA. Therefore, we assume that TSA repressed *MIF* gene transcription through *de novo* protein synthesis.

5.7 Conclusions

The biological activities ascribed to MIF over the last 20 years put this cytokine as a central mediator of cell proliferation and survival, angiogenesis and inflammatory and immune responses. In line with these observations, pre-clinical and clinical studies suggest that MIF represents a therapeutic target for the treatment of immune-related and neoplastic diseases. While our understanding of MIF biology has improved markedly in recent years, little is known about the mechanisms controlling *MIF* gene expression. Very few studies have characterized the DNA-binding elements and cognate transcription factors regulating basal and stimulus-induced *MIF* transcription. Much less is known about the role of epigenetics in regulating *MIF* expression. Most recent data suggest that HDAC activity, but not DNA methylation, strongly impacts on *MIF* transcription. We speculate that the powerful inhibition of MIF expression by HDAC inhibitors contributes, at least in part, to the antitumorigenic and anti-inflammatory activities of these drugs. Further work will be required to more deeply decipher the genetic and epigenetic mechanisms controlling MIF expression in health and diseases. Besides increasing our knowledge on the biology of MIF, these studies may help developing novel MIF-directed intervention strategies for diseases associated with dysregulated MIF expression.

Acknowledgments

The authors are supported by the Swiss National Science Foundation (310000-114073, 310030-118266 and 310030-132744), the Bristol-Myers Squibb Foundation, the Leenaards Foundation and the Santos-Suarez Foundation for Medical Research.

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6 CAMBINOL, AN INHIBITOR OF SIRTUIN1 AND SIRTUIN2 DEACETYLASES, INHIBITS INNATE IMMUNE RESPONSES

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6.1 Introduction

6.1.1 Sirtuins, the class III HDACs

SIR (silent information regulator, initially described as MAR1 (mating-type regulator1) (1)) enzymes were first discovered in the late 70s and early 80s in yeast as proteins responsible for the silencing of the mating type loci and telomeres. Twenty years later five human cDNAs with homology to yeast Sir2 gene were characterized (2). Then yeast Sir2 was shown to exhibit a deacetylase activity that was dependent on oxidized nicotinamide adenine dinucleotide (NAD^+) (3). The substrates of the reaction are NAD^+, H_2O and an acetyl-protein that are transformed by a sirtuin into nicotinamide (NAM), 2'-O-acetyl-ADP-ribose and deacetylated protein (**Figure 6.1A**). Sirtuins also possess a second catalytic activity that is mono-ADP-ribosyl transferases (ART) where NAD^+ and a protein are transformed into nicotinamide and an ADP-ribosylated protein (**Figure 6.1B**). Mammalian Sir2, called SIRT (sir two like proteins) or sirtuins, are classified as class III HDACs due to their deacetylase activities. SIRT1-7 are distinguished according to their sub-cellular localization as well as their enzymatic activities (**Table 6.1**) (4). SIRT1 and SIRT6 are predominantly found in the nucleus, SIRT2 is cytosolic, SIRT3-5 are found in the mitochondria and SIRT7 is a nucleolar protein. Moreover, SIRT1-2 are able to shuttle between cytoplasm and nucleus and SIRT3 between mitochondria and nucleus (5). All sirtuins except SIRT4 and SIRT7 possess NAD^+-dependent deacetylase activities, and all sirtuins except SIRT5 and SIRT7 have a ART activity (6). The biological role of ART activity remains poorly understood. Like for class I, II and IV HDACs, substrates of sirtuins comprise acetylated histones. However the vast majority of their targets are non-histone proteins involved in a variety of cell functions as explained in the following chapter.

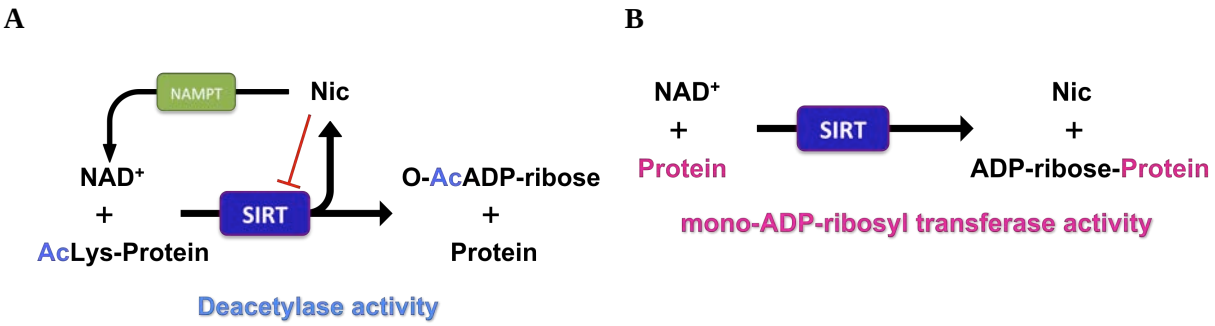


Figure 6.1. Schematic representations of sirtuin enzymatic activities. (**A**) Deacetylase activity transforms NAD^+ and a protein acetylated on a lysine residue into nicotinamide, which inhibits deacetylase activity, 2'-O-acetyl-ADP-ribose as a by-product and a deacetylated protein. (B) Nicotinamide can be recycled into NAD^+ by NAMPT enzyme. Mono-ADP-ribosyl-transferase activity engages NAD^+ and a protein and releases nicotinamide while ADP-ribosylating the protein. NAD^+, oxidized nicotinamide adenine dinucleotide; NAMPT, nicotinamide phosphorybosyltransferase; Nic, nicotinamide.

6.1.2 Targets of sirtuins

SIRT1 is the most documented sirtuin and the list of its targets is constantly growing. SIRT1 is a *bona fide* HDAC that possesses the capacities to deacetylate histone tails on H3K9 and H4K16, to promote the recruitment of histone H1 and its deacetylation on K26 and overall to favor heterochromatin formation and gene silencing (7). The tumor suppressor p53 has been described as a target of SIRT1. Deacetylation of p53 K382 by SIRT1 decreases its transcriptional activity (8). p53 deacetylation by SIRT1 has also been shown to promote cell survival and to decrease apoptosis in response to DNA damage and oxidative stress (9). Premature cellular senescence induced by PML (promyelocytic leukemia protein)-dependent acetylation of p53 is also antagonized by SIRT1 (10). In addition, SIRT1-deficient mice exhibit a hyperacetylated form of p53 and severe developmental heart defects (11). Besides histones and p53, SIRT1 deacetylates numerous proteins involved in the regulation of metabolism such as PGC1α, FOXO transcription factors, the nuclear liver X receptors (LXRs), Acetyl-CoA synthase 1 (AceCS1) and the transcription factors NF-κB p65, STAT3, c-Jun and FOXP3 (12-14).

SIRT1 and SIRT2, acting in couple with CBP/p300, regulate of the acetylation of histone H3 on H3K56 (15,16). SIRT2 deacetylates H4K16 during the cell cycle and has been proposed to favor the condensation of chromatin at the G_2/M transition (17,18). SIRT2 colocalizes on microtubules and cooperates with HDAC6 to deacetylate α-tubulin on lysine 40 (19). SIRT2 was also found to

deacetylate FOXO transcription factors, thereby inhibiting the differentiation of adipocytes (20). FOXO3α is deacetylated by SIRT2 in response to caloric restriction and oxidative stress (21).

Mitochondrial sirtuins modulate the activitiy of metabolic enzymes *via* their deacetylation (SIRT3 and SIRT5) and ADP-ribosylation (SIRT4) activity (22). SIRT3 deacetylates acetyl-coenzyme A synthase 2 (AceCS2) that converts acetate to acetyl-coA. AceCS2 deacetylated by SIRT3 has an increased activity and thus plays a positive role on energy production by promoting acetyl-coA synthesis, the carbon donor used in citric cycle. SIRT3 also interacts with proteins of the complex I of the electron transport chain, reducing ATP production in $Sirt3^{-/-}$ mice. SIRT3 deacetylates H3K56 *in vivo* without affecting global acetyl-H3K56 levels. Thus, SIRT3 probably does not regulate the expression of large portions of the genome (16). SIRT4 ADP-ribosylates and inhibits the function of glutamate dehydrogenase (GDH), an enzyme responsible for the conversion of glutamate in α-ketoglutarate and ammonia. Of note, regulation of GDH is crucial for the control of insulin secretion. SIRT5 deacetylates and activates carbamoyl phosphate synthase 1 (CPS1) a rate-limiting enzyme in the urea cycle and thus indirectly plays a positive role in the amino acid catabolism (22).

SIRT6 was described as a histone H3K9 deacetylase responsible for telomeric chromatin modulation. SIRT6-depleted cells exhibit abnormal telomeric structures, leading to the proposition that SIRT6 participates in telomere maintenance during aging (23). By deacetylating H3K9 on κB promoters, SIRT6 attenuates the occupancy of p65 and transactivation by NF-κB. SIRT6-deficient cells have hyperacetylated H3K9 on κB promoters and enhanced expression of NF-κB-dependent genes (24).

Nucleolar SIRT7 associates with actively transcribed ribosomal RNA (rRNA) genes and interacts with RNA polymerase I. Overexpression of SIRT7 increases RNA polymerase I transcription whereas knockdown of SIRT7 and inactivation of SIRT7 enzymatic activity by mutation or chemical inhibition with nicotinamide decrease transcription of rRNA genes (25).

Table 6.1. Mammalian sirtuins, their subcellular localization, enzymatic activitiy, substrate and biological function. Adapted from Taylor *et al.* 2008 (6) and Haigis & Sinclair, 2010 (4).

Sirtuin	Subcellular localization	Enzymatic activities	Substrates	Biological Functions	Phenotypes associated with knockout mice
SIRT1	Nucleus	Deacetylase	Histones H1(K26), H3(K9, K14), H4(K16), H2A.Z, p53, NF-κB p65, FOXOs, FOXP3, c-JUN, c-FOS, p300/CBP, PGC1-α, HIV tat…	Glucose production, fatty-acid oxidation, cholesterol regulation, fatty-acid mobilization, adipokine regulation, fatty-acid oxidation, insulin secretion, neuroprotection, regulation of cellular differentiation, stress resistance, apoptosis control, mediator for caloric restriction	Perinatal death, retinal, bone and cardiac defects
SIRT2	Cytoplasm	Deacetylase, mono-ADP-ribosyl transferase	α-tubulin, H3(K14), H4(K16), p53, FOXOs	Tubulin deacetylation, cell cycle control	Developmentally normal
SIRT3	Mitochondria	Deacetylase	AceCS2, GDH, electron transport chain Complex I	Mitochondrial protein deacetylation, acetate metabolism regulation, ATP production, regulation of mitochondrial fatty-acid oxidation	Developmentally normal, change in AceCS2 activity and ATP levels, elevated mitochondrial proteins acetylation
SIRT4	Mitochondria	Mono-ADP-ribosyl transferase	GDH	Amino acid-stimulated insulin secretion	Appear healthy, increased mitochondrial GDH activity
SIRT5	Mitochondria	Deacetylase	CPS1, cytochrome c	Urea cycle regulation	Defect in the urea cycle
SIRT6	Nucleus	Deacetylase, mono-ADP-ribosyl transferase	H3(K9), NF-κB p65	Base excision repair, telomeric chromatin structure, NF-κB regulation	Progeroid syndrome, profound hypoglycaemia, death at four weeks
SIRT7	Nucleus	ND	RNA polymerase I	Pol I transcription	Reduced lifespan, cardiomyopathy

6.1.3 Sirtuins, longevity and cancer and age related diseases

The NAD^+ requirement for the enzymatic activity of sirtuins suggested that these enzymes have evolved to sense energy and redox states coupled to the metabolic status of the cell. As studies initiated in the 30s had shown that calorie restriction extend lifespan, sirtuins gained broad interest to the scientific community when Kaeberlein *et al.* demonstrated in 1999 that an extra copy of the *SIR2* gene increased lifespan of *S. cerevisiae* whereas deletion of *SIR2* conferred the opposite phenotype (26). In yeast, fly and mammals, Sir2 expression increases under caloric restriction. Remarkably, transgenic mice overexpressing SIRT1 developed a calorie restriction phenotype characterized by reduced body weight, fat mass and blood cholesterol levels (27). Altogether, these studies suggest a connection between SIRT1, calorie restriction, metabolism and longevity.

These findings generated a considerable interest in the development of SIRT1 modulators, leading to the identification by high-throughput screening of the SIRT1 activator resveratrol, a plant

polyphenol (28). Resveratrol was known to exert anticancer, cardioprotective, anti-inflammatory, antidiabetic and neuroprotective effects (29). Yet, the connection between the pharmacological effects of resveratrol and SIRT1 activation is controversial. Whether resveratrol works through regulation of sirtuin activity remains questionable. In fact, most recent work suggests that resveratrol does not directly enhance the catalytic activity of SIRT1 (30). Additional screenings identified small molecules activator of SIRT1, which improved insulin sensitivity, lowered plasma glucose and increased mitochondrial biogenesis in genetically- and diet-induced obese rodents (31). Some of these activators are currently being evaluated in phase II clinical trials in patients with metabolic diseases. Overall, SIRT1 activation is viewed as an attractive therapy for treating metabolic disorders such as obesity and type II diabetes. Beside SIRT1, SIRT2-4 have been implicated in metabolic processes (6,32-34).

It is commonly assumed that sirtuins have a positive impact on cardiac and neuronal functions. The role of sirtuins in cancer is more debatable. SIRT1 was shown to either inhibit or promote cell apoptosis by targeting p53, p73, E2F1, FOXOs and NF-κB p65. SIRT1 can act both as a tumor promoter and a tumor suppressor, depending on the model studied. Adding a level of complexity, SIRT2, SIRT5 and SIRT7 deacetylate p53. SIRT1-3 and SIRT7 are overexpressed in tumors whereas SIRT2 and SIRT4 are down-regulated in gliomas and myeloid leukemia cells (34-37). Yet, there is up to now no direct correlation between sirtuin activity and a particular type of cancer. Recent studies suggest that SIRT2 protects the integrity of the genome during mitosis and consequently prevents cancer development (38). SIRT2 has been shown to play a role in neurodegenerative disease and its inhibition to have neuroprotective effects against anoxic injury (39). Interestingly, SIRT2 deacetylates α-tubulin, a process that may contribute to increase the efficacy of tubulin poisoning drugs.

6.1.4 Sirtuins and inflammatory disorders

Little is known on the role of sirtuins in regulating inflammatory and immune responses. A correct balance in glucose metabolism is mandatory for adequate immune responses as both hypo- and hyperglycemia are detrimental to immune-cells functions (40). Nicotinamide phosphoribosyltransferase (NAMPT), also called pre-B cell colony enhancing factor (PBEF) or visfatin, functions as the rate-limiting enzyme in the synthesis of NAD. Thus, NAMPT directly

impacts on the activity of NAD-dependent enzymes such as sirtuins. NAMPT is secreted and found to be upregulated in several acute and chronic inflammatory disorders and consequently strongly links NAD metabolism to inflammation (41). Van Gool *et al.* reported that inhibition of NAMPT impairs TNF mRNA translation and TNF biosynthesis by macrophages and DCs in a SIRT6-dependent manner (42). In the same line, Bruzzone *et al.* showed that the NAMPT inhibitor FK866 causes NAD^+ depletion in phytohemagglutinin (PHA)-activated T cells and drastically decreases cell proliferation and release of IFNγ and TNF, probably through the inactivation of SIRT6. In agreement, $Sirt6^{-/-}$ splenocytes secrete less IFNγ in response to concanavalin A (43). FK866 exhibits beneficial effects in experimental models of autoimmune encephalomyelitis (43), collagen-induced arthritis and endotoxemia (44).

MRL/*lpr* mice that spontaneously develop systemic lupus displayed high levels of SIRT1 in $CD4^+$ T cells. Moreover the level of anti-dsDNA antibodies and renal pathological scores are decreased in MRL/*lpr* mice treated with Sirt1 siRNA (45). These results clearly point towards a role of SIRT1 in the pathogenesis of lupus. Sirtinol, an inhibitor of SIRT2 and to a lesser extent SIRT1 decreases airway inflammation and hyper responsiveness in a model of ovalbumin-induced allergic airway disease through an inhibition of the SIRT1/HIF1α/VEGF axis (46). In a model of rat lung trauma-hemorrhage, sirtinol reduces TNF and IL-6 levels and attenuates tissue damage in the lung (47). SIRT1 was also shown to stimulate HIF2α transactivation capacity by a direct interaction during hypoxia both in cultured hepatoma Hep3B cells and in living animals (48). SIRT1 downregualtion is involved in the acute metabolic decline observed in murine peripheral blood leukocytes (PBL) and liver cells of LPS-challenged mice. During the recovery phase, SIRT1 expression returns to normal levels and metabolism to homeostasis. In the liver of $SIRT1^{-/-}$ mice, metabolism also drops following LPS-challenge, but the return to homeostasis is drastically delayed (49). High levels of NAD^+ are associated with the recruitment of SIRT1 and RelB at the TNF promoter during endotoxin tolerance, thereby favoring gene silencing (50). In addition, SIRT1 has been described as a central regulator of autophagy (51) that is known to play key roles in adaptive and innate immunity and to promote effector functions during infection (52). Finally, SIRT1 also regulates the activity of FOXO

transcription factors that regulates innate immune homeostasis in *Drosophila* (53). All these studies argue in favor of a pro-inflammatory role for sirtuins and that sirtuin inhibitors may have potential for the treatment of inflammatory and immune-related disorders.

Unexpectedly however, several studies have reported an anti-inflammatory role for SIRT1. SIRT1 expression is decreased in rats exposed to cigarette smoke extracts and in smoker patients with chronic obstructive pulmonary diseases. SIRT1 expression decreases in monocyte/macrophages and is associated with an increase of both expression and acetylation of NF-κB p65 and increased production of pro-inflammatory mediators (54-56). The reasons why sirtuins promotes both as pro- and anti-inflammatory recations are obscure and remain to be clarified. The discrepancies may originate from the different experimental settings, such as energetic status, timing or tissue and cell-type specific sirtuin expression (57). Of note, there is currently no information available on the role of sirtuins in host response to microbial infections and their possible implication in the pathophysiology of sepsis.

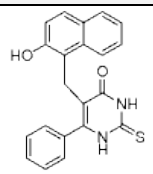
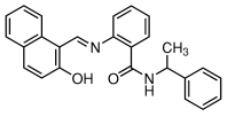
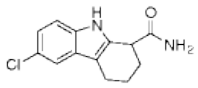
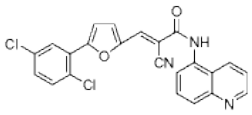
6.1.5 Small molecules inhibitors of Sirtuin activities

The structural differences of the active site of HDAC1-11 and sirtuins render sirtuins insensitive to the action of inhibitors of class I, II and IV HDACs. Several sirtuins inhibitors have been developed (**Table 6.2**). Sirtinol, identified by yeast cell-based phenotypic screening, is more potent towards SIRT2 (IC_{50} 38μM) than SIRT1 (IC_{50} 131μM), but its effects on the other human sirtuins are not known (58,59). Cambinol is a chemically stable sirtuin inhibitors related to splitomicin, a molecule inhibiting Sir2 and Sir2 homologues in yeast (60). Cambinol was identified as a sirtuin inhibitor that competes with the substrate and not with NAD^+, thus cambinol does not interfere with other NAD^+ binding enzymes. Cambinol inhibits SIRT1 (IC_{50} 56 μM), SIRT2 (IC_{50} 59 μM) and, less potently, SIRT5 (IC_{50}>300 μM) (61). Cambinol has antitumor activity in preclinical models, which results at least from an increased acetylation of p53 (62). Furthermore, cambinol-induced inhibition of SIRT1 sensitizes cells to chemotherapeutic drugs (61).

Recently specific inhibitors of SIRT1 and SIRT2 have been developed (**Table 6.2**). EX-527 was identified by high-throughput screen using bacterially expressed human SIRT1 and its activity on

mammalian cells has been confirmed *in vitro* using the Fluor-de-Lys deacetylation assay (63). EX-527 is a selective and highly potent (IC_{50} 98 nM) SIRT1 inhibitor which promotes p53 acetylation (63). AGK2 was identified by *in vitro* screening using a fluorometric assay on a library of structural analogues of known SIRT2 inhibitors. AGK2 is a strong inhibitor of SIRT2 (IC_{50} 3.5 µM) that has a weak effect on SIRT1 (IC_{50} > 50 µM) and SIRT3 (IC_{50} > 50 µM) activity. In a cellular model of Parkinson's disease, AGK2 reduces α-synuclein toxicity. The protective effect is associated with increased acetylation of tubulin (64). The development of specific inhibitors will allow fine temporal inhibition of sirtuins in a variety of experimental systems and a better comprehension of sirtuin function. Unfortunately, despite great efforts, no inhibitor targeting *in vivo* only one specific sirtuin isoform has yet been obtained (58).

Table 6.2. Structure and IC_{50} of selected sirtuins inhibitors. Adapted from Cen *et al., Biochimica et Biophysica Acta, 2009* (58).

Compound	Cambinol	Sirtinol	EX-527	AGK2
				
IC_{50} SIRT1	56 µM	131 µM	98 nM	>50 µM
IC_{50} SIRT2	59 µM	38 µM	Not determined	3.5 µM

6.1.6 Rational for the use of sirtuin inhibitors as anti-inflammatory agents

Depletion of NAD^+ by FK866 has been proposed to decrease TNF and IFNγ secretion by T lymphocytes *via* an inhibition of of SIRT6 activity (43) and to reduce inflammatory parameters in rheumatoid arthritis (44). Morover, we reported in **chapter 2** that inhibition of class I and II HDACs protects animal from toxic shock and severe sepsis (65). As sirtuins share many substrates with HDAC1-11, we hypothesized that sirtuins can play a role in the modulation of innate immune responses. To verify our hypothesis, we tested whether pharmacological inhibition of SIRT1 and SIRT2 modulate innate immune responses to microbial products and infections *in vivo* and *in vitro*.

6.2 Material and Methods

6.2.1 Ethics statement

All animal procedures were approved by the Office Vétérinaire du Canton de Vaud (authorizations n°876.6 and 877.6) and performed according to our institution guidelines for animal experiments.

6.2.2 Mice, cells and reagents

Eight to 12-week-old female BALB/c mice (Charles River Laboratories) were housed under specific pathogen-free conditions and were acclimatized for at least one week before experimentation. Mouse bone marrow precursors were cultured in Iscove modified Dulbecco medium (IMDM), 50 µM 2-mercaptoethanol (βME) and 30% of filtered L929 cells supernatant to obtain bone marrow-derived macrophages (BMDMs). Splenocytes were cultured in RPMI medium containing 2 mM L-glutamine and 50 µM βME. Human whole blood assay was performed as previously described (66). Human PBMCs were isolated from buffy coats of healthy blood donnors using a Ficoll-Paque Plus (GE Healthcare) gradient centrifugation procedure. Mouse RAW 264.7 macrophages (ATCC TIB-71) were cultured in RPMI 1640 medium containing 2 mM L-glutamine. All media contained 10% heat-inactivated fetal calf serum (Amimed, Bioconcept), 100 UI/ml penicillin and 100 µg/ml streptomycin.

Trichostatin A, cambinol and DMSO were from Sigma-aldrich. Sirtinol, EX-527 and AGK2 were from Tocris Bioscience. Microbial products were: *Salmonella minnesota* ultra pure LPS (List Biologicals Laboratories), Pam_3CSK_4 lipopeptide (EMC microcollections), CpG motif containing oligonucleotide (Invivogen) and heat-inactivated *Escherichia coli* (*E. coli*) O18, *Klebsiella pneumonia caroli* (*K. pneumonia*), *Staphylococcus aureus* (*S. aureus*) and *Candida albicans* (*C. albicans*).

6.2.3 RNA analyses by real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using the RNeasy kit (QIAGEN). Reverse transcription of 100 ng to 1µg was performed using the QuantiTect reverse transcription kit (QIAGEN). Relative real-time PCR was performed with a 7500 FAST Real-Time PCR System using Syber FAST mix (Applied Biosystems) and specific primer pairs (**Table 6.3**). Amplifications consisted of 40 cycles of a

denaturation step at 95°C for 3s and an annealing/extension step at 60°C for 30s, with the 7500 Fast mode. Samples were tested in duplicates. mRNA expression was expressed in arbitrary units relative to mRNA expression in untreated cells and normalized with an endogenous control (*Hprt* or *Gapdh*).

Table 6.3. Primers used in RT-PCR

Gene name	Forward	Reverse
Cd40	AGGTTTAAAGTCCCGGATGC	CCTTTGGTTTCTTGACCACCT
Gapdh	CTCATGACCACAGTCCATGC	CACATTGGGGGTAGGAACAC
Hprt	GTTGGATACAGGCCAGACTTTGTTG	GATTCAACTTGCGCTCATCTTAGGC
Ifng	TGAGTATTGCCAAGTTTGAGGTCA	CGGCAACAGCTGGTGGAC
Il1b	TGAAGTTGACGGACCCCAAA	TGATGTGCTGCTGCGAGATT
Il2	TTTGAGTGCCAATTCGATGA	AGGGCTTGTTGAGATGATGC
Il6	AGCAGTAGCAGTTCCCCTGA	AGTCCCTTTGGTCCAGTGTG
Il12b	GGAAGCACGGCAGCAGAATA	AACTTGAGGGAGAAGTAGGAATGG
Irf8	GGGTCCAGAGCAGCTACAAG	TGTCGACCCTGTCTGTTGAG
Klf4	CCAAAGAGGGGAAGAAGGTC	CTGTGTGAGTTCGCAGGTGT
Ly6c1	GCCAATCAGGGATCCTAACA	AGCTCAGGCTGAACAGAAGC
Maf	AAGGAGGAGGTGATCCGACT	TCTCCTGCTTGAGGTGGTCT
Mafb	CATCACCATCATCACCAAGC	AGAAGCGGTCCTCCACACTA
Md2	CAACTCCTCCGATGCAATTA	GGCACAGAACTTCCTTACGC
Nlrp3	CTTCTCTGATGAGGCCCAAG	GCAGCAAACTGGAAAGGAAG
Nos2	CTCTGACAGCCCAGAGTTCC	GAAAGGGAGAGAGGGGAGG
Sfpi1	CTTCCAGTTCTCGTCCAAGC	TTTCTTCACCTCGCCTGTCT
Tlr1	CAATGTGGAAACAACGTGGA	TGTAACTTTGGGGGAAGCTG
Tlr2	AAGAGGAAGCCCAAGAAAGC	CGATGGAATCGATGATGTTG
Tlr4	ACCTGGCTGGTTTACACGTC	CTGCCAGAGACATTGCAGAA
Tlr6	CAGAACTCACCAGAGGTCCAA	CGAGTATAGCGCCTCCTTTG
Tlr9	ACTGAGCACCCCTGCTTCTA	AGATTAGTCAGCGGCAGGAA
Tnf	CCAGGCGGTGCCTATGTCT	GGCCATTTGGGAACTTCTCAT

6.2.4 Cytokine measurements

Cell culture supernatants or plasma were collected to measure the concentration of cytokines. Mouse TNF, IL-6, IL-1β and IFNγ were measured using DuoSet ELISA kits (R&D Systems). For human whole blood and human PBMC/monocyte supernatants, TNF and IL-6 concentrations were measured using WEHI 164 clone 13 mouse fibrosarcoma cells (TNF) or 7TD1 mouse-mouse hybridoma cells (IL-6) bioassays (67).

6.2.5 Nitric oxide assay

Nitric oxide concentration was measured using dilutions of cell culture supernatants into Griess reagent (sulfanilamide 1%, ethylenediamine dihydrochloride N-(1-naphthyl) 0.1%, phosphoric acid (H_3PO_4) 2.5% in H_2O) and compared to serial dilutions of $NaNO_2$ as a standard. Dilutions of

samples and standard were incubated 10 min at room temperature in a microplate and absorbance was read at 550nm using a Versamax Microplate Reader (Molecular Devices).

6.2.6 Flow cytometry

BMDMs were incubated with 2.4G2 monoclonal antibody (mAb) and CD40 monoclonal antibody (3/23-biotin revealed with CyChrome-conjugated streptavidin; BD Biosciences). Flow cytometric analyses were performed using a FACSCalibur™ flow cytometer and data analyzed using FlowJo 8.8.6 software (Tree Star, Inc.).

6.2.7 Western blot analyses

Cytoslic extracts of RAW 264.7 cells were obtained by lysis for 15 minutes on ice with Hepes 10 mM pH 7.9, KCl 10 mM, EDTA 0.1 M, EGTA 0.1 M, DTT 1 mM, PMSF 2.5 mM, NP-40 0.6%. Cells were centrifugated, supernatants contained cytosolic fraction. Pellets that contained nucleus were further rocked at 4°C in Hepes 20 mM, NaCl 0.4 mM, EDTA 1 mM, EGTA 1 mM, glycerol 10%, PMSF 1 mM, DTT 0.1 mM. Nuclear fractions were centrifuged, supernatants were enriched in nuclear proteins. Extraction buffers were completed with cOmplete Mini protease inhibitor cocktail tablets (Roche) and PhosSTOP phosphatase inhibitor cocktail tablets (Roche). Proteins were quantified using the BCA protein assay (Pierce). Cytosolic and nuclear extracts were electrophoresed through 10-12% polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were incubated overnight at 4°C with antibodies specific for phosphorylated (phospho)-extracellular regulated kinase 1/2 (ERK1/2), total-ERK1/2, phospho-p38, total-p38, phospho-MEK1/2, NF-κB p65, MKP-1, acetyl-H3 (Santa Cruz Biotechnology), and α-tubulin (Sigma). After washing, membranes were incubated for 1 h with secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. Signals were revealed using the ECL Western blotting analyses system (GE Healthcare). To detect histones, acid-soluble proteins were extracted and analyzed by Western blotting as described previously (68) using acetylated histones H3 antibodies (06-755 Millipore).

6.2.8 Cell viability assay

Cells were cultured in duplicates or triplicates in 96-well plates in the presence or absence of drugs and stimuli for 18 h. Cell viability was indirectly assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-fromazan assay (Merck).

6.2.9 *In vitro* microbial growth

K. pneumoniae caroli and *E. coli O18* were cultured overnight at 37°C in LB (*K. pneumoniae*) or TSB (*E. coli*) broth. *C. albicans* was cultured overnight at 30°C under agitation in YEPD broth. The following day, 40 µl of microbial suspension were diluted in 4 ml LB broth or YEPD containing 0, 3.1, 12.5, 50 or 200 µM cambinol and incubated at 37°C under agitation. Optical densities (OD_{640}) were read each hour during 6 h.

6.2.10 *In vivo* models

Klebsiella pneumoniae sepsis: 18-60 CFU of *K. pneumoniae caroli* were injected intra-nasally into mice treated with cambinol (10 mg/kg i.p. once per day for three consecutive days starting 24 h before bacterial challenge). Blood was collected 3 days after infection to quantify circulating bacteria. Body weight and survival of mice were followed at least once daily.

LPS-induced shock: Mice were injected with 350 µg LPS i.p. Cambinol (10mg/kg i.p.) was injected 1 h and 24 h after LPS challenge. Blood was collected 1 h and 6 h after LPS challenge. Body weight and survival of mice were followed at least once daily.

6.2.11 Statistical analysis

Comparisons among treatment groups were performed using 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. The analyses were performed using PRISM (GraphPad Software, La Jolla, CA). All reported *P* values are two-sided and values of less than 0.05 were considered to indicate statistical significance.

6.3 Results

6.3.1 Cambinol inhibits cytokine secretion by BMDMs stimulated with microbial products

To characterize the role of sirtuins in modulating innate immune responses, we first analyzed the ability of sirtuin inhibitors to interfere with the response of BMDMs stimulated with LPS and Pam_3CSK_4. We selected for these studies four inhibitors: cambinol that inhibits SIRT1 and SIRT2 with a similar efficiency, sirtinol that inhibits more efficiently SIRT2 than SIRT1, EX-527 that inhibits SIRT1, and AGK2 that inhibits SIRT2 (**Table 6.2**). BMDMs were pre-incubated for 1 h with inhibitors used at concentrations equivalent to 0.25-, 1- and 4-fold theoretical IC_{50}s and then exposed at LPS and Pam_3CSK_4. Cell culture supernatents were collected after 18 h to quantify TNF, IL-6 and IL-12p40 (**Figure 6.2** and data not shown). Cambinol strongly and dose-dependently inhibited cytokine production by BMDMs. Sirtinol inhibited cytokine production only at the highest concentration (200 µM). Unexpectedly, EX-527 and AGK2 did not inhibit but rather enhanced cytokine production. Importantly, cambinol, EX-527 and AGK2 exhibited marginal cytotoxicity at all concentrations tested, whereas sirtinol at 200 µM reduced BMDM viability (measured using the MTT assay) around two-fold (data not shown). Overall, these results suggest that dual, but not single, targeting of SIRT1 and SIRT2 is required to inhibit TNF, IL-6 and IL-12p40 secretion by BMDMs.

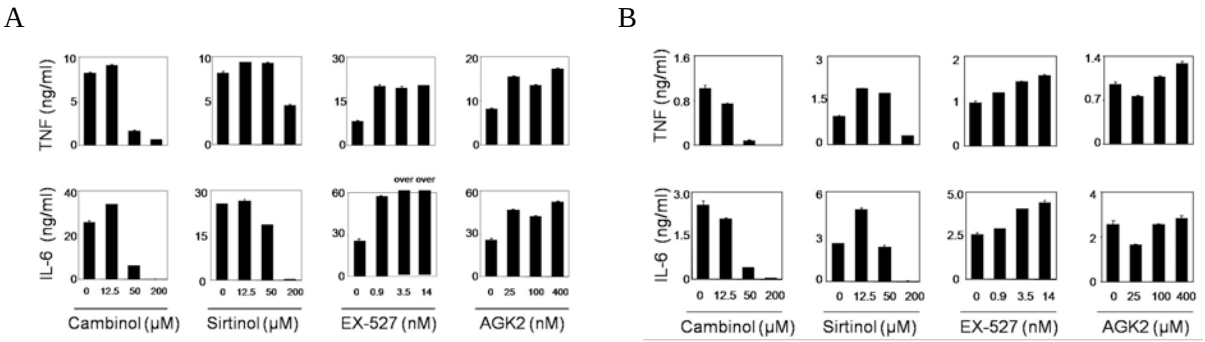


Figure 6.2. Effect of sirtuin inhibitors on TNF and IL-6 production by BMDMs exposed to LPS and Pam_3CSK_4. BMDMs were pre-incubated for 1 h with cambinol, sirtinol, EX-527 and AGK2 prior to stimulation for 18 h with LPS (10 ng/ml) (**A**) and Pam_3CSK_4 (10 ng/ml) (**B**). TNF and IL-6 concentrations in cell-culture supernatants were determined by ELISA. Results are the mean ± SD of triplicate samples from one experiment.

To further determine wether cambinol and sirtinol at non-toxic concentrations (12.5 and 50 µM) could interfere with the response of BMDMs to bacteria and other microbial ligands recognized through TLRs, cells were exposed to *E. coli*, *S. aureus* and to CpG ODN, a TLR9 ligand (**Figure 6.3**). Cambinol efficiently and dose-dependently inhibited TNF and IL-6 secretion triggered by all stimuli. Sirtinol inhibited TNF and IL-6 production induced by *S. aureus* and IL-6 production induced by *E. coli*. As cambinol was the most potent anti-inflammatory compound, we used cambinol in subsequent experiments analyzing the modulation of innate immune and inflammatory responses.

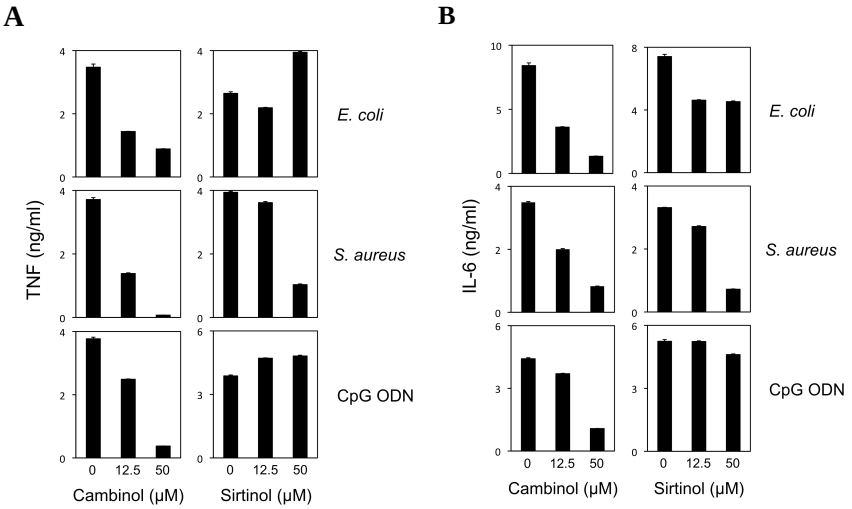


Figure 6.3. Cambinol inhibits TNF and IL-6 production by BMDMs stimulated with bacteria and CpG ODN. BMDMs were pre-incubated for 1 h with cambinol and sirtinol prior to stimulation for 18 h with *E. coli* (10^6 CFU/ml), *S. aureus* ($5x10^6$ CFU/ml) and CpG ODN (2 µg/ml). TNF (**A**) and IL-6 (**B**) concentrations in cell-culture supernatants were determined by ELISA. Results are mean ± SD of triplicate samples from one experiment.

6.3.2 Cambinol inhibits *Tnf*, *Il6* and *Il12b* mRNA synthesis in BMDMs

To define whether cambinol inhibits cytokine production by interfering with gene transcription, *Tnf*, *Il6* and *Il12b* mRNA levels were quantified by RT-PCR in BMDMs pre-treated with cambinol for 1 h and stimulated with LPS or Pam_3CSK_4 for 1, 4 and 18 h (**Figure 6.4** and data not shown). *Tnf* mRNA increased after 1 h of exposition to LPS and Pam_3CSK_4, whereas *Il6* and *Il12b* mRNAs were detected after 4 h of stimulation. Concordant with cytokine secretion, cambinol dose-dependently inhibited LPS- and Pam_3CSK_4-induced *Tnf*, *Il6* ad *Il12b* mRNA up-regulation. These results suggest that cambinol interferes with *Tnf*, *Il6* and *Il12b* transcription.

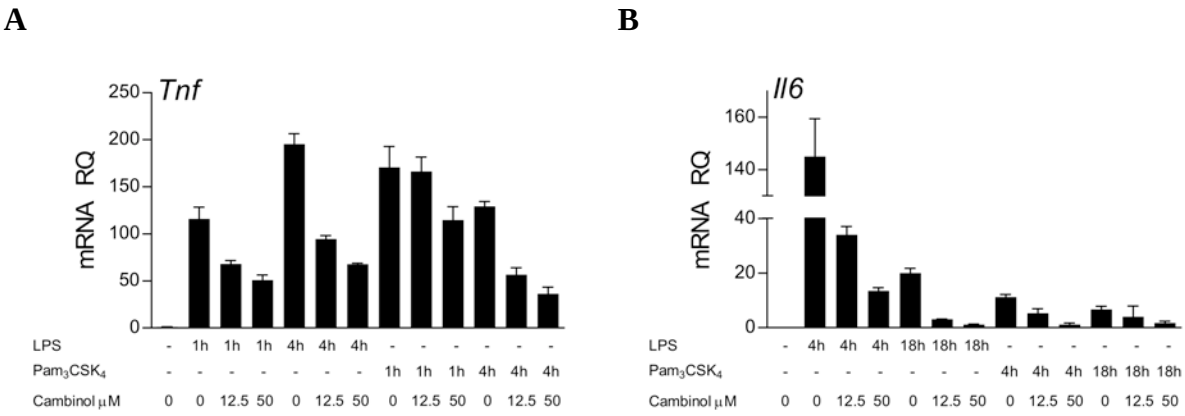


Figure 6.4. Cambinol inhibits LPS- and Pam_3CSK_4-induced *Tnf* and *Il6* mRNA expression in BMDMs. BMDMs were pre-incubated for 1 h with cambinol prior to stimulation for 1, 4 and 18 h with LPS (10 ng/ml) and Pam_3CSK_4 (10 ng/ml). *Tnf* (**A**) and *Il6* (**B**) were quantified by RT-PCR and results expressed as the ratio of cytokine to *Gapdh* mRNA levels. Data are mean ± SD of duplicates and are representative of three independent experiments.

6.3.3 Cambinol inhibits IL-1β production by BMDMs

IL-1β is produced through a multi-step process involving IL-1β mRNA induction and pro-IL-1β synthesis following TLR stimulation, and processing of pro-IL-1β into secreted mature IL-1β through the action of the inflammasome (69). LPS powerfully induced *Il1b* mRNA in BMDMs, a process that was substantially inhibited upon pre-incubation with cambinol (**Figure 6.5A**). To verify that this inhibition affected IL-1β secretion, we measured IL-1β in the cell-culture supernatants of BMDMs primed for 18 h with LPS (to accumulate the pro-form of IL-1β), pre-incubated with or without cambinol and finally exposed for 6 h to octacalcium phosphate (OCP) crystals used as activators of NALP3 inflammasome (70). OCP induced a significant release of IL-1β, which was severely reduced by cambinol (**Figure 6.5B**). These results confirmed the anti-inflammatory activity of cambinol in BMDMs.

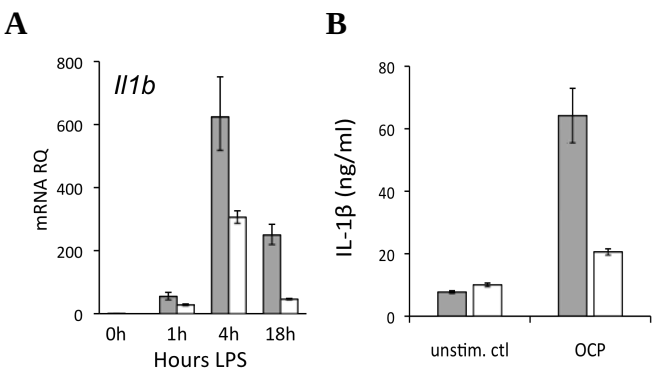


Figure 6.5. Cambinol inhibits IL-1β production by BMDMs. (**A**) BMDMs were pre-incubated for 1 h with cambinol (50 µM) prior to stimulation with LPS (10 ng/ml). *Il1b* and *Gapdh* mRNA levels were quantified by RT-PCR. Data are mean ± SD of duplicate samples. (**B**) BMDMs were primed overnight with LPS (100 ng/ml). Cells were washed, pre-incubated for 1 h with cambinol (50 µM) and stimulated for 6 h with octacalcium crystals (OCP, 500 µg/ml). Secreted IL-1β was quantified by ELISA. White and grey: with and without cambinol pre-incubation, respectively.

6.3.4 Cambinol inhibits NO production by BMDMs

Nitric oxide (NO) is produced during the nitrosative burst through the action of the inducible nitric oxide synthase (iNOS), an enzyme whose expression is induced by inflammatory stimuli. NO plays an important role in the clearing of phagocytosed bacteria and is involved in sustaining the inflammatory response (71). We assessed whether cambinol intereferes with NO production in BMDMs. Whereas LPS, Pam_3CSK_4, CpG ODN, *E. coli* and *S. aureus* promoted NO production by BMDMs primed with IFNγ, cambinol efficiently and dose-dependently decreased NO production (**Figure 6.6**).

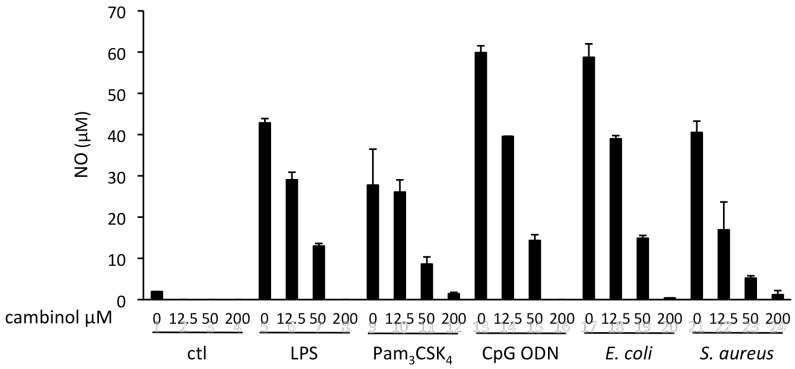


Figure 6.6. Cambinol dose-dependently inhibits the production of nitrites/nitrates by macrophages. BMDMs were primed with IFNγ (100 U/ml, 18 h). Medium was changed and cells were pre-incubated for 1 h with cambinol prior to stimulation for 18 h with LPS (10 ng/ml), Pam_3CSK_4 (10 ng/ml), CpG ODN (2 µg/ml), *E. coli* (10^6 CFU/ml) and *S. aureus* ($5x10^6$ CFU/ml). Nitrite/nitrate concentrations were determined using the Griess reagent. Data are mean ± SD of tripicate samples from one experiment.

6.3.5 Cambinol inhibits CD40 expression by BMDMs

CD40 is a co-stimulatory molecule expressed at the surface of antigen presenting cells (APCs), including macrophages, that mediates interaction with T cells. RT-PCR and flow cytometry analyses showed that *Cd40* mRNA and CD40 membrane expression was strongly up-regulated in BMDMs stimulated with LPS and Pam_3CSK_4 (**Figure 6.7**). Pre-incubation with cambinol powerfully inhibited CD40 up-regulation, suggesting that cambinol may affect APC function of macrophages.

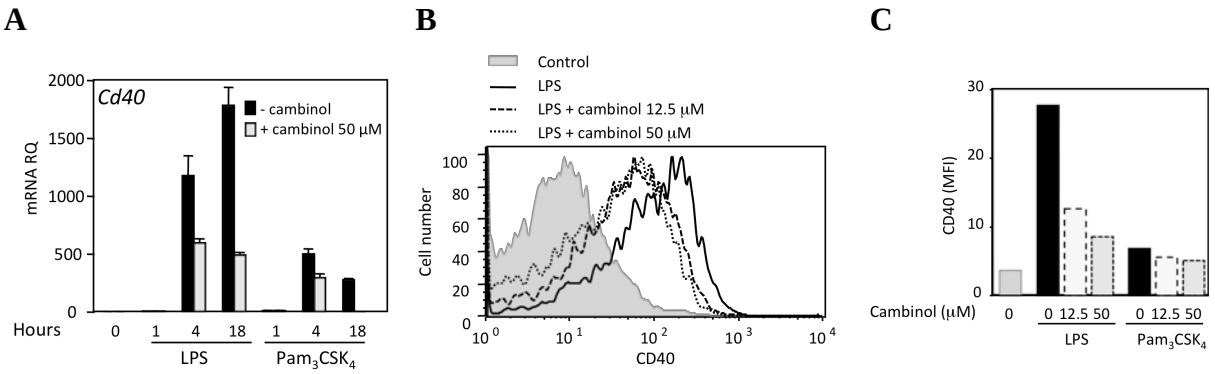
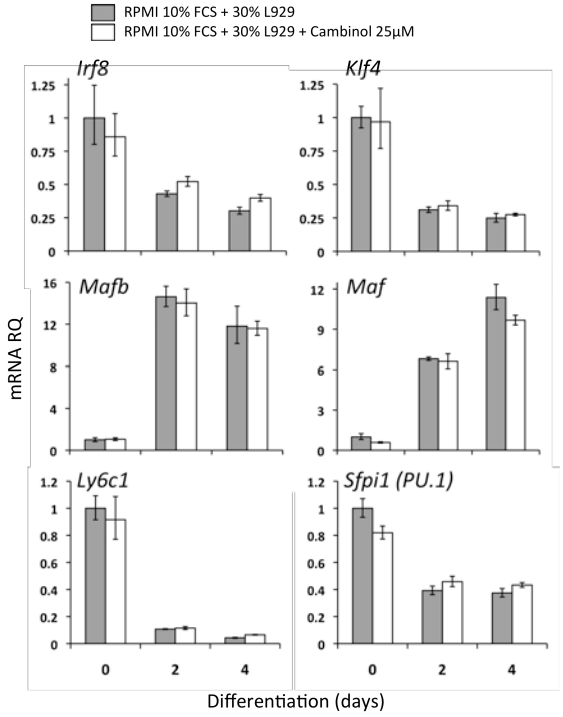


Figure 6.7. Cambinol inhibits CD40 expression in BMDMs. BMDMs were pre-incubated for 1 h with (grey bars) or without (black bars) cambinol prior to stimulation with LPS (10 ng/ml) or Pam_3CSK_4 (10 ng/ml). (**A**) *Cd40* mRNA expression was quantified by RT-PCR and expressed relative to *Gapdh*. (**B-C**) After 18 h of stimulation, CD40 expression was analyzed by flow cytometry and expressed in mean fluorescence intensity (MFI).

6.3.6 Cambinol does not impact on the expression of macrophage differentiation markers

Conditional deletion of Sirt1 in the myeloid compartment generates macrophages with hyperacetylated NF-κB p65 and enhanced transcription of proinflammatory genes (72). Therefore, we analyzed whether cambinol affects monocyte/macrophage differentiation by analyzing the expression of relevant transcription factors and differentiation markers: Mafb and c-Maf involved in the regulation of macrophage differentiation and inhibition of progenitor proliferation, Klf4, Sfpi1, and Irf8 that coordinate the early myeloid commitment in immature progenitors and drive monocytic differentiation, and Ly6c1 a marker of inflammatory macrophages (73). Bone-marrow cells were cultured with macrophage-colony-stimulating factor (M-CSF) in the presence or the absence of cambinol. Gene expression was quantified by RT-PCR at days 0, 2 and 4 of differentiation (**Figure 6.8**). Whereas *Irf8*, *Klf4*, *Spi1* and *Ly6c1* expression declined during M-CSF-induced differentiation of bone-marrow precursors, that of *Mafb* and *c-Maf* increased. Overall, cambinol had no effect on the modulation on gene expression. These preliminary results suggest that cambinol, at anti-inflammatory concentrations, does not affect monocyte/macrophage differentiation or tweak their differentiation toward a proinflammatory phenotype.

Figure 6.8. Cambinol does not affect the expression of macrophage differentiation markers. Bone-marrow cells were cultured in IMDM medium containing 10% FCS and 30% L929 cells supernatant as a source of M-CSF with (white bars) or without (grey bars) cambinol (25 µM). At day 0, 2 and 4 of culture, gene expression was quantified by RT-PCR and expressed relative to *Hprt*. Data are mean ± SD of tripicate samples of one experiment.



6.3.7 Modulation of TLR and Nalp3 expression by cambinol

Pattern recognition molecules are essential for the sensing of microbial and danger signals. To start analyzing the molecular mechanisms whereby cambinol impairs the response of macrophages to microbial and danger products, we first quantified the expression levels of *Tlr* and *Nalp3* mRNA in BMDMs pre-incubated with cambinol and stimulated for 0, 1, 4 and 18 h with LPS. RT-PCR revealed that *Tlr1*, *Tlr2*, *Tlr6* and *Nalp3* were up-regulated in LPS-stimulated BMDMs, *Tlr9* almost not modulated and *Tlr4* transiently down-regulated (**Figure 6.9**). Whereas cambinol did not affect mRNA expression of *Tlr4* and *Tlr6*, it inhibited that of *Nalp3* early on (4 h) and that of *Tlr1* and *Tlr2* later on (18 h). Surprisingly, cambinol up-regulated *Tlr9* expression after 18 h. Therefore, the inhibition of LPS- and CpG-induced cytokine expression by cambinol (**Figure 6.2-4**) probably does not rely on a decreased TLR4 and TLR9 expression. Similarly, it is unlikely that late inhibition of *Tlr1* and *Tlr6* contributes to decrease cytokine response to Pam_3CSK_4 and *S. aureus*. Yet, we should validate these hypotheses at the protein level.

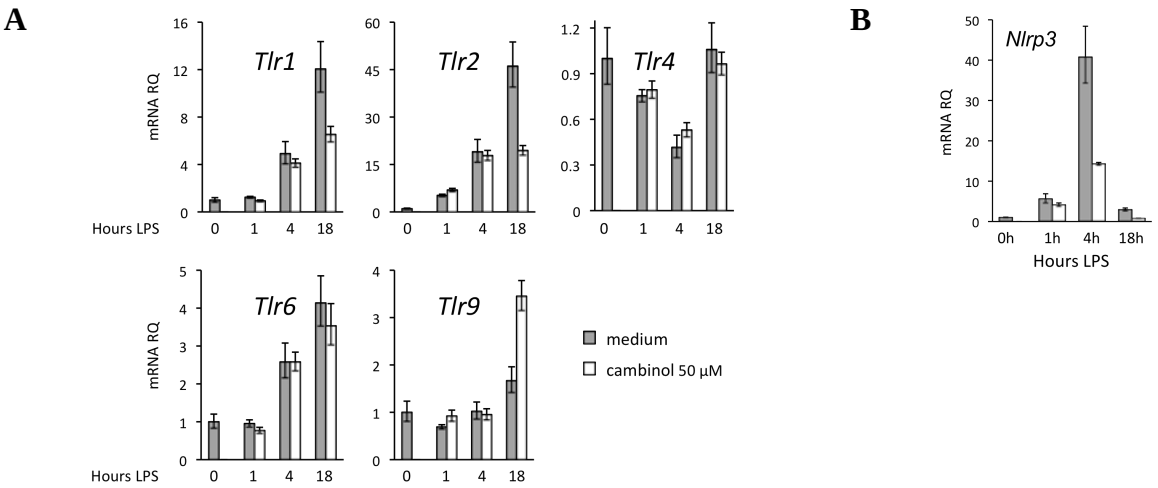


Figure 6.9. Modulation of *Tlr* and *Nalp3* expression by cambinol. BMDMs were pre-incubated for 1 h with cambinol (50 µM) prior to stimulation with LPS (10 ng/ml) for 1, 4 and 18 h. *Tlr1*, *Tlr2*, *Tlr4*, *Tlr6*, *Tlr9* (**A**) and *Nalp3* (**B**) expression was quantified by RT-PCR and results expressed relative to *Hprt* expression. Data are mean ± SD of duplicates determinations.

Considering the above results, we decided to study the effects of cambinol on histone acetylation and signal transduction pathways elicited by microbial products in macrophages. Since these techniques require large amounts of material, we selected the mouse RAW 264.7 macrophage cell line as a model. Indeed, similar to what we observed in BMDMs, cambinol powerfully inhibited cytokine mRNA and protein expression in LPS- and Pam_3CSK_4-stimulated RAW 264.7 macrophages (data not shown).

6.3.8 Cambinol does not increase overall histone H3 acetylation

Inhibitors of class I and II HDACs promote global changes of histone acetylation and balance toward a net hyperacetylation of histones. To check wether global acetylation of histone H3 (acH3), a common target of class I, II and III HDACs, is modified upon cambinol treatment, we analyzed acH3 levels in RAW 264.7 macrophages pre-incubated for 1 h with cambinol and stimulated for 4 and 12 h with LPS (**Figure 6.10**). Neither cambinol nor LPS modified global histone H3 acetylation, suggesting that cambinol does not act through global reprogramming of histone acetylation. As a positive control, trichostatin A (a pan HDAC1-11 inhibitor) strongly increased acH3 levels in LPS-stimulated cells.

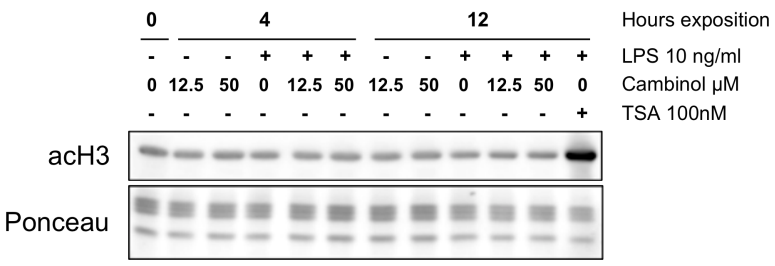


Figure 6.10. Cambinol does not increase global acetylation of histone H3. RAW 264.7 macrophages were pre-incubated for 1 h with (+) or without (-) cambinol or TSA and exposed to LPS for 0, 4 and 12 h. Acetylated-(ac) H3 proteins were analyzed by Western blotting of acid-extracted proteins. Ponceau S staining was used to control for equal loading.

6.3.9 Cambinol does not impair NF-κB p65 translocation, but inhibits MAPK phosphorylation

SIRT1- and SIRT2-dependent deacetylation of NF-κB p65 on Lys310 has been shown to modulate the expression of NF-κB-dependent genes (50,74,75). Since cambinol inhibits SIRT1 and SIRT2 activity, we questioned whether cambinol interferes with NF-κB p65 nuclear translocation. RAW 264.7 macrophages were exposed to LPS for 15-240 minutes, and we analyzed cytosolic and nuclear NF-κB p65 content by Western blotting (**Figure 6.11**). LPS promptly stimulated NF-κB p65 nuclear translocation, a process that was not affected by cambinol pre-treatment. We will test the possibility that cambinol affects NF-κB signaling at the DNA level (*i.e.* docking of NF-κB to DNA and NF-κB-mediated transcriptional activity).

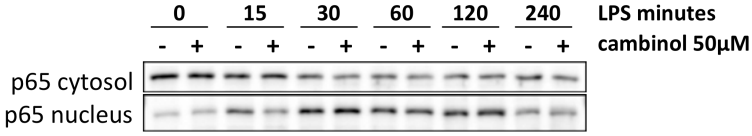


Figure 6.11. Cambinol does not interfere with NF-κB nuclear translocation. RAW 264.7 macrophages were pre-incubated for 1 h with (+) or without (-) cambinol (50 µM) prior to stimulation with LPS (10 ng/ml). Cytosolic and nuclear NF-κB p65 was analysed by Western blotting. Results are representative of two independent experiments.

Activation of MAPKs triggered by TLR stimulation is required for active transcription of pro-inflammatory genes. To test whether MAPKs were targetted by cambinol, we assessed the phosphorylation of p38, ERK1/2 and JNK in RAW 264.7 macrophages (**Figure 6.12**). Phosphorylation of p38, ERK1/2 and JNK was detected 30 minutes upon LPS stimulation and elevated up to 240 minutes. Strikingly, cambinol almost fully abrogated LPS-induced p38, ERK1/2 and JNK phosphorylation.

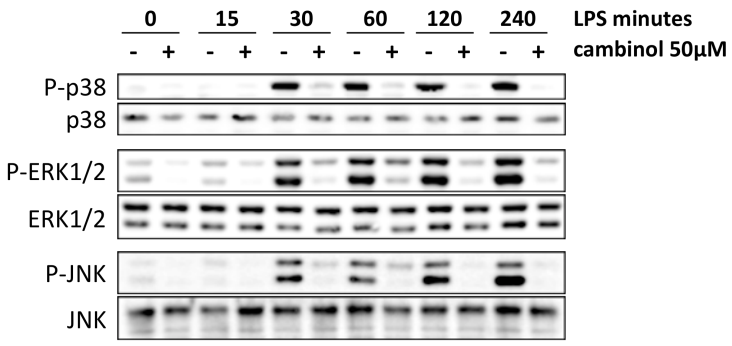
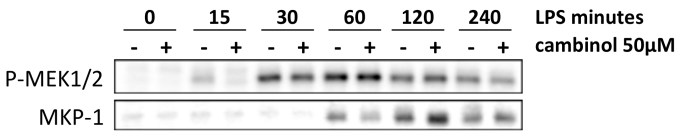


Figure 6.12. Cambinol inhibits MAPK phosphorylation. RAW 264.7 macrophages were pre-incubated for 1 h with (+) or without (-) cambinol (50 µM) prior to stimulation with LPS (10 ng/ml). Expression levels of phospho (P-) and total p38, ERK1/2 and JNK were analysed by Western blotting. Results are representative of two independant experiments.

Impaired phosphorylation of MAPKs mediated by cambinol may rely on the inhibition of the upstream MAPK kinase (MAPKK) MEK1/2, or the induction/activation of MAPK phosophatases (MKPs, also known as dual-specific phosphatase or DUSP). In preliminary experiments, we focused on MKP-1 (DUSP1), which plays a key role in the feedback negative control of MAPK activation upon microbial challenge (76,77). To our surprise, cambinol neither inhibited MEK1/2 phosphorylation nor induced MKP-1 expression in RAW 264.7 macrophages (**Figure 6.13**). Thus, the mechanism by which cambinol interferes with MAPK phosphorylation remains to be identified. In preliminary experiments, we observed that cambinol potentiates the expression of several LPS-inducible DUSPs. Additional work will be required to confirm the relevance of these observations at the protein level in relation to host response to microbial stimulation.

Figure 6.13. Cambinol does not interfere with MEK1/2 activation and MKP-1 expression. RAW 264.7 macrophages were pre-incubated for 1 h with (+) or without cambinol (50 µM) prior to stimulation with LPS (10 ng/ml). Phospho (P-) MEK1/2 and MKP-1 were analysed by Western blotting. Results are representative of two independent experiments.



6.3.10 Cambinol impairs cytokine production by splenocytes, whole blood and PBMCs

The experiments described above were performed using BMDMs and RAW 264.7 mouse macrophages. To verify that cambinol affects the response of immune cells other than macrophages, we measured the cytokine response of mouse splenocytes following polyclonal stimulation with the superantigen toxic shock syndrome toxin 1 (TSST-1) and concanavallin A (**Figure 6.14**). TSST-1

strongly induced *Il2* and *Ifng* mRNA levels in splenocytes. Pre-incubation for 1 h with cambinol at 25 µM or above decreased *Il2* and *Ifng* mRNA expression. Moreover cambinol dose-dependently inhibited the secretion of IFNγ by splenocytes stimulated with concanavalin A. These results indicate that the anti-inflammatory effect of cambinol is not restricted to monocytes/macrophages, but is also efficient on splenocytes.

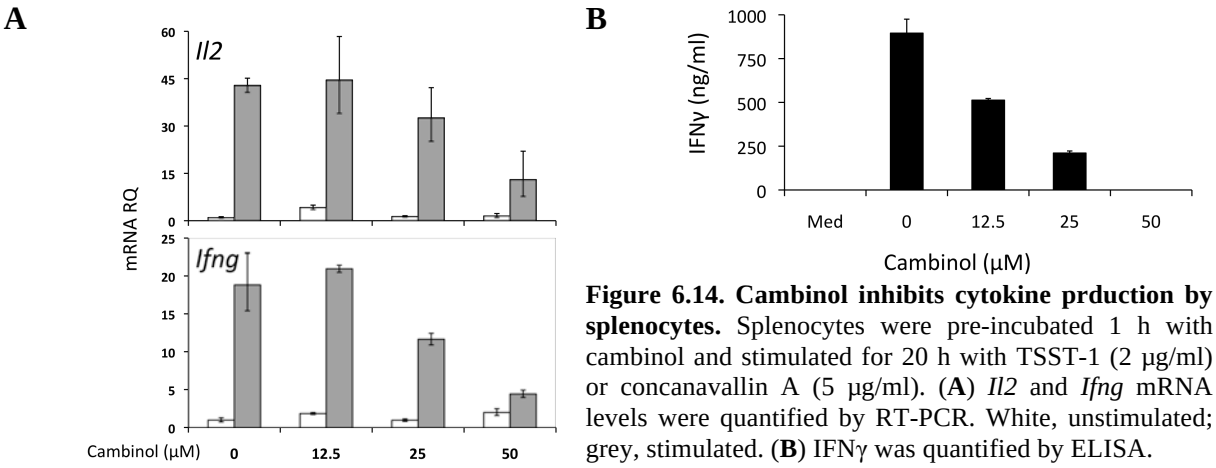


Figure 6.14. Cambinol inhibits cytokine prduction by splenocytes. Splenocytes were pre-incubated 1 h with cambinol and stimulated for 20 h with TSST-1 (2 µg/ml) or concanavallin A (5 µg/ml). (**A**) *Il2* and *Ifng* mRNA levels were quantified by RT-PCR. White, unstimulated; grey, stimulated. (**B**) IFNγ was quantified by ELISA.

To test the effect of cambinol on cytokine production by human primary immune cells, we developed a human whole blood assay and analyzed the response of peripheral blood mononuclear cells (PBMCs) obtained from healthy blood donors (**Figure 6.15**). As anticipated, stimulation of whole blood and PBMCs with LPS promoted the production of great quantities of TNF and IL-6 that were dose-dependently inhibited by cambinol. Thus, cambinol reduces cytokine production induced by microbial products in both murine and human primary innate immune cells.

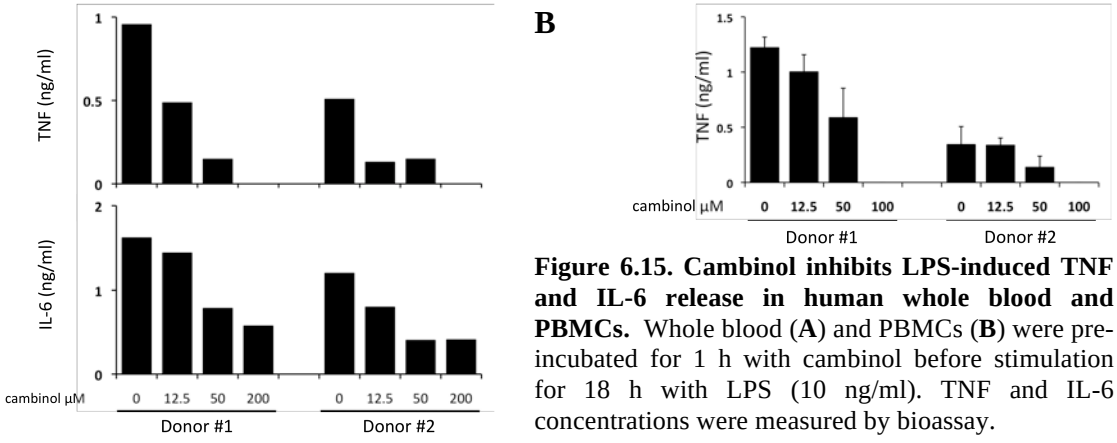


Figure 6.15. Cambinol inhibits LPS-induced TNF and IL-6 release in human whole blood and PBMCs. Whole blood (**A**) and PBMCs (**B**) were pre-incubated for 1 h with cambinol before stimulation for 18 h with LPS (10 ng/ml). TNF and IL-6 concentrations were measured by bioassay.

6.3.11 Cambinol protects mice from lethal endotoxemia

We developped a model of endotoxic shock to establish whether cambinol has anti-inflammatory properties *in vivo*. Mice were injected intra-peritoneally (i.p.) with LPS (17.5 mg/kg). In a first series of experiments, animals were sacrified 0, 1, 4, 8 and 24 h post-innoculation to quantify *Sirt1* and *Sirt2* mRNA expression in the spleen, liver and kidney (**Figure 6.16**). In both spleen and kidney, *Sirt1* and *Sirt2* gradually decreased during the course of the analysis. On the contrary, *Sirt1* and *Sirt2* mRNA slightly increased early on in the liver (t=1-4 h for *Sirt1* and t=1 h for *Sirt2*) and then returned to basal levels after 24 h. Thus, *Sirt1* and *Sirt2* expression is modulated in a time- and tissue-dependent fashion during endotoxic shock. The results argue for a possible regulatory role of situins during endotoxemia.

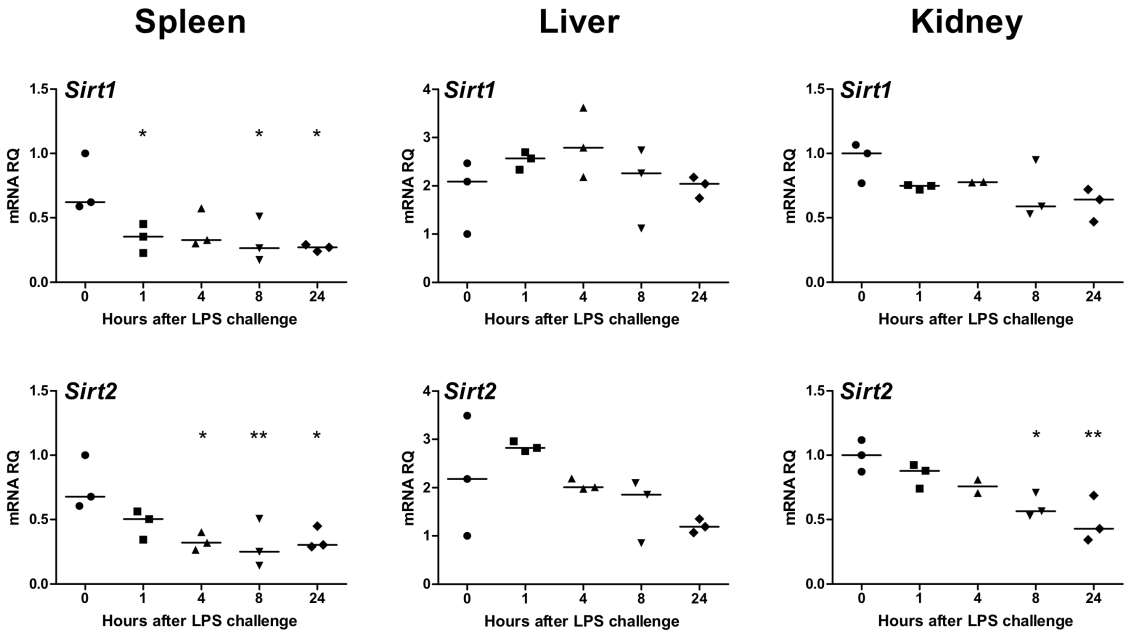


Figure 6.16. Expression levels of *Sirt1* and *Sirt2* mRNA in spleen, liver and kidney of endotoxic mice. BALB/c mice were injected i.p. with LPS (17.5 mg/kg). Spleen, liver and kidney were collected after 0, 1, 4, 8 and 24 h. *Sirt1* and *Sirt2* mRNA levels were quantified by RT-PCR and expressed relative to *Hprt* mRNA levels. *, $0.05 > P > 0.005$, ** $P < 0.005$ by one-way ANOVA and Dunnett's multiple comparison test for group comparisons,

To establish whether cambinol has anti-inflammatory properties *in vivo*, cambinol (10 mg/kg) was administrated i.p. 1 h before and 24 h after LPS injection. Blood was collected 1 h after LPS administration to quantify TNF levels. Remarkably, administration of cambinol significantly

decreased TNF blood levels (P = 0.049) and increased survival from 8.3% to 46.1% (P = 0.035) (**Figure 6.17**).

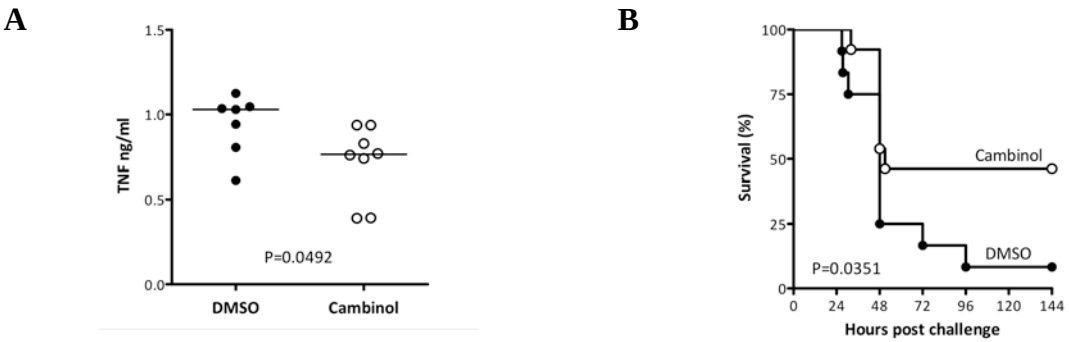
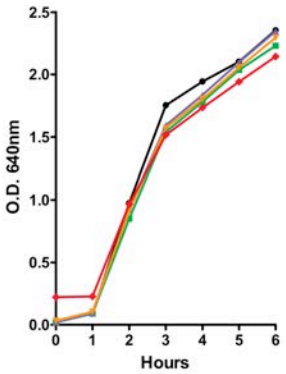


Figure 6.17. Cambinol protects from endotoxemia. BALB/c mice were injected i.p. with LPS (17.5 mg/kg). Cambinol (10 mg/kg) or diluent (DMSO) was administrated i.p. 1 h before and 24 h after LPS challenge. (**A**) TNF concentrations in blood collected 1 h after LPS challenge were measured by ELISA (n=7-8 per group). (**B**) Survival of mice (n=12-13 per group).

6.3.12 Cambinol confers protection to severe sepsis

Next, we developped a model of *Klebsiella pneumoniae* (*K. pneumoniae*) pneumonia to analyze the impact of cambinol in a model of bacterial infection. Prior performing *in vivo* experiments, we tested wether cambinol possesses any intrinsic microbicidal or microbiostatic activity. Growth curve of *K. pneumoniae* cultured with increasing concentrations of cambinol ranging from 0 to 200 µM demonstrated that cambinol had no impact on bacterial growth *in vitro* (**Figure 6.18**). Similar results were obtained when analyzing *E. coli* and *C. albicans* (data not shown).

Figure 6.18. Cambinol does not impair the growth of *Klebsiella pneumoniae*. *K. pneumoniae* were seeded in Luria-Bertani (LB) broth containing 0 (black), 3.1 (green), 12.5 (blue), 50 (orange) or 200 µM (red) cambinol and grown at 37°C under agitation. Bacterial growth was measured as the optical density read at 640 nm.



Mice were infected intranasally with 18-63 CFU *K. pneumonia* and treated with cambinol (10 mg/kg) or DMSO 24 h before, at the onset and 24 h and 48 h after infection. Blood was sampled 3 days after bacterial innoculation. Bacterial loads did not significantly differ between control and

cambinol-treated mice, albeit being 2-fold lower in cambinol treated animals (**Figure 19A**). Mice started to die 3 days post-infection in the control group. Whereas only 13.3% of mice survived infection in the control group, 60% of mice survived in the cambinol group (P = 0.015; **Figure 19B**). Thus, cambinol protects mice from lethal *K. pneumoniae* infection.

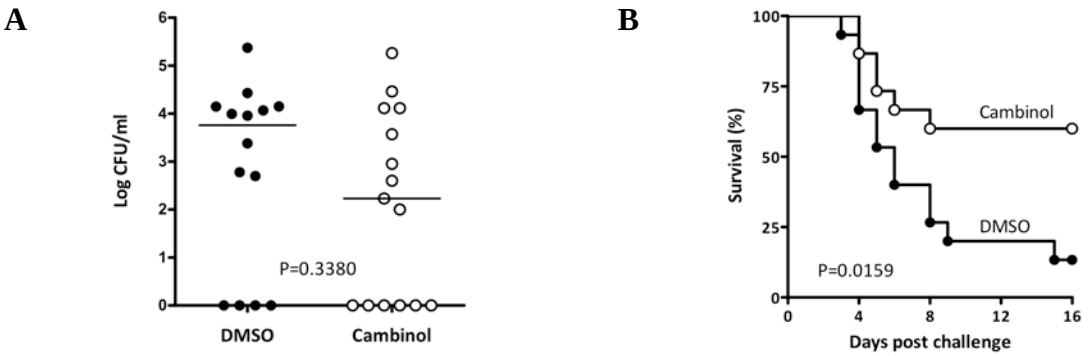


Figure 6.19. Cambinol confers protection against *Klebsiella pneumoniae* pneumonia. BALB/c mice (n=15 per group) were challenged intranasally with 18-63 CFU *K. pneumonia*. Cambinol (10 mg/kg) or diluent (DMSO) was administrated i.p. 24 h before, at time of challenge, 24 h and 48 h after *K. pneumonia* challenge. (**A**) Blood was sampled 3 days after infection to enumerate circulating bacteria. (**B**) Survival of mice.

6.4 Discussion

The inflammatory response can be regulated by different mechanisms that can be divided into gene-specific, signal-specific and cell-specific (78). Acetylation and deacetylation can influence the inflammatory response at these three levels. Histone modifying enzymes including HATs and HDACs have been shown to be important regulators of pro-inflammatory gene transcription in a gene-specific manner by modifying chromatin structure (79).

In the present study, we report that cambinol, an inhibitor of SIRT1 and SIRT2, inhibits the expression of cytokines, nitric oxide and CD40 induced by different microbial products in macrophages, splenocytes, whole blood and PBMCs. Moreover, cambinol reduces the expression levels of TNF and protects animals from endotoxic shock. Cambinol also increases survival rate of mice in a model of severe *K. pneumoniae* pneumonia. In line with our results, reduction of NAD bioavailability decreased TNF production in mice challenged with LPS (44).

Numerous studies have shown that acetylation modifies the structure or the activity of signal transducers and chromatin (80-83). Cambinol inhibits *Tnf*, *Il1b*, *Il6*, *Il12b* and *Cd40* mRNA expression in LPS- and/or Pam_3CSK_4-stimulated macrophages, suggesting that cambinol interferes with the transcription of immune related genes. Surprisingly however, cambinol does not modify the nuclear translocation of NF-κB, a well-known target of acetylation-dependent regulation (84,85). Yet, cambinol drastically inhibits the phosphorylation of p38, ERK1/2 and JNK without affecting the activation of the upstream MAPKK MEK1/2. At first glance, these results are surprising because NF-κB and MAPKs pathways are usually activated concomitantly by microbial products in innate immune cells (86). Our result could be explained by a specific action of cambinol on the MAPK pathway. Supporting this assumption, SIRT1 interacts with c-Jun and reduces AP-1 transcriptional activity (80). Moreover, sirtinol induces senescence-like growth arrest and disrupts the Ras-MAPK pathway in human cancer cell lines stimulated with epidermal growth factor (87). Finally, nicotinamide inhibits the accumulation of phosphorylated ERK1/2 during the activation of primary murine B lymphocytes with LPS, anti-IgM, anti-CD40 or anti-CD38 (88). Further studies will be devoted to clarify the role of sirtuins and sirtuin inhibitors in the regulation of MAPK activity.

Although there is growing evidences that sirtuins modulate immune responses, their influence on inflammation remains poorly understood and controversial (57). Sirtuins, and primarily SIRT1, are involved in the pathogenesis of lupus, experimental autoimmune encephalomyelitis, collagen-induced arthritis, allergic airway disease and trauma-hemorrhage (43-47). Moreover, SIRT6 is required for optimal TNF synthesis by macrophages and DCs (42) and TNF and IFNγ production by PHA-stimulated peripheral blood lymphocytes (43). In line with these studies, cambinol powerfully inhibits the production of pro-inflammatory cytokines induced by microbial products and heat-killed bacteria in macrophages and splenocytes.

We observed that cambinol inhibits IL-1β production by macrophages at least in part by decreasing *Il1b* gene expression. The NALP3 inflammasome plays a key role in controlling IL-1β secretion (69). SIRT1 has been reported to deacetylate and activate PGC-1α, a process that in turn promotes mitochondrial activity and biogenesis (89). Interestingly, activated NALP3 inflammasome relocalizes from the endoplasmic reticulum (ER) to perinuclear environment and co-localize with ER and mitochondria (90). NALP3 activation is regulated by reactive oxygen species (ROS) (91) and SIRT1 promotes mitochondrial biogenesis. Thus, it would be important to analyze the effects of cambinol and SIRT1 inhibition on ROS production by mitochondria as well as NALP3 mitochondrial relocalization, as it may participate to impede IL-1β production.

Together with ROS, reactive nitrogen species (RNS) produced by phagocytes create a hostile milieu for pathogens (71). Deficiency in inducible nitric oxide synthase (iNOS) impairs anti-microbial host defenses (92,93). Our observation that cambinol inhibits the generation of RNS in response to LPS suggests that inhibition of sirtuins may affect bactericidal activity of phagocytes as recently shown for inhibition of class I and II HDACs (65,94).

The role of sirtuins in the differentiation of immune cells is yet obscure. One study reported that conditional deletion of SIRT1 in myeloid cells in mice favors the development of pro-inflammatory macrophages (72). In a model of monocyte/macrophage differentiation from bone marrow precursors, we could not detect any effect of cambinol in shaping the expression of key

transcription factors or differentiation markers specific of the monocyte/macrophages lineage. The relevance of these observations will be assessed by analyzing the proportions and the phenotypes of monocytes/macrophages in mice treated with cambinol.

Several studies described anti-inflammatory properties of SIRT1, which have been associated with acetylation-dependent modulation of the activity of NF-κB and AP-1 (80,84,85). Going well along with these observations, we observed that the SIRT1 and SIRT2 specific inhibitors EX-527 and AGK2, contrary to cambinol, increase cytokine production induced by LPS and Pam_3CSK_4 in macrophages. Moreover, sirtinol, which inhibits SIRT2 more efficiently than SIRT1, has not the same ability as cambinol to reduce cytokine secretion. Several explanations may reconcile these contradictory results.

First, sirtuins probably act in concert to control the expression of cytokine genes. The net effect on gene expression may result from a delicate balance between the opposing action of pro- and anti-inflammatory sirtuins or sirtuin-associated molecules, depending on the chromatin context. Thus highly specific sirtuin inhibitors like AGK2 and EX-527 may not act similar to more broad-spectrum inhibitors such as cambinol. Up to now, SIRT2 has not been implicated in the regulation of immune or inflammatory responses. Although catalytic inhibition of SIRT2 with AGK2 does not reduce LPS-induced TNF and IL-6 release by macrophages, targeting SIRT1 and SIRT2 using cambinol impairs *Tnf* and *Il6* gene expression. Moreover, depleting SIRT2 expression with shRNA in RAW 264.7 macrophages inhibits LPS-, Pam_3CSK_4- and CpG ODN-induced TNF release (data not shown). It is possible that SIRT2 develops anti-inflammatory in conjunction with SIRT1. In the same line, Peck *et al.* have reported that inhibition of both SIRT1 and SIRT2, and not of SIRT1 alone, induces p53 acetylation *in vivo* (95). We are currently developing a colony of Sirt2 knockout mice to be able to definitively address the role of SIRT2 in the regulation of the inflammatory and innate immune responses.

A second explanation that could account for the contradictory results reported in this study relies on the specificity of the sirtuin inhibitors, which might be broader than claimed. Although

cambinol had no activity against SIRT3 and SIRT5 (61), it was not tested against other sirtuins, notably SIRT6 that was recently shown to participate to the post-transcriptional control of TNF production by dendritic cells (42). We cannot exclude a role of cambinol in inhibiting SIRT4 and SIRT7. In fact, combined treatment with of EX527 and AGK2 did not reproduce the effect of cambinol with the same efficiency (data not shown). Interestingly, cambinol showed activity when used at concentrations lower than its theoretical IC_{50}. Yet, one should also recall that the IC_{50}s of sirtuin inhibitors have been defined *in vitro* in experimental systems comprising solely sirtuins, sirtuin substrates and sirtuin inhibitors, which do not reflect the complexity of the environment of the chromatin to which sirtuins are recruited *in vivo*. Moreover, there is up to now no data on the stability, metabolism and bioavailability of sirtuin inhibitors *in vivo*.

Overall, we have demonstrated that cambinol inhibits pro-inflammatory cytokine production by innate immune cells through a mechanisms affecting gene transcription. The precise upstream mechanisms used cambinol remains to be defined. Most importantly, cambinol inhibits TNF synthesis and increases survival during endotoxic shock and augments survival to lethal *K. pneumoniae* infection, suggesting that cambinol or other drugs of this class could be used as adjunctive therapy in pathologies characterized by acute or chronic inflammatory responses and in sepsis.

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7 CONCLUDING REMARKS & PERSPECTIVES

In contrast to our extensive knowledge of the effects of HDAC inhibitors (HDACi) in the field of cancer, much less is known about the influence of these drugs on the innate immune system. In this work, we report compelling evidences indicating that HDACi have powerful anti-inflammatory and immunomodulatory activities *in vitro* and in *vivo*.

In **chapter 1 and 2**, we present a first comprehensive study of the effects of HDAC1-11 inhibitors (therefater called HDACi) on innate immune responses. As summarized in the illustration below, we have seen that HDACi affect numerous facets of the innate immune response. Strikingly, expression studies revealed that HDACi act essentially as negative regulators of basal and microbial product-induced expression of critical immune receptors and antimicrobial products by innate immune cells. These results strengthen the notion of a certain specificity of HDAC inhibition in altering gene expression. Indeed, a global analysis of the effects of HDACi showed that they affected the expression of only a small proportion (less than 10%) of all cellular genes in macrophages with similar proportions of genes up-regulated and down-regulated.

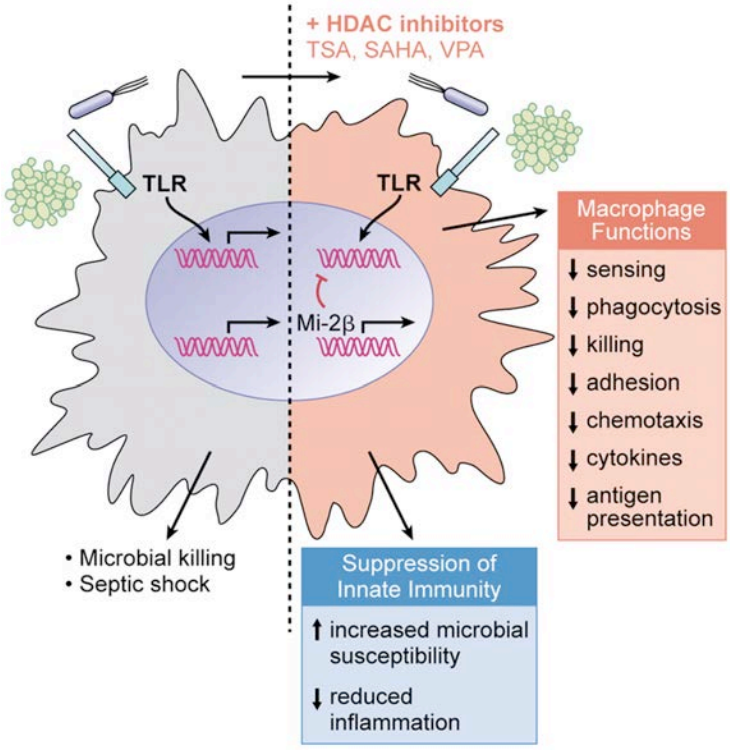

Figure 7.1 HDAC inhibitors block innate immunity. Addition of broad-spectrum HDAC inhibitors to macrophages stimulated through TLRs results in a dominant inhibition of gene expression (up to 60% of TLR-induced genes) encompassing suppression of all major functions of macrophages. Consequently, *in vivo* susceptibility to infections is increased when the HDAC inhibitor valproate is administered to mice, whereas overreactivity of innate immunity as observed in septic shock is decreased. (Professional illustration by Kenneth X. Probst)
From Bode KA & Dalpke AH. **Blood** 2011;117(4):1102-3.
Comment on Roger T. *et al.* **Blood** 2011;117(4):1205-17.

The molecular mechanisms by which HDACi interfere with gene expression are not fully understood, but are likely multiple depending on the cell type, the drug and the stimulus analyzed. In line with previous studies, HDACi did not reduce histone H4 acetylation and phosphorylation or activation of signal transduction pathways (NF-κB, MAPKs, IRFs, STAT1, AP-1) playing a crucial role in the initiation of inflammatory and innate immune responses. We identified a new molecular mechanism by which HDACi mediate their effects through the induction of the trancriptional repressor Mi-2β and its recruitment to the promoter region of the *Il6* gene analyzed as prototypical HDACi-targetted cytokine. Whether HDACi act preferentially or not through Mi-2β to inhibit immune gene expression is presently unknown and will require broad scanning technology such as ChiP on chip analyses.

Treatment with VPA remarkably reduced cytokine levels and protected mice from Pam_3CSK_4-induced fulminant toxic-shock. Furthermore, VPA protected animals from cecal ligation and puncture, one of the most stringent models of bacterial sepsis. Whereas major advances in antimicrobial therapy and supportive care have reduced short-term mortality from sepsis in the last twenty years, the prognosis of patients with severe infections continues to be grim. Therefore, the identification of new treatment options for septic patients remains imperative. In light of the results reported here, we propose that HDACi could represent an efficacious adjunctive therapy of severe sepsis. These drugs may have the advantage over therapies targeting a single molecule (*i.e.* antibodies) to broadly dampen pro- and anti-inflammatory reactions by reshuffling gene expression.

Histones themselves possess intrinsic pro-inflammatory properties. Histones H3 and H4 released by necrotic cells during endotoxic shock are highly toxic and promote the death of endothelial cells. It will be of interest to study the post-translational modifications of histones circulating in septic animals and to analyze whether HDACi treatment modifies the acetylation status of blood histones. Indeed, it may represent an additional mechanism by which HDACi could be beneficial by promoting detoxifying and cytoprotective effects. Furthermore, it will be of primary interest to identify the exact DAMP(s) related to circulating histones (for example histones alone or histones complexed with DNA) and the molecules or PRR(s) involved in their recognition by endothelial and immune cells.

Considering that pro-inflammatory mediators released during the course of an infection coordinate the development of innate and adaptive immunity, one may anticipate that HDACi may interfere with the generation of pathogen-specific adaptive immune responses (*i.e.* the generation of pathogen-specific T and B cells). Several studies have shown that HDACi diminish the APC function of macrophages and dendritic cells, notably through down-regulating the expression of costimulatory, adhesion and antigen presentation molecules. We have shown that microbial uptake by phagocytes is impaired by HDACi. Altogether these data suggest possible adverse effects of HDACi on the generation of adaptive response to microbes and vaccines.

VPA increased bacterial burden and mortality of mice infected with a sub-lethal inoculum of *K. pneumoniae*, and accelerated death rate and increased overall mortality in a model of disseminated candidiasis. These experiments definitely prove that HDACi hamper innate immune responses under pathophysiological situations, as can be expected from any treatment blocking or inhibiting the activity of innate receptors and cytokines. Although we used VPA as a model HDACi because of its low toxicity, it can be argued that VPA is employed since decades to treat epileptic patients with no major side effect. Yet, higher concentrations of VPA have been tested for cancer chemotherapy, moreover in combination with immunosuppressive therapies.

Thus, one obvious matter is to define whether HDACi affect natural host defenses in patients. Although numerous phase I and II clinical trials have been performed, no definitive answer can be given to this crucial question for several reasons. First, clinical trials testing HDACi are typically performed in patients with advanced cancer who usually have confounding underlying components and reduced life expectancy. Second, few patients have been treated for long time periods with HDACi (except VPA), so that long-term toxicity is not known. Third, whether patients were treated prophylactically with antimicrobial agents and whether episodes of infection occurred may not have been always accurately reported, as the objectives of clinical trials were to assess the bioavailability, toxicity and response of cancer to the drugs. Nonetheless, it is worthwhile to mention that patients treated with HDACi such as MS-275, VPA, SAHA and ITF2357 commonly experienced hematologic toxicity characterized by thrombocytopenia, leucopenia and neutropenia which are major risk factors

for developing severe infection. In fact, episodes of severe infection have been reported in patients treated with HDACi with or without neutropenia. Large prospective studies will be required to settle the impact of HDACi on host immune status and susceptibility to infection, especially in immunosuppressed cancer patients.

MIF is an important mediator of the inflammatory and innate immune responses, playing also a role in promoting cell proliferation and tumorigenesis. In **chapter 4 and 5**, we report that HDACi decrease *MIF* gene transcription through a local deacetylation of *MIF* promoter-associated histones that affects the recruitment of basal transcriptional machinery. This deacetylation is intriguing since HDACi are supposed to inhibit class I, II and IV HDACs and that these enzymes are responsible for deacetylation of histone tails. We speculate that this effect could be ascribed to the recruitment of sirtuins to the MIF promoter following treatment with HDACi. This hypothesis is relevant for two reasons. First, sirtuins are not sensitive to classical HDACi. Second, sirtuins expression is dose-dependently increased by TSA in HeLa cells (results from exploratory RT-PCR analyses not shown in this manuscript) and down-regulation of *MIF* expression by TSA was dependent on *de novo* protein synthesis. To test our hypothesis, we will investigate the recruitment of sirtuins to the MIF proximal promoter in cells treated with HDACi. Another unexplored feature is that TSA did not impact on the expression levels of Sp1 and CREB, which control *MIF* gene basal transcription. Further work will be required to test whether TSA displaces the binding of Sp1 and CREB from the MIF promoter through a direct modification of these proteins affecting their DNA binding capacity, or through the recruitment of a transcriptional repressor competing with, or disrupting, Sp1 and CREB DNA binding.

Most interestingly, infusion of TSA reduced MIF blood levels in mice, leaving open the possibility that HDACi impair tumorigenesis by reducing MIF bioavailability. This assumption could be verfied by analyzing tumor development and MIF content in a murine model of carcinogenesis in which animals would be treated with HDACi with or without concomitent supplementation with recombinant MIF. Moreover, it would be interesting to analyze MIF production by macrophages of

the tumor microenvironment since it is well established that the proinflammatory milieu generated by macrophages sustains tumorigenesis.

Sirtuins are histone deacetylases whose role in immunity just begins to be explored. In **chapter 6**, we provide the results of experiments suggesting that inhibition of SIRT1 and SIRT2 using cambinol have strong anti-inflammatory repercussions as illustrated by reduced cytokine production. In line with these results, RAW 264.7 macrophages, in which *Sirt1* or *Sirt2* was silenced, produced lower levels of TNF and IL-6 in response to microbial products (data not shown). Having established a colony of SIRT2 knockout mice, we will be able to verify the relevance of these observations by using SIRT2 knockout cells. We will also use SIRT2 knockout animals to establish whether SIRT2 deficiency influences the differentiation of monocyte/macrophages and other immune cell types *in vivo*.

If the net anti-inflammatory effect of cambinol seems similar to the one obtained with HDACi, the mechanism underlying the action of the different drugs is probably different. Indeed, cambinol profoundly interfered with MAPK activation in macrophages stimulated with LPS, which was not the case for HDACi. As cambinol did not affect LPS-induced phosphorylation of the upstream MAPKK MEK1/2, we favor the possibility that cambinol acts through the induction of a dual-specific phosphatase (DUSP). MKP1 being excluded from a preliminary screen, we will persue by analyzing the expression of DUSPs in macrophages treated with cambinol and the effect of cambinol in macrophages in which DUSP expression will be silenced using siRNA. The binding partners of SIRT1 and/or SIRT2 in the MAPK pathway will also be explored in co-immunoprecipitation studies.

NF-κB p65 is a well-known target of SIRT1 and SIRT2. Even though we could not observe any effect of cambinol on LPS-induced NF-κB p65 nuclear translocation, it does not exclude an effect of cambinol on NF-κB p65 DNA binding or transactivating activity. These particular points will be verified in electrophoretic mobility shift assays, transfection studies using a NF-κB luciferase reporter system and chromatin immunoprecipitation of cytokine promoters.

Up to now, we have analyzed the effects of cambinol on only a few aspects of innate immune responses *in vitro*. Obviously, we will have to extend our analyses on dendritic cells, and to assess the impact of sirtuin inhibition on the expression of PRRs, the production of reactive oxygen and nitrogen species (ROS and RNS), and the phagocytosis and the killing of pathogens by phagocytes. The effect of cambinol and SIRT1 inhibition on the production of ROS by mitochondria will be particularly scrutinized as it has recently been reported that ROS are important for NALP3 inflammasome activation and thus for the secretion of IL-1β. Moreover, we have shown that inducible *Il1b* gene expression is reduced by cambinol.

Unfortunately, almost nothing is known on the stability, metabolism and bioavailability of sirtuin inhibitors *in vivo*. Pharmacokinetics experiments are drastically needed in order to establish efficient drug administration protocols in animal models. Nevertheless, we developped pre-clinical models demonstrating that cambinol protects mice from endotoxic shock and lethal *K. pneumoniae* infection. Although we should extend our analyses of the pathophysiological parameters in septic animals and verify that cambinol impairs host defenses to otherwise non-lethal infection, our preliminary data with cambinol miror the ones obtained using HDACi. Therefore, the same concerns about the utilization of HDACi (*i.e.* increasing susceptibility to infection particularly in immunodeficient patients) may apply to sirtuin inhibitors, especially since several sirtuin inhibitors have enterred phase II clinicals trials.

HADCi and surtuin inhibitors have very promising therapeutic potential for the treatment of a large spectrum of diseases. Our present data suggest that these drugs may increase the susceptibility to infections, especially in the immunocompromissed host. A possible way to avoid these potential adverse effects is to persue the development of new generation isoform-specific inhibitors, which may lead to the discovery of inhibitors selectively blocking the pathological action of HDACs without interfering with antimicrobial host defences.