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MODULATION OF INNATE IMMUNE RESPONSES AND HOST DEFENSES AGAINST INFECTIONS BY PROPIONATE, SIRTUINS (SIRT 2, 3 AND 5) AND TRAINING

Heinonen Tytti

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Département de Médecine
Service des Maladies Infectieuses

MODULATION OF INNATE IMMUNE RESPONSES AND HOST DEFENSES AGAINST INFECTIONS BY PROPIONATE, SIRTUINS (SIRT 2, 3 AND 5) AND TRAINING

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par

Tytti HEINONEN

Master de l'Université de Lausanne

Jury

Prof. Sanjiv Luther, Président
Prof. Thierry Roger, Directeur de thèse
Prof. Ping-Chih Ho, Expert
Prof. Salomé LeibundGut-Landmann, Experte

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Expert·e·s	Monsieur	Prof.	Ping-Chih	**Ho**
	Madame	Prof.	Salomé	**LeibundGut-Landmann**

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SUMMARY

The innate immune system is the first line of defense against invading pathogens. Innate immune cells are classified into subpopulations that show high plasticity to modulate their functions in response to external stimuli. Recently, the reprogramming of innate immune cells has been described to afford protection against infections, a process named “training”. Histone deacetylases (HDACs) form a family of enzymes comprising classical HDACs (HDAC1-11) and sirtuins (SIRT1-7). HDACs regulate metabolic and immunologic pathways. Inhibition of HDACs has a context-dependent effect with reports showing either pro-inflammatory or anti-inflammatory effects. While HDACs share common location and targets, their crosstalk is poorly understood. In this project, we combined different approaches using propionate as an inhibitor of HDACs (HDACi), sirtuin knockout mice and a model of trained immunity to study the modulation of innate immune responses and host defenses.

Propionate is a short-chain fatty acid with anti-inflammatory properties produced by the gut microbiota during fiber fermentation. Since inflammatory responses are required to fight pathogens, we tested whether food supplementation with propionate increases susceptibility to infection. Propionate reduced the production of inflammatory cytokines by innate and adaptive immune cells. However, propionate had no effect on the survival of mice in a panel of preclinical models of infections and toxic shock. These observations support the safety of using propionate-based therapies in the treatment of inflammatory diseases without increasing the risk of infections.

In the second part, we characterized the innate immune status of mouse lines deficient in SIRT2, SIRT3, SIRT5, SIRT2/3 and SIRT3/5. We show that SIRT2 deficiency increased phagocytosis by macrophages and protected from staphylococcal infection, while SIRT3 and SIRT5 deficiencies had no effect on host susceptibility to infections. SIRT2/3 and SIRT3/5 deficiencies increased cytokine production. SIRT2/3 deficiency reduced glycolysis and protected mice from endotoxemia. SIRT3/5 deficiency increased the production of reactive oxygen species (ROS) and modulated immune cell frequencies in mice with little effect on host responses to infections. Altogether, our results suggest a crosstalk between sirtuins affecting innate immune responses.

In the last part, we analyzed the breadth of protection and the cellular mechanisms underlying the protection conferred by trained immunity. We show that training with a fungal cell wall preparation rich in ß-glucan protected from systemic, peritoneal, gastrointestinal and pulmonary infections. Training increased the number of bone marrow and splenic hematopoietic progenitors as well as blood monocytes and neutrophils. *In vitro*, training increased macrophage effector functions. Thus, training afforded a broad protection by increasing the number and the reactivity of innate immune cells.

Overall, our study uncovers crosstalk between sirtuins affecting innate immune responses that should be considered during the development of therapeutic drugs. Moreover, they support the development of drugs to target cells and cellular pathways involved in trained immunity to fight against infectious and inflammatory diseases.

RÉSUMÉ

Le système immunitaire inné représente la première ligne de défense contre l'invasion de pathogènes. Les cellules immunitaires innées expriment des récepteurs leur permettant de reconnaître un grand nombre de structures microbiennes et sont capables de moduler leur fonctionnalité en réponse à des stimuli externes. Des études récentes montrent que la reprogrammation des cellules immunitaires innées, un processus appelé « training », protège des infections. Les déacétylases d'histones (HDACs) forment une famille d'enzymes composée de 18 membres: HDAC1-11 et SIRT1-7. Les HDACs régulent des voies métaboliques et immunologiques. L'inhibition des HDACs induit une réponse pro-inflammatoire ou anti-inflammatoire selon le type de cellule, d'organe et de maladie étudié. Alors que la localisation et les cibles des HDACs se chevauchent, l'interaction entre HDACs est méconnue. Dans ce projet, nous avons étudié l'impact du propionate comme inhibiteur d'HDAC1-11, de déficiences en sirtuines (SIRT) et du training sur la réponse immunitaire innée et les défenses de l'hôte.

Le propionate, un acide gras à chaîne courte avec des propriétés anti-inflammatoires, est produit par les bactéries intestinales durant la fermentation de fibres. La réponse inflammatoire étant nécessaire à l'élimination de pathogènes, le propionate pourrait augmenter la susceptibilité aux infections. Dans nos expériences, le propionate inhibait la production de cytokines inflammatoires par les cellules immunitaires innées et adaptatives. Cependant, le propionate n'influençait pas la survie des souris dans des modèles d'infection et de choc toxique. Ces observations suggèrent que des thérapies utilisant du propionate dans le traitement de maladies inflammatoires n'augmentent pas le risque infectieux.

Dans la deuxième partie de ce travail, nous avons caractérisé des lignées de souris déficientes en SIRT2, SIRT3, SIRT5, SIRT2/3 et SIRT3/5 en terme de réponse immunitaire innée. Nous avons montré que la déficience en SIRT2 augmentait la phagocytose et protégeait les souris dans un modèle d'infection chronique à *Staphylococcus aureus*, alors que les déficiences en SIRT3 et SIRT5 n'avaient aucun effet sur la réponse anti-infectieuse. Les déficiences en SIRT2/3 et SIRT3/5 augmentaient la production de cytokines. La déficience en SIRT2/3 réduisait la glycolyse et protégeait de l'endotoxémie. La déficience en SIRT3/5 augmentait la production d'espèces réactives d'oxygène par les mitochondries et modulait les proportions de cellules immunitaires sans influencer la survie dans des modèles d'infection. En résumé, nos résultats montrent une interaction entre sirtuines affectant la réponse immunitaire innée.

Dans la dernière partie de cette thèse, nous avons analysé l'ampleur et les mécanismes cellulaires de la protection médiée par le training. Nous avons montré que le training induit par un composé fongique riche en ß-glucan protégeait d'infections systémiques, péritonéale, gastro-intestinale et pulmonaires. Le training augmentait le nombre de progéniteurs hématopoïétiques dans la moelle osseuse et la rate ainsi que de monocytes et neutrophiles dans le sang. *In vitro*, le training augmentait les fonctions effectrices des macrophages. Par conséquent, la protection induite par le training résulte de l'augmentation du nombre et de la réactivité des cellules immunitaires innées.

Dans l'ensemble, nos études révèlent des interactions entre sirtuines influençant la réponse immunitaire innée qu'il est important de considérer lors du développement de médicaments. De plus, nos résultats montrent que les cellules et les voies de signalisation impliquées dans le training sont des cibles intéressantes pour le traitement de maladies infectieuses et inflammatoires.

RÉSUMÉ DESTINÉ À UN LARGE PUBLIC

Le système immunitaire regroupe des barrières physiques (par exemple notre peau), des composés humoraux (les anticorps) et des cellules immunitaires (les globules blancs). Ces éléments protègent notre organisme face aux microbes. Les cellules immunitaires expriment des récepteurs reconnaissant les microbes, ce qui les active afin de mettre en place des mécanismes de défense. Les cellules immunitaires dites « innées » reconnaissent un large éventail de microbes. Au contraire, les cellules immunitaires dites « adaptives », connues comme étant les lymphocytes, reconnaissent des structures spécifiques à chaque microbe et génèrent des cellules mémoires, notamment lors de la vaccination, permettant une réponse rapide et efficace en cas de réexposition au même microbe.

Des études récentes ont montré que les cellules immunitaires innées peuvent aussi moduler leur réactivité selon les signaux qu'elles reçoivent de microbes ou d'autres cellule, augmentant ainsi leur réponse face à un agent infectieux. Ce phénomène est appelé « training », comme un apprentissage des cellules immunitaires innées à éliminer les microbes extrêmement rapidement.

Dans ce travail, nous avons étudié différents mécanismes modulant la réponse des cellules immunitaires innées et permettant leur apprentissage. Dans un premier temps, nous nous sommes focalisés sur une famille d'enzymes appelées les déacétylases d'histone (HDACs) et qui est composée de 11 HDACs (HDAC1-11) et de 7 sirtuines (SIRT1-7). Ces enzymes modifient d'autres protéines et régulent différents processus cellulaires. Nous avons montré que la répression d'HDAC1-11 avec un inhibiteur naturel produit par les bactéries de l'intestin (*i.e.* le propionate) ou l'absence de sirtuines chez des souris modifiées génétiquement influençait marginalement la fonction des cellules immunitaires et la survie des souris dans des modèles d'infections. Cependant, les souris chez lesquelles nous avons inactivé deux sirtuines (SIRT2/3 ou SIRT3/5) avaient une réponse immunitaire augmentée. Globalement, nos résultats suggèrent que les sirtuines peuvent avoir des activités complémentaires et que des médicaments ciblant plusieurs sirtuines, qui sont actuellement en essai clinique, pourraient induire des effets non anticipés lors de l'étude d'une seule sirtuine.

Par la suite, nous nous sommes concentrés sur les mécanismes d'apprentissage des cellules immunitaires innées. Nous avons montré que l'injection d'un composé de la paroi des champignons à des souris induit le training, ce qui les protégeait contre un grand nombre d'infections telles que la septicémie, la péritonite, la pneumonie et la gastro-entérite, et ceci pour une longue période. Ce traitement augmentait le nombre de cellules immunitaires innées dans le sang et augmentait la réactivité de ces cellules face à des microbes pathogènes.

Dans l'ensemble, ce travail démontre que les sirtuines peuvent, sous certaines conditions, moduler la réponse immunitaire innée et que le training affecte tant le nombre que la fonctionnalité des cellules immunitaires innées. Ainsi, nous avons identifié des cibles attractives pour le développement de nouveaux médicaments visant à traiter les infections et les maladies inflammatoires chroniques.

MAIN ABBREVIATIONS

ATP	Adenosine triphosphate
BCG	Bacillus Calmette-Guérin
CDS	Cytosolic DNA sensor
CLP	Cecal ligation and puncture
CLR	C-type lectin receptor
CMP	Common myeloid progenitor
CSF	Colony-stimulating factor
DAMP	Danger associated molecular pattern
DC	Dendritic cells
DNA	Deoxyribonucleic acid
ETC	Electron transport chain
FAO	Fatty acid oxidation
FDA	Food and Drug Administration (US)
FOXO	Forkhead transcription factor of class O
Foxp3	Forkhead box protein 3
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G-protein coupled receptor
GSDMD	Gasdermin D
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Inhibitor or histone deacetylase
HFD	High fat diet
HIF-1α	Hypoxia inducible factor 1α
HIV	Human immunodeficiency virus
HSC	Hematopoietic stem cell
IDH	Isocitrate dehydrogenase
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
LPS	Lipopolysaccharide
LT-HSC	Long-term hematopoietic stem cell
MAMP	Microbial-associated molecular pattern
M-CSF	Macrophage colony-stimulating factor
MDSC	Myeloid-derived suppressor cell
MPP	Multipotent progenitor
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor κB

NK	Natural killer cell
NLR	NOD-like receptor
OXPHOS	Oxidative phosphorylation
PRR	Pattern recognition receptor
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCFA	Short chain fatty acid
SIRT	Sirtuin
SOD	Superoxide dismutase
ST-HSC	Short-term hematopoietic stem cell
TCA	Tricarboxylic acid cycle (Krebs cycle)
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
TNF	Tumor necrosis factor

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1 INTRODUCTION

1.1 Innate immunity

Mammals are constantly exposed to potentially harmful microbes. The innate immune system represents the first line of defense with constitutive barriers, specialized cells and soluble mediators. Innate immune cells express pattern recognition receptors (PRRs) that sense the presence of microbial associated molecular patterns (MAMPs) or danger associated molecular patterns (DAMPs) released by injured or dying cells [1, 2]. To sense the broadest possible range of signals, PRRs are expressed at the surface (Toll-like receptors (TLRs) and C-type lectin receptors (CLRs)), in endosomes (TLRs) and in the cytoplasm (NOD-like receptors (NLRs), RIG-I like receptors (RLRs) and cytosolic DNA sensors (CDSs)) [3, 4]. PRRs exhibit some specificity towards different biochemical structures accounting for the overall vast panel of recognized microorganisms. When PRRs bind their ligands, they initiate a signaling cascade leading to the production of effectors molecules such as interleukins, interferons, chemokines, growth factors and antimicrobial molecules and the establishment of effector functions. Innate immune cells eradicate invading microorganisms through phagocytosis and killing, and stimulate the recruitment of innate and adaptive immune cells [5]. Innate immune cells are also instrumental for inflammation resolution and tissue repair [6]. Therefore, their activity has to be tightly regulated to avoid imbalanced, life-threatening, immune responses [7, 8].

1.1.1 Innate immune cells

The innate immune system is evolutionary ancient since it is present in all multicellular organisms from mammals to plants, and to crustaceans and insects. Innate immune cells were first described in the 19th century by Metchnikoff and Ehrlich [9] and classified as phagocytes and granulocytes based on their morphology observed using a microscope and on functional tests. Further studies on cellular ontogeny subdivided these cells into basophils, dendritic cells (DCs), eosinophils, mast cells, monocytes, macrophages and neutrophils. Innate immune cells were described to originate from common myeloid progenitors (CMPs) to terminally differentiated cells with little plasticity and no

memory. However, these ancient paradigms are constantly challenged, for instance following the identification of lymphoid cells with innate features (*i.e.* innate lymphoid cells (ILCs) including natural killer (NK) cells) [10] and memory characteristics in innate immune cells (see part 1.4 Trained immunity) [11]. Moreover, single cell analyses revealed that each group of innate immune cells shows substantial plasticity giving rise to heterogeneous cell populations that can be subdivided according to functional, metabolic and phenotypic variants [12]. Finally, accounting for the overall complex and diverse landscape of innate immune cells [13], novel cell types are regularly characterized, for example ILCs, myeloid-derived suppressor cells (MDSCs) and plasmacytoid DCs (pDCs) that all play a role in inflammation and infections.

Innate immune cells are tissue-resident or patrolling cells with different origins dictating their localization and function. Most tissue-resident macrophages and skin Langerhans cells originate from the yolk sac or derive from fetal liver monocytes during embryonic development and have self-maintaining abilities during adulthood [14, 15]. Other immune cells develop from self-renewing and multipotent hematopoietic stem cells (HSCs) and progenitors present in the bone marrow [16] (**Figure 1**). HSCs comprise long-term HSCs (LT-HSCs) that are mainly quiescent under steady-state [17] and give rise to short-term HSCs (ST-HSCs) with limited self-renewal potential and multipotent progenitors (MPPs) that are biased towards a specific cell lineage [16, 18]. According to mathematical and experimental models, LT-HSC undergo four divisions before losing their self-renewal and repopulation potentials [19]. At steady-state, HSCs poorly contribute to the production of blood cells, which is mainly supported by MPPs [20, 21]. The MPP pool comprises myeloid-biased (MPP2 and MPP3) and lymphoid biased (MPP4) cells [18]. Despite this lineage bias, MPPs maintain some plasticity to differentiate into both common myeloid progenitors (CMPs) and common lymphoid progenitors. CMPs and common lymphoid progenitors are lineage restricted progenitors that undergo successive maturation states to ultimately give rise to all cells circulating in the bloodstream [22, 23]. At homeostasis, the bone marrow releases mainly mature myeloid cells and immature B cells, but also

progenitors of T cells and DCs that will differentiate in the thymus or during their migration to peripheral tissues.

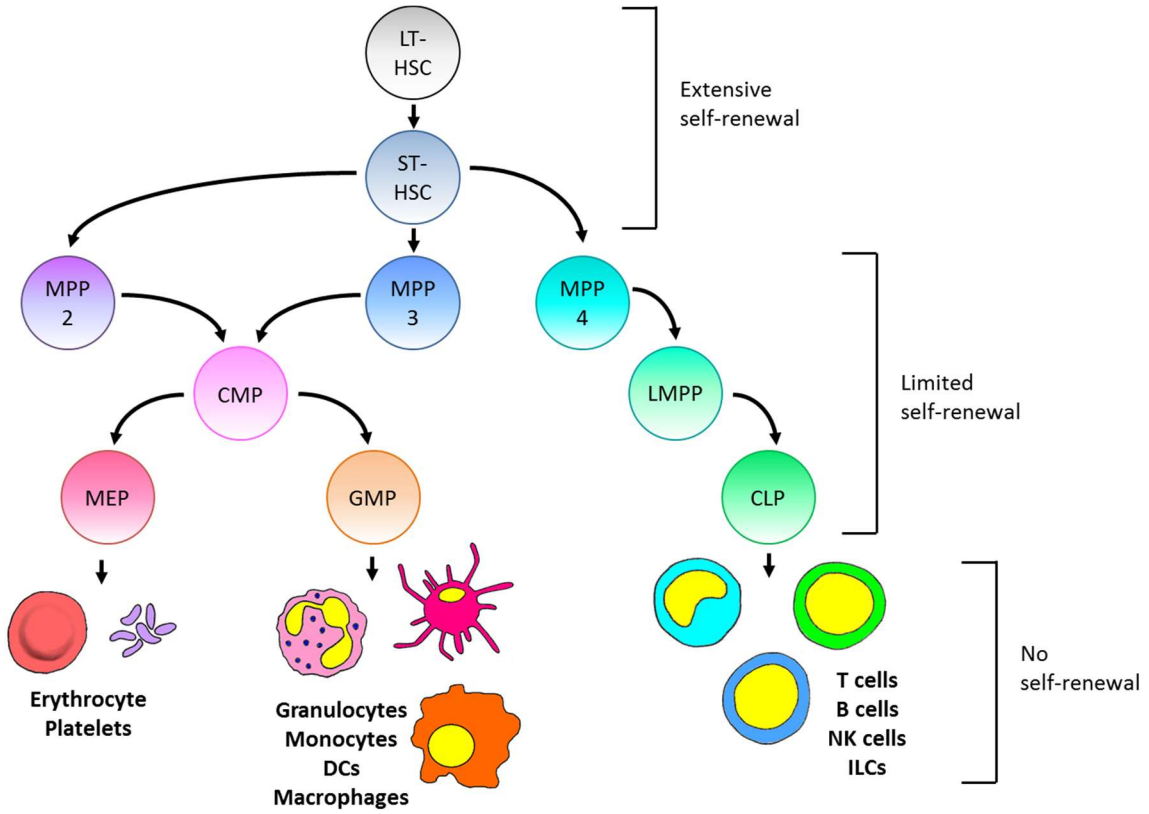


Figure 1: Hematopoiesis of immune cells. Hematopoietic stem cells (HSC) differentiate into lineage-restricted multipotent progenitors (MPPs) that give rise to common myeloid progenitors (CMPs) and lymphoid-primed-multipotent progenitors (LMPPs). These progenitors further differentiate into lineage-biased progenitors: megakaryocyte-erythrocyte progenitors (MEPs), granulocyte-macrophage progenitors (GMPs) and common lymphoid progenitors (CLPs). Finally, these cells generate mature innate and adaptive immune cells.

In this work, we mainly focused on neutrophils and macrophages. Neutrophils are quickly and massively recruited to the site of infection where they activate effector functions including degranulation, phagocytosis, production of reactive oxygen species (ROS) and release of neutrophil extracellular traps (NETs) [24]. NETs are composed of chromatin scaffolds containing histones and trapping antimicrobial peptides and enzymes (elastase, myeloperoxidase) that immobilize and kill pathogens [25]. Neutrophils display some degree of diversity with multiple activation states and functions in both inflammation and tissue repair [26]. Being unable to proliferate, neutrophils rely for

their heterogeneity on the subsequent activation and silencing of genes during their maturation. These genetic and epigenetic modifications are believed to underlie the heterogeneity of neutrophils with additional alterations according to environmental signals. Since the lifespan of neutrophils in the blood is under 24 hours, the mouse bone marrow produces daily an estimate of 10^7 mature neutrophils that egress into blood [26]. The release of neutrophils from the bone marrow is regulated by two surface molecules: CXC-chemokine receptor 2 (CXCR2) and CXCR4 [27]. Bone marrow stromal cells express CXC-chemokine ligand 12 (CXCL12) that interacts with CXCR4 expressed by pre-neutrophils and retains them in the bone marrow [28]. Mature neutrophils downregulate CXCR4 to leave the bone marrow and upregulate CXCR2 to sense chemoattractants (CXCL1-8) leading to their mobilization into tissues [27]. Neutrophils not recruited to organs within a day become "aged" with a hyper-reactive phenotype [29] and are eliminated from the circulation. Aged neutrophils upregulate CXCR4 that leads them back to the bone marrow triggering their removal and the release of young neutrophils [30]. These cycles of release and clearance follow a circadian rhythm at steady-state based on the need of the organism, including protection against pathogens during the active period and tissue regeneration during the resting period [31].

Macrophages form a family of specialized tissue-resident cells, including microglia, Kupffer cells, osteoclasts, peritoneal macrophages, red pulp macrophages and alveolar macrophages. Contrary to the long believed assumption that all tissue macrophages originate from circulating blood monocytes in adults, some tissue macrophages (microglia, alveolar macrophages and part of Kupffer cells) are long-lived self-maintaining cells seeded during fetal development by early embryonic precursors [32]. Nonetheless, blood monocytes are the main source of cells to increase the macrophage pool during an infection [33]. Macrophages are professional phagocytes constantly sampling their environment for MAMPs/DAMPs [34]. Numerous studies have highlighted the plasticity of macrophages. According to the signals they encounter, macrophages are activated to generate proinflammatory or anti-inflammatory responses through the production of molecules among which cytokines, ROS, reactive nitrogen species (RNS) and enzymes (arginase, indoleamine 2,3-dioxygenase

(IDO)). This led to the concept of M1-M2 macrophage polarization, paralleling the concept of T-helper (Th) cell polarization [35]. Macrophages activated with LPS (± IFNγ) are known as classically activated or M1 macrophages while macrophages activated with IL-4 and IL-13 are known as alternatively activated or M2 macrophages. M1 macrophages display powerful inflammatory and antimicrobial activities, while M2 macrophages are involved in the resolution of inflammation and promote adipose tissue homeostasis, tissue repair and resistance to helminth infection [36]. M1 polarizing signals activate STAT1/5, IRF5 and NF-κB transcription factors to regulate the expression of genes [37, 38], while M2 polarizing signals mainly induce STAT3/6 and IRF4 transcription factors [39, 40]. The resulting gene expression patterns induce different metabolic states dictating macrophage phenotype [41]. M1 macrophages shift their metabolism towards glycolysis comparably to cancer and activated T cells in a process called Warburg effect [42], while M2 macrophages generate ATP mainly through oxidative phosphorylation (OXPHOS) accounting for their role in restoring homeostasis [43]. Obviously, the M1 and the M2 polarization states of macrophages are only the extremes of a continuum of activations states [44, 45]. During an infection, both M1 and M2 macrophages are important to clear the pathogen and to restore homeostasis. Numerous diseases have been linked with an unbalanced proportion of proinflammatory and anti-inflammatory macrophages inducing persistent states of hyper-inflammation or hypo-inflammation [46].

1.1.2 Pattern recognition receptors (PRRs)

PRRs are expressed by progenitors, immature and mature immune cells [47] and non-immune cells (endothelial, epithelial and stromal cells) [3, 48]. Each cell type expresses a specific set of receptors at steady-state that can be modulated during inflammatory conditions. The intracellular pathways activated by the interaction between PRRs and MAMPs/DAMPs are partly or fully shared between PRRs accounting for similar immune responses to structurally different pathogens. Moreover, one MAMP/DAMP can be recognized by multiple PRRs and some PRRs show crosstalk leading to synergistic effects. Therefore, the activation of one PRR may be required for the full activation of another PRR or

its pathway [49]. We describe here the main families of PRRs and some of their molecular features and targets (**Figure 2, Table 1**).

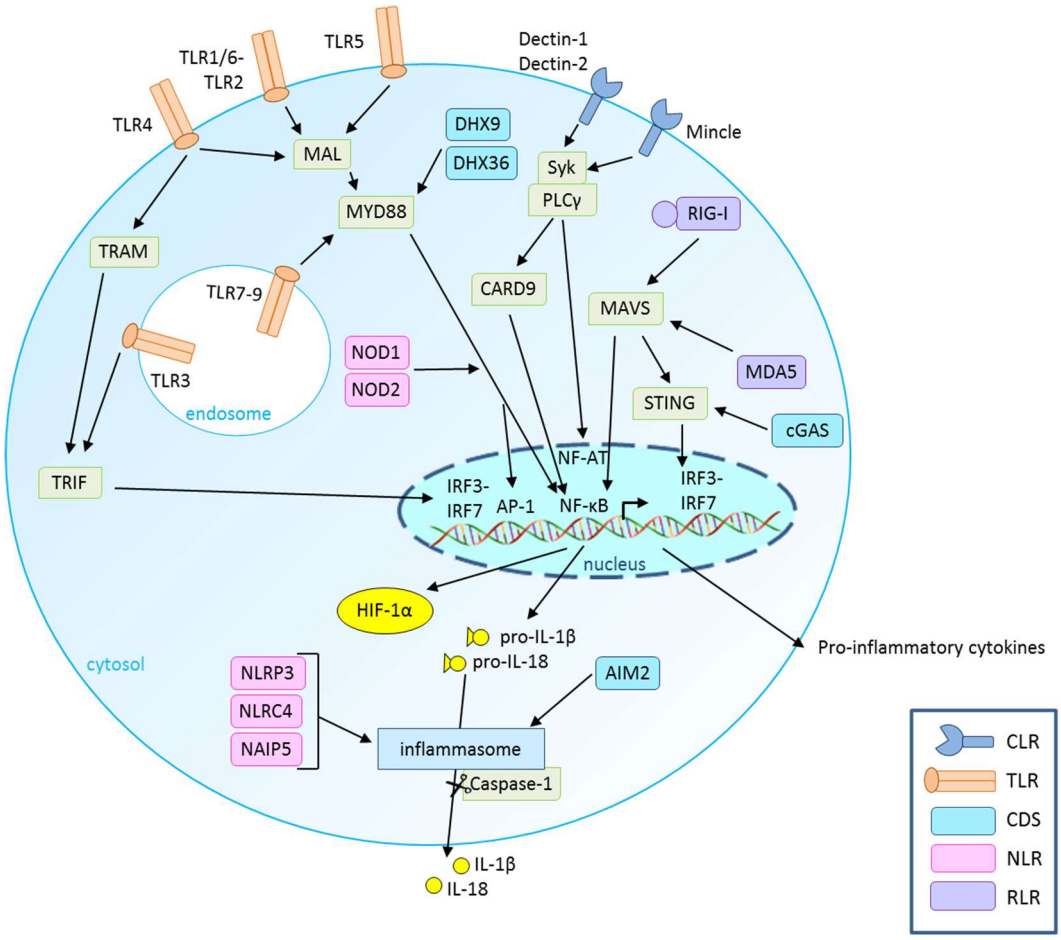


Figure 2: Representation of the five main families of pattern recognition receptors (PRRs). Toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-I like receptors (RLRs), NOD-like receptors (NLRs) and cytosolic DNA sensors (CDSs) are grouped based on functional and structural similarities.

TLRs. TLRs were the first PRRs described in the 1980s [50]. The TLR family comprises 10 members in human (TLR1-10) and 12 in mouse (TLR1-9 and TLR11-13) [51]. TLRs are expressed at the cell surface (TLR1-2, TLR4-6 and TLR10) or in endosomes (TLR3, TLR7-9 and TLR11-13) as homo- or heterodimers. Surface TLRs mainly recognize microbial cell wall structures (lipopolysaccharide (LPS), lipoteich acid, β-glucans, mannans, flagellin) and endosomal TLRs sense microbial nucleic acids (DNA or RNA). Upon ligand binding, TLRs recruit adaptor proteins (MyD88, MAL, TRIF, TIRAP or TRAM) to the cell membrane [52]. This interaction leads to the activation of mitogen-activated protein kinases (MAPKs),

nuclear factor κB (NF-κB) and interferon (IFN) regulatory factors (IRF) signaling pathways controlling the production of cytokines [53].

Table 1: Structures recognized by PRRs.

	PRR	Ligand	Pathogen or organism
CDS	cGAS	DNA	Microbes, self
	AIM2	DNA	Microbes, self
CLR	DC-SIGN	Mannose, N-mannan	HIV, fungi
	Dectin-1	β-glucans	Fungi
	Dectin-2	α-mannans, glycoproteins	Fungi, mycobacteria
	Mincle	α-mannose	Fungi, mycobacteria
NLR	NAIP5	Flagellin	Intracellular bacteria
	NLRC4	Flagellin	Intracellular bacteria
	NLRP3	ATP, crystals, pore forming toxins, K^+ efflux, $Ca2^+$ mobilization, cathepsin, ROS	Bacteria, viruses, self
	NOD2	MDP	Bacteria
RLR	LGP2	Unknown	RNA viruses
	MDA5	Long dsRNA	RNA viruses
	RIG-I	Short dsRNA	RNA and DNA viruses
TLR	TLR2/6	Lipopetides, zymosan	Gram positive bacteria, fungi
	TRL3	dsRNA	Viruses
	TLR4	LPS	Gram negative bacteria
	TLR5	Flagellin	Bacteria
	TLR7, 8	ssRNA	Viruses
	TLR9	CpG ODN	Bacteria, viruses

DNA: deoxyribonucleic acid; RNA: ribonucleic acid; ATP: adenosine triphosphate; MDP: muramyl dipeptide; dsRNA: double stranded RNA; ssRNA: single stranded RNA; CpG ODN: CpG oligodeoxynucleotides.

NLRs. NLRs are with TLRs the PRRs that have generated the greatest interest. The family of NLRs comprises intracellular receptors sharing conserved structural domains. The human genome encodes for 22 NLRs and over 30 *Nlr* genes have been described in mice [54]. NLRs are involved in the detection

of intracellular bacteria [54], viral components, and products of sterile inflammation [55, 56]. NLRs are expressed in the cytoplasm at steady-state in an auto-inhibitory conformation that upon stimulation turns active and allows the oligomerization of the molecules. Triggering NLRs leads either to the direct activation of downstream NF-κB or MAPK signaling pathways or to the activation of the receptor-related multiprotein complex called inflammasome [57, 58]. The two best described inflammasomes, NLRC4 and NLRP3, do not act as direct receptors of MAMPs/DAMPs. Instead, their activation is initiated through indirect mechanisms. The NLRC4 inflammasome requires the sensing of flagellin by NAIP5 which forms a platform enabling NLRC4 oligomerization [59]. NLRP3 oligomerization is initiated by a broad range of MAMPs/DAMPs (ATP, crystals, pore forming toxins, K^+ efflux, Ca^{2+} mobilization, cathepsin and ROS among others), suggesting that NLRP3 does not directly interact with these signals but responds to general stress signals (**Figure 3**). Oligomerized NLRs recruit trough their caspase recruitment domain (CARD) or pyrin domain (PYD) the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) that in turn recruits pro-caspase-1 protease through CARD-CARD interaction [60]. Autoproteolysis of pro-caspase-1 leads to its activation into caspase-1 that cleaves its substrate gasdermin D (GSDMD) into C-terminal GSDMD (GSDMD-C) and GSDMD-N, and pro-interleukin 1β (IL-1β) and pro-IL-18 into IL-1β and IL-18. GSMD-Ns oligomerize and form pores in cell membrane, allowing the release of IL-1β and IL-18. The pores also allow the release of other intracellular small molecules and mediate pyroptosis, a programmed cell death [61]. Besides the canonical inflammasomes decribed above, non-canonical inflammasomes can also induce pyroptosis and NLRP3 inflammasome activation (**Figure 3**). To trigger non-canonical inflammasome, intracellular LPS activates mouse caspase-11 or human caspase-4 and 5 that cleave GSDMD and induce pore formation. This leads to pyroptosis and an efflux of K^+ that triggers NLRP3 inflammasome, caspase-1 activation and pro-IL-1β and pro-IL-18 maturation and secretion [62].

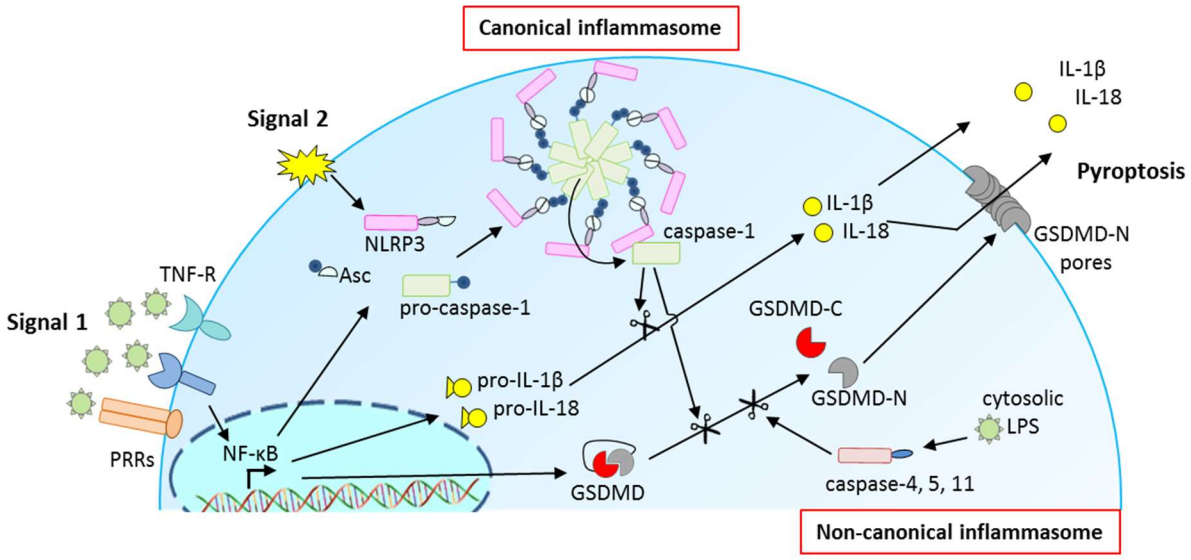


Figure 3: Canonical and non-canonical inflammasome activation. Signal 1 (PAMPs, DAMPs) is sensed by PRRs or TNF receptor (TNF-R) activating NF-κ signaling and enhancing the transcription of inflammasome components (caspases, NLRP3, Asc), gasdermin D (GSDMD) and pro-cytokines (IL-1β and IL-18). Signal 2 (ATP, crystals, ROS, K^+ efflux,…) induces canonical inflammasome oligomerization and pro-caspase-1 autoproteolysis. Active caspase 1 cleaves pro-IL-1β and pro-IL-18 into their mature form and GSDMD. Cytosolic LPS activates non-canonical inflammasomes (caspase-11 in mouse, caspases-4 and 5 in human) that cleave GSDMD leading to N-terminal GSDMD (GSDMD-N) pore formation and pyroptosis.

CLRs. CLRs are represented by over 1'000 receptors subdivided in 17 subfamilies according to their structure [63]. CLRs are either soluble or membrane-bound at the cell surface. Soluble CLRs, including collectins (Mannose binding lectin (MBL), surfactants) opsonize microorganisms to promote phagocytosis [64]. Membrane-bound CLRs are well known to interact with fungal structures, but they also recognize DAMPs and motifs from bacteria, viruses and parasites [65]. Indeed, dectin-1, dectin-2 and mincle are membrane-bound CLRs recognizing polysaccharides expressed at the surface of fungi, bacteria or viruses. Dectin-1 forms a homodimer to bind β-glucans, the most abundant polysaccharides of fungal cell walls. Dectin-2 and mincle interact with dectin-3 as heterodimers to bind mannans [66]. All three dimers signal through their intrinsic or adaptor-recruited immunoreceptor tyrosine-based activation motifs (ITAMs) to activate the SYK kinase and induce an intracellular signaling cascade [67]. These pathways lead to the activation of NF-κB or nuclear factor of activated T

cells (NF-AT) inducing the production of pro-inflammatory or regulatory cytokines, respectively. CLRs can also induce the production of ROS promoting the activation of the NLRP3 inflammasome that controls the maturation and secretion of IL-1β. Moreover, dectin-1 was described to form a non-canonical inflammasome with SYK, caspase-8 and Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) to promote IL-1β maturation [68].

RLRs. RLRs play a major role during viral infection. The RLR family comprises two functional receptors, retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5), and one regulatory receptor, laboratory of genetics and physiology 2 (LGP2) [69]. The three receptors have different affinities for viral double-stranded RNA (dsRNA) structures implying that one virus can be recognized by one or multiple RLRs [70]. The detection of viral dsRNA induces a conformational change and activates RLRs. Activated RIG-I and MDA5 bind their adaptor protein MAVS (also known as IPS-1/VISA/Cardif) to induce a signaling cascade leading to the production of type I IFNs and other cytokines to mount antiviral host defense mechanisms [71]. The expression of RLRs is upregulated during a viral infection through IFN-dependent and independent signals leading to an increased sensitivity in viral detection [72].

CDSs. The presence of DNA in the cytosol underlies the presence of an invasive microorganism or cellular stress. These nucleic acid structures are sensed by CDSs that comprises various intracellular proteins that can be structurally unrelated among which absent in melanoma 2 (AIM2), cyclic GMP-AMP synthase (cGAS), DAI, DDX41, DHX9, DHX36, IFI16 [73]. AIM2 and cGAS are reported to directly induce the production of type I IFNs [74, 75]. Moreover, after induction with type I IFNs, AIM2 forms an inflammasome allowing a concerted mode of action between cGAS and AIM2 [76]. CDSs also support other PRRs to induce innate responses. For instance, the RNA polymerase III converts cytosolic dsDNA rich in AT into RNA recognized by RIG-I [77, 78].

1.1.3 Cytokines in defense mechanisms

The recruitment and activation of immune cells are controlled by intercellular activating and inhibiting signaling molecules called cytokines acting in autocrine, paracrine and endocrine fashion [79]. Cytokines include colony-stimulating factors (CSF), chemokines, interferons (IFNs), interleukins (ILs) and tumor necrosis factor (TNF) and transforming growth factor beta (TGF-β) families [80]. The magnitude and temporality of cytokine production is cytokine, pathogen and signaling-dependent. During an uncontrolled infection, cytokines travel through the bloodstream to reach the bone marrow, where they induce the proliferation and differentiation of myeloid cells in a process called "emergency myelopoiesis" [81]. This process leads to the egress of a large number of mature and immature innate immune cells from the bone marrow to fight pathogens and replace dying leukocytes. Progenitors migrate to lymphoid tissues, including spleen and liver, where they promote extramedullary hematopoiesis niches [82].

CSFs promotes myelopoiesis towards a specific cell type, for instance granulocyte-CSF (G-CSF) and macrophage-CSF (M-CSF) bias cell differentiation towards granulocytes and macrophages, respectively [83]. Chemokines are divided in C, CC, CXC, CX_3C and C subfamilies according to the position of the N-terminal cysteine (**Figure 4**) [84]. Chemokine receptors allow the migration of target cells through chemokine gradients [85]. Immune cell subpopulations express specific panels of chemokine receptors and modulate their expression upon environmental cues suggesting a dynamic migration pattern [86-88]. During the acute phase of an infection, CXCL1-8 and CCLs bind CXCR2 expressed by neutrophils and CCR2 expressed by inflammatory monocytes, respectively.

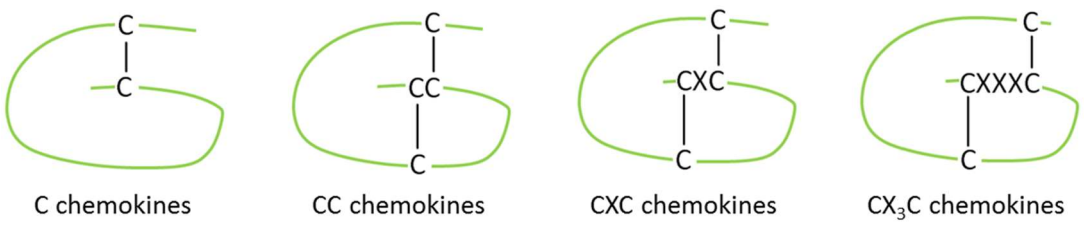


Figure 4: Cysteine and disulfide bonds in chemokine subfamilies. C: cysteine; X: amino acid other than cysteine.

TNF, IFNs and ILs recruit and activate immune cells in a cytokine, cell and context dependent manner. TNF, originally known as cachectin, is one of the first cytokines produced during an infection. The main source of TNF is monocytes/macrophages, while it is also produced at low levels by other cell types such as dendritic cells, mast cells, lymphoid cells, endothelial cells, cardiomyocyte and, adipocytes. TNF acts through two receptors: TNFR1 expressed in most tissues and TNFR2 expressed mainly by immune cells [89]. Accordingly, TNF is involved in systemic inflammation and has broad biological roles including acute phase response, fever, apoptotic cell death and vasodilatation [90].

Type I IFNs (IFNα, β, κ, ε, τ, ω, χ) and type III (IFNλ1-3, *i.e.* IL-29/28A/28B) are mainly associated with antiviral mechanisms while type II IFN (IFNγ) plays a major role in the fight against bacterial, fungal and parasitic infections [91]. IFNα and IFNβ are mainly produced by pDCs and fibroblasts, respectively, albeit lymphocytes, macrophages, endothelial cells and osteoclasts can also produce type I IFNs. Nearly all cells of the body have receptors to sense IFNα and IFNβ enabling them to reduce viral replication. IFNγ is produced primarily by activated T-cells and NK cells and acts as a potent activator of macrophage functions.

Since more than 40 ILs have been described, we will not discuss all of them but just pinpoint to some important features. Of note, the family of ILs also include the chemokine CXCL8 (IL-8) and the type III IFNs mentioned above (IL-29/28A/28B). ILs have pleiotropic activities and act locally or systemically. ILs are particularly important to shape innate and adaptive immune responses by mediating pro-inflammatory, anti-inflammatory and regulatory functions. IL-1β, IL-12 and IL-18 are pro-inflammatory cytokines that activate antimicrobial functions and promote immune cell proliferation and differentiation. IL-6 activates macrophages and lymphocytes and is a main driver of the acute phase response [92]. TGF-β, IL-10 and IL-1RA counterbalance pro-inflammatory responses by promoting an alternative activation of immune cells with anti-inflammatory and tissue repair properties or by blocking the activity of inflammatory cytokines. ILs are also determinant in the differentiation of naïve CD4 T cells into T helper cells (Th1, Th2, Th17) and T regulatory (Treg) cells

through IL-12 (Th1), IL-2 and IL-4, (Th2), IL-6, IL-21, IL-23 and TGF-β (Th17) and IL-10 and TGF-β (Treg). Th1, Th2 and Th17 cells are characterized by the production of IL-2, TNF and IFNγ (Th1), IL-4, IL-5, IL-6, IL-13 and IL-25 (Th2) and IL-17, IL-21 and IL-22 (Th17). These mediators are important to fight against intracellular pathogens (Th1), extracellular parasites (Th2) and extracellular bacteria and fungi (Th17). Treg cells produce IL-10, IL-35 and TGF-β to regulate immune responses and induce immune tolerance. The speed at which cytokine genes are transcribed depends on the signaling cascade, but also on their chromatin conformation. The promoters of primary response genes, *i.e.* genes rapidly expressed in response to a signal such as *TNF* and *IL1* genes, are in a state of constitutive open chromatin allowing a quick transcriptional response [93]. Secondary response genes such as *IL6* and *IL12* genes require nucleosome remodeling to enable transcription.

Improper control of pathogens and dysregulation of the immune system with an imbalance between pro- and anti-inflammatory contributions can lead to organs failure and a clinical syndrome known as sepsis [94]. According to a consensus definition released in 2016, sepsis is defined as a "life-threatening organ dysfunction due to a dysregulated host response to infection" [95]. Sepsis is the leading cause of death from infection affecting an estimate of 20-30 million people per year worldwide with around 20% mortality during the first days after sepsis onset [96]. Moreover, sepsis survivors suffer from long-term sequelae. Unfortunately, sepsis incidence is rising due to aging of the population and increasing burden of chronic diseases, number of immunocompromised patients, use of invasive procedures for treatments and resistance of microorganisms to antimicrobials. Susceptibility to sepsis is influenced by the pathogen (type, load, virulence, infectivity, antimicrobial resistance), the host (age, sex, genetic, immune status, comorbidities, nutrition, vaccine, medication) and the environment (temperature, pollution, nutritional context, health care access, therapy). Patients with sepsis show a concomitant inflammatory and immunoparalysis status associated with important metabolic disturbances [97]. Current research based on single-cell techniques aims at unravelling the immune cells involved in sepsis, characterizing their metabolism, functionality and genetic or epigenetic markers to use them as biomarkers or targets for sepsis adjunctive therapies [98].

1.2 Histone deacetylases (HDACs)

Acetylation of histone tails is a major epigenetic modification affecting chromatin structure, recruitment of chromatin-binding proteins and gene transcription [99]. Histone acetylation is a dynamic process regulated by histone acetyl transferases (HATs) and histone deacetylases (HDACs). Histones acetylation is linked to transcriptionally active chromatin (euchromatin) with facilitated recruitment of polymerase II (Pol II) and its coactivators, while deacetylation compacts the chromatin (heterochromatin) and represses gene transcription (**Figure 5**) [100]. Mammalian genomes encode for eighteen HDACs divided in eleven Zn^{+}-dependent classical HDACs (HDAC1-11) and seven NAD^{+}-dependent sirtuins (SIRT1-7). HDACs have been further divided in five classes according to their sequence homologies to yeast proteins, their localization and their enzymatic activity [101]. Class I HDACs (HDACs 1, 2, 3, 8) are homologous to yeast *Rpd3*, class IIa (HDACs 4, 5, 7, 9) and class IIb HDACs (HDACs 6, 10) to *Hda1*, class III sirtuins (SIRT 1-7, see **section 1.3**) to *Sir2*. Class IV HDAC (HDAC 11), the smallest HDAC, has similarities with both class I and II HDACs. All classical HDACs are mainly located in the nucleus although class II HDACs can shuttle to the cytoplasm (**Table 2**). Sirtuins are located in the nucleus, cytoplasm or mitochondria. Albeit HDACs were first reported to target histones, it has become clear that they also target thousands of non-histone proteins and catalyze other protein modifications including succinylation, malonylation and glutarylation [102]. Therefore, HDACs regulate numerous biological processes linked to epigenetics, metabolism and immunity [103].

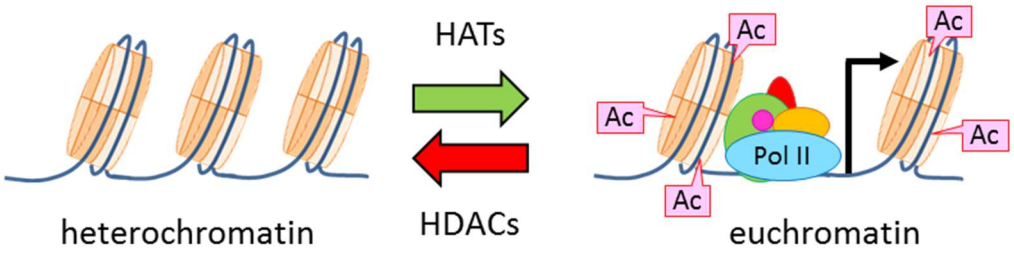


Figure 5: Histone acetylation and deacetylation regulate chromatin opening and pendant gene transcription. Acetylation by histone acetyltranferases (HATs) opens chromatin leading to recruitment of polymerase II (Pol II) and its coactivators and enables gene transcription. Histone deacetylases (HDACs) remove acetyl groups from histones hindering gene transcription. Ac: acetylation. Adapted from [104].

Table 2: Localization and knocking out of HDACs in mouse.

Class	Enzyme	Localization	Enzymatic activity	KO mouse phenotype
Class I	HDAC 1	Nucleus	Deacetylase, decrotonylase	Embryonic lethal [105]
	HDAC 2	Nucleus	Deacetylase, decrotonylase	Perinatal lethal [105]
	HDAC 3	Nucleus	Deacetylase, decrotonylase	Embryonic lethal [106, 107]
	HDAC 8	Nucleus	Deacetylase, de-fatty-acylase	Perinatal lethal [108]
Class IIa	HDAC 4	Nucleus / Cytoplasm	Deacetylase	Perinatal lethal [109]
	HDAC 5	Nucleus / Cytoplasm	Deacetylase	Viable [110]
	HDAC 7	Nucleus / Cytoplasm	Deacetylase	Embryonic lethal [111]
	HDAC 9	Nucleus / Cytoplasm	Deacetylase	Viable [112]
Class IIb	HDAC 6	Cytoplasm / Nucleus	Deacetylase	Viable [113]
	HDAC 10	Cytoplasm / Nucleus	Deacetylase	Not reported
Class III	SIRT 1	Nucleus	Deacetylase, decrotonylase	Perinatal lethal [114]
	SIRT 2	Cytoplasm / Nucleus	Deacetylase, demyristylase, decrotonylase	Viable [115]
	SIRT 3	Mitochondria	Deacetylase, decrotonylase	Viable [116]
	SIRT 4	Mitochondria	ADP-ribosyltransferase, lipoamidase	Viable [117]
	SIRT 5	Mitochondria	Deacetylase, demalonylase, desuccinylase, deglutarylase	Viable [118]
	SIRT 6	Nucleus	Deacetylase, ADP-ribosyltransferase, deacylase	30-day lethal [119]
	SIRT 7	Nucleolus	Deacetylase	Viable, short lifespan [120]
Class IV	HDAC 11	Nucleus	Deacetylase, demyristoylase	Viable [121]

1.2.1 Role of classical HDACs

Despite their chromatin remodeling function, HDACs lack a DNA binding motif requiring their integration in multiprotein complexes to modulate histone acetylation supporting an interaction and possible regulation of non-histone proteins. Class I HDACs interact with nuclear proteins to form activating or inhibiting complexes and directly affect the transcription of HDAC-specific gene subsets [122]. HDAC1 and HDAC2 have some redundant functions and can substitute each other. They play a role in cell cycle regulation, DNA damage repair and cell development appraised by the observation that germline deletion of any class I HDAC in mouse is lethal (**Table 2**). Class IIa HDACs have a structurally larger catalytic site compared to other HDACs and are therefore unable to produce an

effective deacetylation reaction [123]. However, class IIa HDACs were not silenced during evolution and are hypothesized to repress gene expression independently of their catalytic site, for instance by serving as a scaffold for the recruitment of proteins including HDACs of other classes.

HDAC6 is the best-described class IIb HDAC promoting DNA repair in the nucleus and deacetylating tubulin in the cytoplasm [124, 125]. Tubulin acetylation stabilizes the microtubule network linking the activity of HDAC6 to cell motility but also to defective intracellular transport underlying neurodegenerative diseases [126]. HDAC10, the second class IIb HDAC, promotes autophagy and polyamine metabolism by directly deacetylating components of these pathways [127, 128]. The characterization of HDAC11, the only class IV HDAC, is elusive with little knowledge on possible targets. HDAC11 has been reported to promote inflammation in immune cells and to reduce lipid oxidation [121, 129]. Mice deficient in HDAC11 show a decreased weight gain when fed a high fat diet (HFD).

1.2.2 Classical HDACs in innate immune cells and during infections

MAMPs/DAMPs affect the expression of HDACs in an enzyme, cell and stimulus-dependent manner [130, 131]. In macrophages, LPS rapidly upregulates HDAC1 and HDAC9 expression and transiently reduces the expression of HDACs 4-8 and HDACs 10-11 [130]. However, measuring the impact of HDACs on cellular immune responses is complex and controversial results have been reported. HDACs affect multiple cellular pathways with complex stimulatory and inhibitory networks omitted in studies focusing one specific pathway or molecule. Moreover, HDACs have been mainly studied using inhibitory (pan) drugs (HDACi, see **section 1.2.3**) with limited understanding on the mechanism of action and the impact on other cellular components, at the expense of targeting specific HDACs.

Dynamic epigenetic modifications play an important role during immune cell development and lineage commitment for subsequent activation and silencing of specific genes. HSCs show increased histone acetylation in line with low levels of HDACs. Several HDACs are expressed in progenitors at different stages of immune cell development accounting for progenitor-specific

acetylation patterns [132]. Inhibiting single or multiple HDACs affects cell development and progenitor commitment. HDACi promote the development of monocytes and granulocytes over erythrocytes [133] and impair the maturation of DCs [134]. Class I HDACs promote cell proliferation and deficiency in either HDAC 1, 2 or 3 results in cell cycle arrest and impaired cellular differentiation [135, 136]. Mice deficient in HDAC 1-4 show cell proliferation defects during development and are non-viable [137]. The role of HDACs in immune cell development implies that their expression is tightly regulated at the transcriptional and post-translational levels [138].

In macrophages, inhibition of HDACs reduces bacterial phagocytosis and killing and decreases cytokine production [139, 140]. HDACi powerfully inhibit the expression of secondary response genes encoding for inflammatory cytokines (*IL6*, *IL12b*) but marginally impact on the expression of the primary response genes *TNF* and *IFNb* [141, 142]. HDAC3 promotes M1 over M2 polarization of macrophages by repressing the expression of M2 related genes [143, 144]. However, HDAC11 and HDAC6 inhibit the production of IL-10 in a concerted way [145]. Treatment of neutrophils with HDACi before stimulation induces hyperacetylated NETs with increased capacity to activate macrophages [146]. HDAC11 limits neutrophil migration, phagocytosis and production of inflammatory cytokines [147]. HDAC11 was also proposed to regulate the expansion and function of MDSCs in tumor models. However, HDAC11 suppression concomitantly increases the inflammatory response of neutrophils and the immunosuppressive phenotype of MDSCs [148].

1.2.3 Classical HDAC inhibitors (HDACi)

An epigenetic mark found in multiple cancers is the loss of acetylation on histone 4 at lysine 16 (H4K16) [149]. Overexpression or mutation of multiple HDACs are observed in tumors and often associated with disease progression and poor patient outcome [150]. Dysregulated HDAC expression is generally associated with altered gene expression and aberrant cell functionality. However, contradictory observation have been reported in the literature, suggesting HDAC and cell type-dependent effects [151]. Besides tumorigenesis, several HDACs have been associated with neurodegenerative diseases.

Histone acetylation is important in memory formation with dynamic variations upon neuronal activity [152]. Overexpression of HDAC2 impairs synaptic plasticity and memory formation while HDAC2 deficiency reverses the phenotype [153]. Patients with Alzheimer's diseases have increased levels of HDAC2 [154] while HDAC5 and HDAC9 are linked to schizophrenia [155, 156]. These findings support the clinical development of HDACi, with numerous molecules in the pipeline.

Valproic acid (VPA) was first synthesized in 1882 and licensed to treat epilepsy and bipolar disorder in 1967 in France, then approved by the Food and Drug Administration (FDA) in 1978. VPA was shown later to inhibit HDACs [157] and thus represents the first HDACi ever marketed for clinical applications. More recently, HDACi have been developed primarily as anticancer agents. The second marketed HDACi is SAHA (also called vorinostat), approved by the FDA in 2006 for the treatment of cutaneous T cell lymphoma [158]. Three other HDACi have been approved since then: romidepsin and belinostat for the treatment of cutaneous and/or peripheral T-cell lymphoma, and panobinostat as a third line treatment for multiple myeloma [159]. Many other HDACi are tested in clinical trials, including the short-chain fatty acids (SCFAs) produced by the gut microbiota (see **section 1.2.4**). The small number of approved drugs is linked to the limited understanding of their mechanisms of action and to side effects due to the large number of processes impacted by HDACs [160]. Current research aims at developing not only HDAC-specific inhibitors and pan-HADCi, but also inhibitors targeting the multiprotein complexes HDACs are part of [161]. However, it is important to test the effect of HDACi on non-targeted diseases and opportunistic infections to foresee possible adverse reactions (reviewed in [104]).

HDACi are protective in murine models of cecal ligation (CLP) and puncture with decreased mortality [162], lung injury [163] and memory loss in survivors [164]. Mice treated with VPA are protected against lethal CLP, and lipopeptide or LPS-induced toxic shock, but are more sensitive to non-severe infections with *Klebisella pneumoniae* and *Candida albicans* [141, 165]. In viral infections with human immunodeficiency virus (HIV) or herpesvirus, inhibition of HDACs reverses viral latency

and accelerate viral replication [166, 167]. Therefore, HDACi are promising latency-reversing agents to purge HIV reservoir. Other HDACi directly abrogated viral replication [168-170].

1.2.4 Short chain fatty acids (SCFAs) inhibit HDACs

SCFAs are chemically defined as organic molecules with a backbone of one to six carbons. SCFAs are abundant metabolites of the mammalian gut, primarily produced by the fermentation of complex carbohydrates in the large intestine by the gut microbiota [171]. The most abundant SCFAs in human are acetate (C2), propionate (C3) and butyrate (C4) (**Figure 6**), reaching all together 50-100 mM in the colonic lumen [172]. Most of SCFAs are absorbed in the colon since only 5-10% of their production are found in the feces [173]. SCFAs can enter the portal circulation to act as a source of SCFAs in the bloodstream and reach any tissue. At the cellular level, SCFAs are sensed by membrane bound G protein coupled receptors (GCPRs), or enter the cell through passive diffusion or active transport [174]. SCFAs increase the acetylation of histones, with butyrate exhibiting the strongest activity [175-177]. SCFAs act as HDACi by reducing HDAC expression or through direct binding to HDACs [178, 179].

Butyrate is mainly used as an energy source in the colon while propionate is metabolized in the liver, leading to rather low concentrations of these SCFAs in the blood when compared to acetate. Acetate crosses the blood brain barrier (BBB) to regulate satiety [180]. From an immunological perspective, SCFAs are immunosuppressive compounds. SCFAs binding to the GPCRs GPR41, GPR43 and GPR109a inhibit p38 and c-Jun N-terminal kinase (JNK) MAPK pathways to reduce pro-inflammatory cytokine production while enhancing the release of anti-inflammatory cytokines [181, 182]. GPR43 and GPR109a deficient mice exhibit increased production of inflammatory mediators and recruitment of immune cells, mainly granulocytes, in the intestine [181, 183].

SCFAs maintain homeostasis and reduce inflammation in multiple tissues and cell types (**Figure 6**). In the gut, SCFAs promote epithelial barrier integrity and the production of mucus by goblet cells [184]. The mucus layer creates a gradient of SCFAs decreasing from the lumen to the laminal surface enabling epithelial cells to sense variations in overall SCFAs concentration. SCFAs also interact

with the immune cells present in the lamina propria. Butyrate modulates histone acetylation and reduces the secretion of pro-inflammatory cytokines by macrophages [185]. Butyrate reduces the glycolytic capacity and mTOR activation in macrophages but promotes bacterial killing through histone acetylation-mediated increased production of antimicrobial effector molecules [186]. Butyrate and propionate, acting as HDACi on HDAC6 and HDAC9, increase the acetylation of the transcription factor forkhead box 3 (Foxp3) to promote the proliferation and the suppressive function of Tregs [187, 188]. The homeostatic effects of SCFAs are transmitted to the offspring reinforcing the notion that SCFAs act as epigenetic modulators [189].

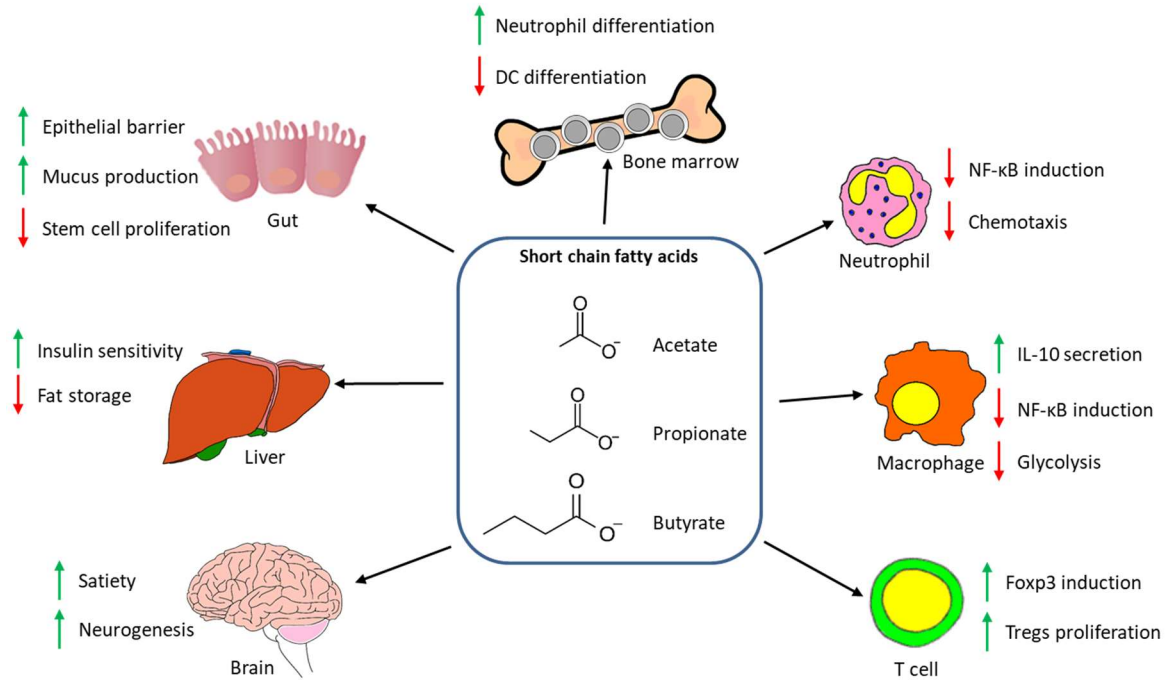


Figure 6: Impact of short chain fatty acids (SCFAs) on organs and immune cells. The main SCFAs are propionate, acetate and butyrate. SCFAs promote mucus production and the integrity of the epithelial barrier in the gut protecting the organism from pathogen invasion and keeping the microbiota at distance from the cells. SCFAs circulate through the bloodstream to maintain insulin sensitivity and reduce fat storage in the liver. Acetate is transported to the brain where it promotes the feeling of satiety and induces the maturation of microglia. In the bone marrow, SCFAs promote granulopoiesis in detriment of DC maturation. At the cellular level, SCFAs reduce the pro-inflammatory profile of immune cells and promote the secretion of anti-inflammatory cytokines. In T cells, SCFAs promote regulatory T cells (Tregs) proliferation and activity.

The immunosuppressive effects of SCFAs promoted their study in inflammatory diseases and in carcinogenesis [190], and treatments are in development with SCFAs supplementation or drugs targeting GPR41 and GPR43. Oral supplementation of butyrate reduces NF-κB activation, increases the number of Tregs and protects mice against colitis [191, 192]. SCFAs are also protective against colonic cancer through their HDACi activity and as an unfavorable source of energy for cancer cells that mainly use glucose [180]. The dissemination of SCFAs in the organism leads to its protective effect on distal organs, including liver, lungs and brain. Supplementation with butyrate of mice fed with HFD increases insulin sensitivity and protects against obesity [193]. SCFAs reduce the severity of airway allergic diseases including asthma by downregulating the Th2 and DC responses and neutrophil recruitment in the lungs [194-196]. Butyrate has shown controversial results in cancer prevention [197, 198] and recent research suggests that the individual response to butyrate treatment could be microbiota dependent [199]. The anti-inflammatory effect of SCFAs questions their impact on infectious diseases. Acetate and butyrate are protective during infection with *Citrobacter rodentium* [186, 200]. SCFAs show a protective effect against major foodborne pathogens including *Salmonella* spp, *Escherichia coli*, *Shigella* spp and *Listeria monocytogenes* [201]. The protective effect of SCFAs could be mediated by direct inhibition of bacterial growth, promotion of antimicrobial function of immune cells or maintenance of epithelial barrier integrity [186].

1.3 Sirtuins (SIRT)

The human and murine genomes encode for seven sirtuins mainly located in the nucleus (SIRT1, SIRT6 and SIRT7), the cytoplasm (SIRT2) and the mitochondria (SIRT3, SIRT4 and SIRT5). However, many sirtuins have been observed in other organelles implying that they can shuttle between subcellular compartments [202]. Although sirtuins were first described as deacetylases, several sirtuins have a weak deacetylation activity and preferably remove other functional groups including succinyl, malonyl, acyl and glutaryl. Sirtuins target thousands of histone and non-histone proteins and affect multiple cellular pathways.

Sirtuins gained interest in the 90's when their yeast homologs, the SIR proteins, were shown to increase the lifespan of *Saccharomyces cerevisiae* [203, 204]. This effect was later linked to caloric restriction in yeast [205] and generalized to other organisms including *Drosophila* [206] and mice [207, 208]. During caloric restriction, the metabolism of carbohydrates and fats is altered leading to an increased level of intracellular NAD^+ and promoting the activity of sirtuins [209]. In mice, caloric restriction increases the expression of sirtuins while feeding HFD decreases their expression in muscle and heart tissues. Yet, caloric restriction increases the lifespan of organisms independently of sirtuin expression [210, 211]. In humans, multiple studies addressed the association between genetic variations in *Sirtuin* genes and longevity with controversial results. SIRT1 and SIRT3 were decreased with age and few allelic variations were linked to a reduction of age-associated diseases with no correlation with mortality [212, 213]. As we will summarize later, sirtuins act both as inducers and inhibitors of oncologic and inflammatory diseases, which prompted the development of sirtuin modulators. Multiple clinical trials are currently testing the potential of sirtuin activators, the most studied being resveratrol, for the treatment of inflammatory and metabolic disorders with encouraging results [214]. Overall, sirtuins are promising targets to alleviate age-associated diseases, without affecting lifespan. Since numerous observations from *in vitro* studies and *in vivo* preclinical models use pharmacological modulators of sirtuins, we will first briefly summarize the development of activators and inhibitors of sirtuins.

1.3.1 Modulation of sirtuins

Resveratrol is a polyphenolic compound naturally occurring in grapes and berries identified by high-throughput screening as an activator of human SIRT1 and of yeast Sir2 that increases the lifespan of *S. cerevisiae* [215]. In mice and humans, resveratrol increases the expression and activity of SIRT1 [216] and SIRT3 [217] to promote homeostasis and reduce inflammation. In fact, resveratrol quickly attracted the attention of not only the scientific community but also of the media, the public and the industry following its possible role in protecting people consuming animal fat from cardiovascular disease, a phenomenon known as the French paradox [218]. Overall, resveratrol has been suggested

to exert anticancer, cardioprotective, anti-inflammatory, antidiabetic and neuroprotective effects. Synthetic resveratrol derivatives and additional screenings identified small molecules activator of sirtuins that increase mitochondrial biogenesis, improve insulin sensitivity, lower plasma glucose and cholesterol and protect from atherosclerosis [219-222]. Numerous clinical trials are in progress to test the usage of resveratrol or its derivatives in age-associated inflammatory diseases. Overall, this field is largely focused on SIRT1 activation with few compounds targeting other sirtuins directly or indirectly through SIRT1 activation.

Sirtuins have a pathogenic role in several diseases. By testing natural compounds and small molecule libraries and by rational drug design, sirtuin inhibitors have been developed with the potential to target dysregulated sirtuin expression and/or activity. The majority of the compounds available today inhibit SIRT1 and SIRT2. They include the physiologic inhibitor nicotinamide, the hydroxynaphthaldehyde (sirtinol, salermide and cambinol), derivatives of splitomicin, the indole derivative EX 527 and tenovins (**Table 3**). While these inhibitors act through different mechanisms, they have in common anti-tumor activity *in vitro*, which have been confirmed in animal models for cambinol and tenovins [219]. The inhibitors are designed to bind selectively the catalytic domain of sirtuins [223]. However, depending on the dose used, inhibitors affect multiple sirtuins. Additionally, inhibiting the activity of one sirtuin may modulate pathways ultimately affecting the expression and function of other sirtuins. Given the broad range of biological processes that sirtuins influence on, this limitation may give rise to important side effects *in vivo*. Moreover, the role of sirtuins in infections is largely uncharacterized. Therefore, considering the anti-inflammatory properties of some sirtuins, the newly developed drugs should be tested for their effect on infections to overrule the risk of infections during treatment.

Table 3: Selection of sirtuins modulators.

Molecule	Sirtuin(s)	Concentration (µM)	Effect	Clinical trial	Ref
Activators					
Resveratrol	SIRT1 SIRT3 SIRT5	5 50 50	↑ cancer cell death ↑ autophagy ↓ inflammation	Phase III	[224] [225] [226]
SRT2104	SIRT1	3	↑ neuroprotection ↓ inflammation	Phase IIb	[227]
UBCS039	SIRT6 SIRT5	75-100 100	↑ cancer cell death	no	[228] [229]
Inhibitors		**IC_{50}**			
AGK2	SIRT2 SIRT1, SIRT3	3.5 > 50	↑ neuroprotection	no	[230]
AK-7	SIRT2 SIRT1, SIRT3	15.5 > 50	↑ neuroprotection	no	[230]
Cambinol	SIRT1 SIRT2 SIRT5	56 59 42% at 300	↑ cancer cell death ↓ inflammation	no	[230]
EX-527	SIRT1 SIRT2, SIRT3	0.1-1 20-49 µM	↑ inflammation	no	[230]
Nicotinamide	SIRT1, SIRT2 SIRT3 SIRT5, SIRT6	100-120 µM 50 µM 150-184 µM	↑ cancer cell death	Phase III	[231]
Sirtinol	SIRT1 SIRT2 SIRT3	38-131 µM 38-58 µM 24% at 50 µM	↑ cancer cell death ↓ gluconeogenesis	no	[232]
Tenovin-6	SIRT1 SIRT2	37.5 µM 10.4 µM	↑ cancer cell death ↑ p53	no	[233]

Information on clinical trials obtained from https://clinicaltrials.gov, on May 1st 2019.

1.3.2 Targets of sirtuins

Sirtuins promote cellular homeostasis by modulating epigenetic, inflammatory and metabolic pathways. Several studies have applied mass spectrometry techniques to assess protein modifications in wild type and sirtuin deficient cells [234, 235]. These studies revealed thousands of differentially regulated genes and proteins between wild type and deficient cells with as many potential targets for sirtuins. However, the observed phenotype might change drastically according to the method used to target sirtuins, for instance by using small-interfering RNA (siRNA), short hairpin RNA (shRNA), cells isolated from whole body or conditional knockout mice or drugs targeting sirtuins. Moreover, sirtuins share targets and the deletion of one sirtuin may be compensated by others. We present here some commonly admitted targets and cellular roles for sirtuins (**Figure 7**).

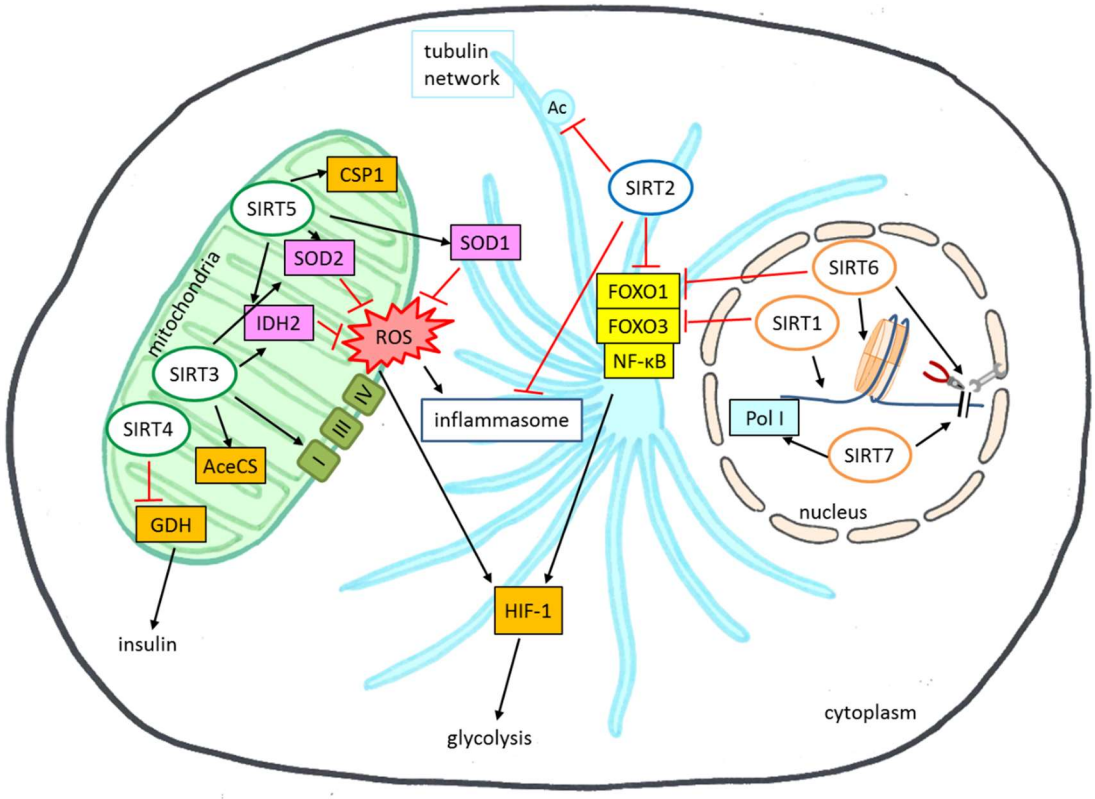


Figure 7: Sirtuins target unique and shared proteins to modulate gene expression, homeostasis, metabolism and inflammation. The seven sirtuins are mainly localized in the nucleus (SIRT1, SIRT6, SIRT7), the cytoplasm (SIRT2) and the mitochondria (SIRT3, SIRT4, SIRT5). Among others, sirtuins regulate gene transcription (Pol I), DNA repair, transcription factors (yellow), structural proteins (tubulin network), metabolic pathways (orange), electron transport chain (ECT) components (green) and ROS detoxifying enzymes (pink). Several sirtuins may target similar enzymes to affect common cellular pathways. Ac: acetyl; AceCS: acetyl-CoA synthetase; CSP1: carbamoyl phosphate synthase 1; FOXO: forkhead box protein O; GDH: glutamate dehydrogenase;HIF-1α: hypoxia inducible factor 1α; IDH: isocitrate dehydrogenase; NF-κB: nuclear factor κB; Pol I: polymerase I; ROS: reactive oxygen species; SOD: superoxide dismutase; I, II, III: ECT complex I, II, III.

SIRT1. The nuclear SIRT1 is the most extensively studied sirtuin with numerous identified targets including histones H1, H3 and H4 and the transcription factors NF-κB, forkhead box protein O 1 (FOXO1) and FOXO3 [236]. SIRT1 acts directly on gene expression and impacts on inflammatory, metabolic and cellular survival pathways. During caloric restriction, the expression of SIRT1 is upregulated to promote autophagy and hinder glycolysis by repressing hypoxia inducible factor 1α (HIF-1α) mediated gene expression [237, 238]. On the contrary, SIRT1 was suggested to stabilize HIF-1α during hypoxia to promote glycolysis and cell survival [239]. SIRT1 was reported to both sensitize

and protect cells to TNF-induced apoptosis through inhibition of NF-κB and FOXO1, respectively [240, 241]. SIRT1 promotes mitochondrial homeostasis by activating the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) possibly linking the activity of SIRT1 to mitochondrial sirtuins [242]. In tumorigenesis, SIRT1 has deleterious and protective effects by inactivating tumor suppressors but also reducing DNA damage [243]. These results suggest a cell-type and context dependent effect for SIRT1 with regulatory mechanisms by other nuclear proteins.

SIRT2. SIRT2 is the only sirtuin primarily located in the cytoplasm where it deacetylates tubulin [244]. Tubulin deacetylation decreases tubulin stability and renders microtubules more prone to mechanical breaks [245], linking SIRT2 to axonal degeneration in neurons [246], inhibition of mitosis, and NLRP3 inflammasome assembly [247, 248]. SIRT2 has been reported to translocate into the nucleus during the G2/M phase, where it deacetylates histones H3 and H4 [249, 250], NF-κB and members of FOXO transcription factor family [251, 252]. SIRT2 interacts with and deacetylates p65 to regulate the expression of NF-κB dependent genes, with reports suggesting both activating and inhibiting outcomes [240, 252, 253]. SIRT2 deacetylates FOXO3 which promotes the transcription of ROS detoxifying enzymes and inhibits oxidative stress [251]. SIRT2 deacetylates FOXO1, reducing adipocyte differentiation [254], promoting autophagy [255] and inducing gluconeogenesis [256]. SIRT2 deacetylates and promotes signaling of MAPK phosphatase-1 (MKP-1), enhancing the production of inflammatory mediators[257, 258].

SIRT3. SIRT3 is the main mitochondrial deacetylase and is highly expressed in mitochondria-rich tissues including liver, heart and brown adipose tissue. SIRT3 maintains cellular homeostasis by binding to and activating the ROS detoxifying enzymes superoxide dismutase (SOD) 2 and isocitrate dehydrogenase (IDH) 2 [259, 260]. Reduction of ROS destabilizes HIF-1α [261] promoting OXPHOS for energy production. SIRT3 directly targets other metabolic enzymes including acetyl-CoA synthase (AceCS) [262], and interacts with components of the electron transport chain (ETC) complex I [263] to sustain ATP production. SIRT3 modulates thermogenesis in brown adipose tissue by deacetylating

uncoupling protein 1 (UCP1) to increase mitochondrial inner membrane conductance and to produce heat [264]. During stress, SIRT3 activates optic atrophy 1 (OPA1) protein to promote mitochondrial fusion [265] and heat-shock protein 10 (Hsp10) to control folding and function of fatty acid oxidation (FAO) enzymes [266]. SIRT3 also induces nuclear translocation of PGC-1α [267] and FOXO3 [268] and the expression of their target genes to promote mitochondrial functions.

SIRT4. The mitochondrial SIRT4 is one of the least studied sirtuins. SIRT4 downregulates glutamine metabolism through inhibition of glutamate dehydrogenase (GDH) [269, 270]. This mechanism promotes DNA damage repair, which in turn activates SIRT4 in a positive feedback loop [270], and reduces insulin secretion [117]. More recently, SIRT4 was reported to promote leucine metabolism, a second pathway downregulating insulin secretion [271].

SIRT5. SIRT5 mainly localizes in the mitochondria where it exerts a major desuccinylation activity and shows a weak deacetylase activity [272]. SIRT5 was first shown to target and activate carbamoyl phosphate synthase 1 (CPS1) to regulate the urea cycle [273]. SIRT5 can localize both inside and outside the mitochondria and regulates the succinylation state of 12% of all cellular succinylation sites [274]. SIRT5 targets multiple metabolic enzymes including components of FAO and ETC in the mitochondrial matrix [272, 275, 276] and glycolytic enzymes in the cytoplasm [277]. SIRT5 activates ammonia detoxifying enzymes and, like SIRT3, ROS detoxifying enzymes including SOD2, its cytosolic homolog SOD1, and IDH2 [278-280]. During starvation, SIRT5 promotes mitochondrial fusion sustaining OXPHOS [281] but also deacetylates STAT3 to inhibit its mitochondrial translocation and metabolic regulation [282].

SIRT6. SIRT6 targets histone H3 thus increasing genomic stability and decreasing gene expression [283, 284]. Like SIRT1 and SIRT2, SIRT6 targets FOXO1 and FOXO3 and represses FOXO-mediated gene expression [285]. SIRT6 represses HIF-1α to promote OXPHOS over glycolysis [286]. SIRT6 also maintains lipid homeostasis through repression of the lipogenic transcription factors SREBP1 and SREBP2, and activation of the AMP-activated protein kinase (AMPK) [287].

SIRT7. SIRT7 interacts with RNA polymerase (Pol) I and promotes its transcriptional activity [288]. Further studies showed that SIRT7 potentially interacts with Pol II and Pol III [289, 290]. SIRT7 directly impacts on DNA damage repair through histone modification and presence in multi-protein chromatin remodeling complexes [291, 292]. SIRT7 targets transcription factors including osterix (osx) to promote bone formation [293].

1.3.3 Sirtuins in immune cells

Hematopoietic progenitors and mature immune cells express all sirtuins at different levels as illustrated in **Figure 8** with embryonic stem cell (ESC), HSC and CMP. The nuclear sirtuins SIRT1, 6 and 7 play an important role during development and their deletion in mice is lethal (**Table 2**, [294]). SIRT1 is highly expressed in stem cells [295] where it maintains stemness by directly targeting p53 translocation to the nucleus and by modulating gene expression pattern through chromatin silencing and through FOXO activation [296-298]. SIRT6 regulates HSC proliferation through epigenetic control of the Wnt signaling pathway [299] while SIRT7 controls HSC differentiation by enhancing mitochondrial stability [300].

The expression and potential role of mitochondrial sirtuins depends on mitochondrial mass. ESC and HSC rely mainly on glycolysis for energy production with little OXPHOS [301]. However, mitochondria are important to maintain HSC through mitophagy with a continuous mitochondrial biogenesis and are required for the differentiation of HSC into progenitor cells, mainly lymphoid-biased progenitors [302]. In line, SIRT3 is expressed at higher level in CLP than in myeloid progenitors [303]. During adulthood, SIRT2 and SIRT3, but not SIRT1, promote the maintenance of HSC by reducing oxidative stress and NLRP3 inflammasome activation [304, 305]. As we will see later, in this project, we focused on the role of SIRT2, SIRT3 and SIRT5 in innate immune cells, mainly macrophages and neutrophils.

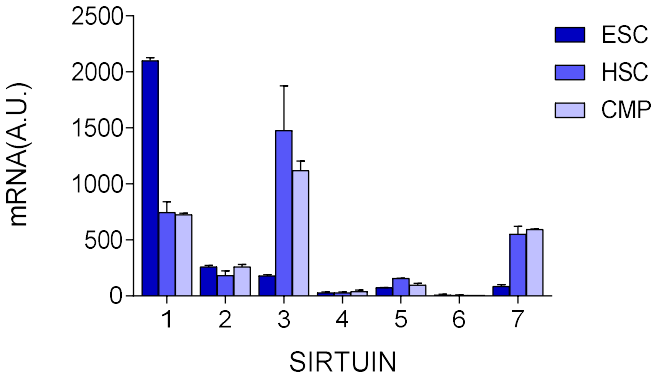


Figure 8: Expression of sirtuins in embryonic stem cell (ESCs), hematopoietic stem cells (HSCs) and common myeloid progenitors (CMPs). Data extracted from biogps.org

Macrophages. The role of SIRT2 in macrophages is controversial with reports of pro-inflammatory and anti-inflammatory profiles, and no impact. SIRT2 promotes a M1 phenotype with increased NF-κB activation, iNOS expression and ROS production while decreasing arginase-1 transcription [306]. In contrary, SIRT2 deacetylates p65, inhibits NF-κB activity and reduces the transcription of NF-κB target genes and the pro-inflammatory response in macrophages [115]. SIRT2 activity has been associated with reduced TLR signaling and the establishment of a tolerant phenotype [307]. SIRT2 deacetylates tubulin in macrophages, and inhibition of SIRT2 promotes NLRP3 assembly and subsequent IL-1β secretion [248]. Finally, it has been shown in the laboratory that selective inhibitors of SIRT2, AK-7 and AGK2, do not modulate cytokine production by macrophages exposed to various TLR ligands [230].

SIRT3 induces a M2 phenotype in macrophages by promoting mitochondrial function and reducing ROS levels. Peritoneal macrophages deficient in SIRT3 produce higher levels of pro-inflammatory cytokines and ROS [308, 309]. This effect is linked to a decreased SOD2 activity and OXPHOS, and increased inflammasome activation in SIRT3 deficient cells. In line, SIRT3 promotes autophagy which reduces NLRP3 inflammasome activation and consequently IL-1β secretion [310]. SIRT3 deacetylates the autophagy component ATG5, promotes autophagosome formation and antimicrobial defenses in macrophages incubated with *Mycobacterium tuberculosis* [311]. SIRT3 also reduces the accumulation of lipid in macrophages decreasing the formation of foam cells [312].

SIRT5 sustains the production of pro-inflammatory cytokines by promoting p65 acetylation and NF-κB activity [307]. SIRT5 rescues the tolerant phenotype of macrophages mediated by SIRT2, reinforcing the pro-inflammatory impact of SIRT5 [307]. In contrary, SIRT5 decreases IL-1β production through the desuccinylation and inhibition of the glycolytic enzyme pyruvate kinase M2 [313]. Moreover, SIRT5-deficient mice are susceptible to DSS-induced colitis [313]. A similar phenotype is observed using mice deficient in either SIRT2 or SIRT3 [115, 314].

Neutrophils. During granulopoiesis, the sequential expression and repression of SIRT1 is important to regulate the acetylation level of CCAAT/enhancer-binding proteins (C/EBP) that play a central role in neutrophil development [315]. The role of sirtuins in mature neutrophils is scarce with only two studies addressing the role of SIRT3. SIRT3 increases ROS production in neutrophils, but has an uncertain effect on the production of NETs [316, 317].

The role of sirtuins in neutrophil recruitment has been studied in inflammatory models. SIRT2 promotes LPS-induced renal tubular expression of CXCL2 and CCL2 and the recruitment of neutrophils to kidneys, and SIRT2 aggravates post-ischemic liver injury increasing proinflammatory cytokines, hepatocellular necrosis and apoptosis, and neutrophil infiltration [257, 258]. In contrary, SIRT3 reduces neutrophil recruitment to the lungs of mice subjected to LPS-induced acute lung injury and during mycobacterial infection [309, 311]. Treatment of rats with resveratrol reduces neutrophil accumulation in sepsis-induced myocardial injury and spinal cord injury-induced lung damages [318, 319].

DCs, T cells and B cells. SIRT1 modulates DC function and promotes the induction of Tregs [320-322]. The role of other sirtuins in DCs, T cells and B cells is less studied. SIRT2 decreases TNF production by DCs through inhibition of NF-κB [252]. *Salmonella* infection increases the expression of SIRT2 in DCs, inhibits T cell proliferation and promotes bacterial survival in $CD8^+$ T cells [253]. In contrast, SIRT2 deficiency increases the proportion of activated T cells in lymph nodes of mice subjected to DSS-induced colitis, worsening diseases outcome [115]. Like SIRT1, SIRT3 promotes the

function of Tregs and protects against allograft rejection [323]. SIRT1 reduces memory T cells proliferation through targeting FOXO1 and decreases glycolysis [324]. The role of SIRT2, SIRT3 and SIRT5 in T cell activation and memory formation has not been reported. Yet, since SIRT2, SIRT3 and SIRT5 affect metabolic pathways and gene transcription, one may anticipate that they affect T cell functionality at baseline or during stress conditions.

1.3.4 Sirtuins in age-associated, inflammatory and infectious diseases

The homeostatic role of sirtuin and their association with longevity prompted research on their function in inflammatory and age-related diseases. Inflammation and aging are connected since aging is a major risk factor for inflammatory diseases and age-associated diseases have an inflammatory component [325]. Sirtuins have been extensively studied in various human tumors and in preclinical mouse models of cancer with controversial results (**Table 4**). The expression of sirtuins is increased or reduced in human cancers in an enzyme and cell dependent manner [326]. In line, sirtuins regulate cellular components to act either as pro-tumorigenic or tumor suppressor. For example, SIRT2 downregulates the tumor suppressor p53 [327] but stabilizes the oncoprotein Myc [328]. Moreover, the antioxidant effects of SIRT3 and SIRT5 reduce oxidative damage-mediated cancer development, but protect cancer cells against oxidative stress induced apoptosis [329]. SIRT3 interacts with p53 to either reduce [330] or promote [331] cell cycle arrest in bladder and lung cancers. Finally, SIRT5 promotes the expression of drug resistance genes [332] and tumor suppressors [333].

Sirtuins in neurodegeneration. An important alteration during aging is memory loss. The expression of SIRT2 and its impact on tubulin stability are linked with neurodegenerative diseases including Parkinson and Alzheimer's diseases [334], while SIRT3 has a protective effect through its antioxidant and homeostatic functions [335]. SIRT3 expression correlates with neuronal survival in a mouse model of AD but with disease occurrence in humans [336]. Since overproduction of mitochondrial ROS is associated with neurodegeneration, the authors hypothesized that AD patients upregulate SIRT3 expression in temporal neocortex in an attempt to control the oxidative burst.

Table 4: Sirtuins in inflammation and human diseases.

		Pro-inflammatory effect	Anti-inflammatory effect	Expression in human diseases	Ref
SIRT1	↑	Myc	DNA repair, mitochondrial function	cancer, HD, CVD	[326, 337-339]
	↓	p53	NF-κB	cancer, obesity, AD, PD, MS, T2D, heart failure, psoriasis	
SIRT2	↑	NO production, Myc, chemokines	genomic integrity	cancer, AD	[326, 339-341]
	↓	tubulin stability, autophagy, p53	NF-κB, inflammasome assembly	cancer, obesity, (RA), psoriasis	
SIRT3	↑		oxidative metabolism	cancer, RA	[326, 335, 338-340]
	↓	oxidative stress, p53	oxidative stress, HIF-1α, cytokines	cancer, obesity, AD, psoriasis	
SIRT4	↑	lipogenesis	DNA repair, oxidative metabolism		[326, 339, 342-344]
	↓		insulin secretion, glutamine metabolism, NF-κB	cancer, T2D, obesity, psoriasis	
SIRT5	↑	NF-κB	PKM2, oxidative metabolism	cancer, AD	[313, 326, 338, 339, 345]
	↓	oxidative stress	oxidative stress	psoriasis, obesity	
SIRT6	↑	protection against chemotherapy, TNF	genomic stability, DNA repair, autophagy	cancer, psoriasis	[326, 339, 346-348]
	↓		NF-κB, HIF-1α	cancer, CVD, atherosclerotic lesions, AD, heart failure	
SIRT7	↑	NF-κB, oncogenes	genomic stability, DNA repair, HSC regeneration	cancer, psoriasis	[326, 339, 349, 350]
	↓	neuronal viability	Myc	cancer, obesity	

AD: Alzheimer's disease; CVD: cardiovascular disease; HD: Huntington's disease; MS: multiple sclerosis; PD: Parkinson disease; RA: rheumatoid arthritis; T2D; Type II diabetes. Parenthesis indicate a trend.

Sirtuins in metabolic disorders. Sirtuins regulate cellular pathways and modulate the development of metabolic disorders. SIRT2 and SIRT3 protect mice against HFD-induced obesity and insulin resistance [351, 352]. Mechanistically, SIRT2 promotes the activity of glucokinase protecting against impaired glucose metabolism [353]. It also reduces the infiltration of inflammatory M1 macrophages in atherosclerotic plaques [354]. SIRT3 reduces pro-inflammatory cytokine production and promotes

intestinal barrier permeability [355]. SIRT5 was neither protective nor harmful during HFD [118]. However, in ob/ob obese mice developing type 2 diabetes (T2D), SIRT5 reduces lipid accumulation in the liver and promotes liver homeostasis by increasing OXPHOS [356].

Sirtuins in infection and sepsis. The expression of sirtuins is modulated during the course of sepsis [357]. In obese mice subjected to CLP-induced sepsis, SIRT2 expression decreases during hyper-inflammation while it increases during the hypo-inflammatory phase [358]. SIRT3 acts in concert with SIRT1 and the NF-κB subunit RelB to induce a metabolic switch from glycolysis to OXPHOS initiating inflammation adaptation [359]. During LPS induced septic shock, SIRT3 increases the survival of mice by limiting inflammation-induced damage [360]. SIRT2 is protective in a model of CLP [361] while SIRT3 has no effect on mouse survival [362]. Oppositely, SIRT2 decreases the survival of ob/ob mice during CLP [358]. Few studies addressed the role of SIRT2, SIRT3, and SIRT5 in pure bacterial infections. During infection with *Listeria monocytogenes*, SIRT2 is dephosphorylated and translocates to the nucleus to acetylate H3K18 and promote bacterial infection [250]. In line, SIRT2 deficient mice are protected against *Salmonella* Typhimurium infection with lower bacterial burden and increased survival [253]. SIRT3 protects mice against *M. tuberculosis* infection by promoting autophagy and reducing lung inflammation [311].

1.4 Innate immune memory and trained immunity

Since the first vaccine against smallpox developed in 1796 by Dr Edward Jenner, it has become clear that the host has the ability to keep a memory of the pathogens it has encountered. This role was exclusively attributed to the adaptive immune system with the generation of memory T and B cells [363, 364]. These memory cells are generated during a primary infection to express unique T cell and B cell receptors recognizing specific antigenic epitopes. Therefore, the ability to remember a previous infection has been a key feature discriminating innate and adaptive immune cells.

This dogma has been challenged with the observation that invertebrates and plants, which do not have an adaptive immune system, display some degree of recall of previous infections [365]. In plants, a primary inoculation of a pathogen leads to epigenetic modifications at the promoter of defense genes. These modifications were observed at distal parts of the plants and were stable as demonstrated by their heritability [366, 367]. Early studies in mice showed that heterologous agents could afford protection against infections with pathogens independently of adaptive immune memory [368, 369]. In the 80's, Bistoni, Cassone and colleagues (University of Perugia, Perugia, Italy) performed pioneer studies showing that mice injected intravenously (i.v.) with a low dose of *Candida albicans* were protected against a lethal dose of *Staphylococcus aureus* given i.v. [370]. This protection was linked to an increased number of peripheral blood polymorphonuclear cells (PMN) and cytotoxicity of splenocytes. In humans, the Bacillus Calmette-Guérin (BCG) vaccine, primarily used against tuberculosis, protects against various infectious diseases [371, 372]. In all these situations, the structure of the first and second challenges are different, suggesting that memory T cells and B cells are not involved in the protective process.

In the recent years, studies have unraveled the modulation or remembering capacity of innate immune cells. In 2011, Netea and colleagues (Radboud University Medical Center, Nijmegen, Netherlands) proposed to group these memory features under the term of "trained immunity" [373]. His group showed that intraperitoneal (i.p.) challenges with β-glucans, a cell wall component of *C. albicans*, protects T/B cell-defective Rag1-deficient mice against a subsequent systemic infection with *C. albicans* or *S. aureus* [374]. They proposed a model where the exposition to a first agent modulates the immune state and the ensuing response to pathogen (**Figure 9**). The capacity of immune cells to reprogram their immune response after the first contact is called "innate immune memory", and defined as "training" when the immune response is increased and "tolerance" when it is decreased. During the past years, research in this field has increased exponentially with studies showing memory capacity in numerous innate immune cells and unravelling molecular mechanisms.

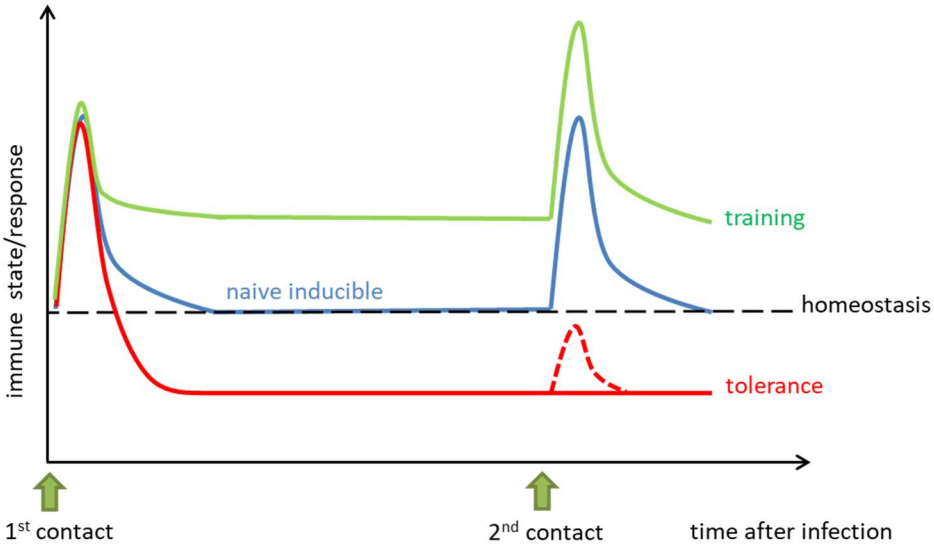


Figure 9: Model of innate immune memory. The exposition to a first agent (1st contact) modulates the immune program giving rise to an increased (trained) or a decreased (tolerant) immune response when encountering a second agent (2nd contact). Adapted from [375].

1.4.1 Defining memory

Traditionally, immune memory is defined as the ability of the immune system to recognize a previously encountered antigen to mount a faster and stronger secondary response upon re-exposure to the same antigen. T and B cells express receptors with a variable (VDJ) region conferring antigen specificity (**Table 5**). In the situation of an infection, a primary contact induces the selection of lymphocytes with receptors conferring the highest antigenic affinity that undergo a rapid phase of clonal expansion. Expansion is followed by a phase of contraction during infection resolution, and finally a phase of memory during which a small amount of cells are conserved as dormant memory cells with an increased proliferative capacity upon restimulation [376-378].

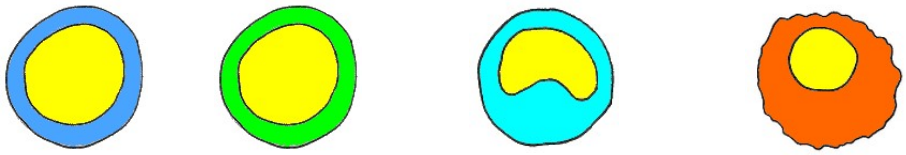
NK cells were the first non-T, non-B cells reported to have memory features lasting up to 4 weeks [379] and associated with epigenetic modifications [380]. The acquisition of memory features by NK cells follows phases that parallel those of T cells and B cells (expansion, contraction and memory). Memory NK cells differentially express inhibitory and activating NK receptors. NK cell

memory can be induced by murine cytomegalovirus (MCMV) [381], haptens [379] and cytokines [382]. This interaction is mediated by the Ly49 receptors in mice and the homologous KIR receptors in humans. Priming of NK cells activates STAT1 and STAT4 pathways affecting NK cell functionality and receptor expression. The combination of NK cell surface receptors leads to antigen specific but also non-specific recognition [383, 384] (**Table 5**).

In monocytes, macrophages and DCs, memory features are independent of specific antigen recognition (**Table 5**). Exposure of human PBMCs to β-glucans before their differentiation into macrophages induces epigenetic and metabolic modifications ultimately triggering the production of higher levels of pro-inflammatory cytokines. Two seminal studies described the epigenetic landscape characterizing trained monocytes and identified the dectin-1/Akt/mTOR/HIF-1α pathway as a main driver of the Warburg effect occurring in monocytes trained with β-glucans [385, 386]. Genome-wide ChIP-seq analyses in trained monocytes/macrophages reveals the enrichment of histone 3 lysine 4 trimethylation (H3K4me3, a mark associated with active gene expression) at the promoter of genes encoding for PRRs, signaling molecules and inflammatory cytokines. These observations enhance the link between metabolic changes and their impact on immune responses with a long-lasting effect enabled by epigenetic modifications [387]. Innate immune memory can either increase (trained) or decrease (tolerized) secondary innate immune responses.

Of note, the notion of innate memory is not restricted to monocyte-derived macrophages and DCs as it was recently demonstrated with mouse brain-resident macrophages (microglia) as an important modifier of neuropathology [388] and with mouse alveolar macrophages [389]. Moreover, non-immune cells showed long-term adaptation following priming. For example, mouse skin epithelial stem cells developed memory features dependent on the AIM2 inflammasome [390] while human respiratory epithelial stem cells reprogramed cell differentiation accounting for “allergic memories” during lung allergic inflammation [391].

Table 5: Memory remodulation of immune cells.



	T cells	B cells	NK cells	Monocytes
Priming	Antigen	Antigen	Virus, hapten, cytokine	MAMPs, DAMPs
Receptor	TCR	BCR	NK cell receptors	PRRs
Pathways	Akt/NF-κB	TRAF/NF- κB	STAT	Akt/HIF-1α
Antigen specific	Yes	Yes	Yes/No	No
Protection	Homologous	Homologous	Homo/heterologous	Heterologous
Proliferation	Clonal expansion	Clonal expansion	Clonal expansion	Progenitors
Metabolism	FAO, OXPHOS, glycolysis	Autophagy	Citrate-malate	Glycolysis, cholesterol synthesis
Epigenetic modifications	Yes	Yes	Yes	Yes

Akt: protein kinase Bα; BCR: B cell receptor; FAO: fatty acid oxidation; HIF-1α: hypoxia inducible factor 1α; MAMP: microbial associated molecular pattern; NF-κB: nuclear factor κB; NK: natural killer; OXPHOS: oxidative phosphorylation; STAT: signal transducer and activator of transcription; TCR: T cell receptor; TRAF: TNF receptor associated factor.

1.4.2 Molecular mechanisms of trained immunity

The trained phenotype of cells or organisms is assessed by measuring the response of immune cells to stimulation. This phenotype is the result of four important features: the inducer, metabolism and epigenetics (**Figure 10**).

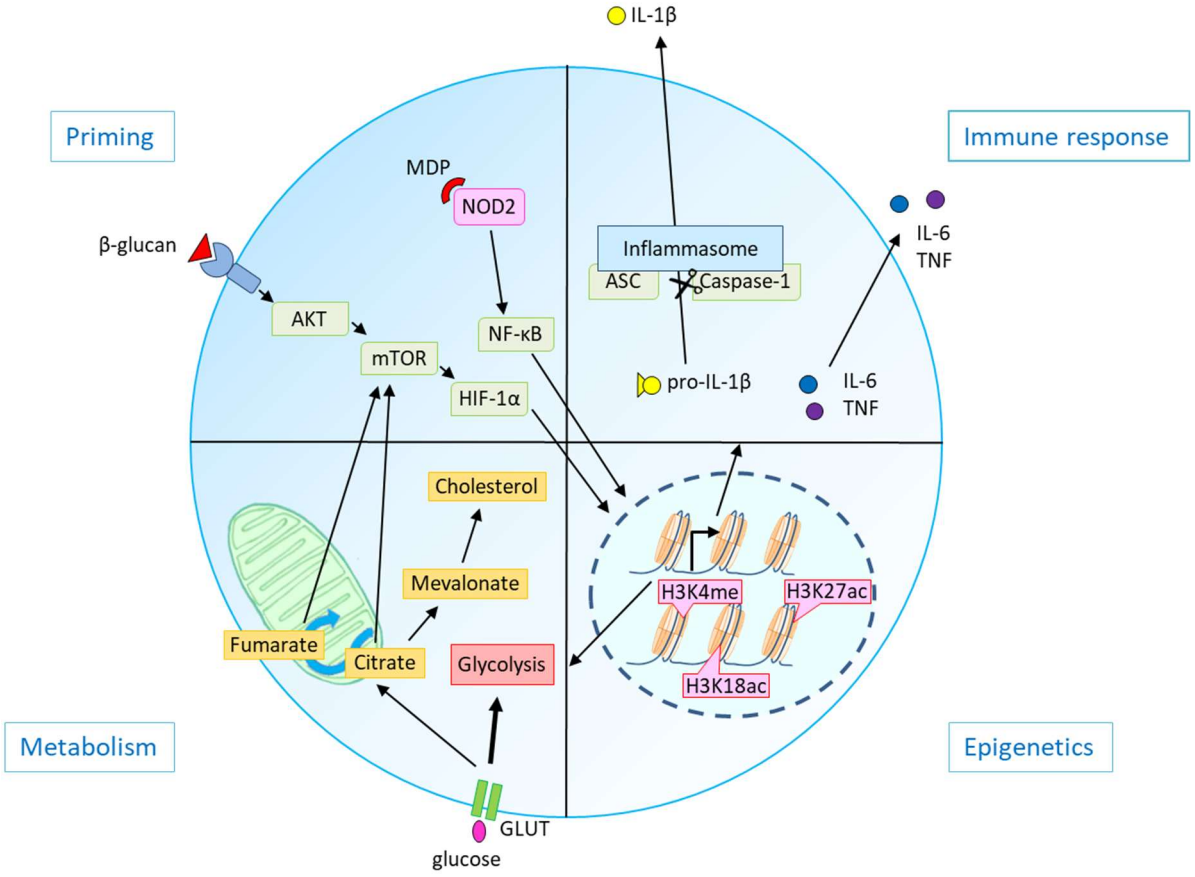


Figure 10: The trained phenotype is the result of priming agent, metabolic reprogramming, epigenetic modifications and immune responses. The priming agent activates a signaling pathway leading to the transcription of metabolic and immune genes. The metabolism of trained cells switches towards glycolysis and cholesterol synthesis with multiple intermediates acting as signaling molecules. The immune response increases the production of cytokines promoting cell recruitment, proliferation and antimicrobial functions. These effects are long-lasting with epigenetic marks.

Training inducers. Various agents induce trained immunity, but not all reprogram the same cells in the same fashion. The nature, dose and duration of exposure to MAMPs/DAMPs influences the reprograming of innate immune cells. Ifrim and colleagues showed that the same stimulus can induce training or tolerance depending on the concentration applied for priming [392]. This also applies to LPS, generally considered to induce tolerance, which can provide both tolerance and protection according to the model used. Indeed, Yoshida and colleagues reported that mice challenged 3 weeks previously with LPS are protected from *S. aureus* infection, suggesting that long-term epigenetic changes induced by LPS also drive innate immune memory. In peritoneal macrophages, these

epigenetic changes result from the release of ATF7, a member of ATF-CREB superfamily, from the chromatin and a decrease of H3K9me2 repressive mark at the promoter of immune genes [393]. Although BCG and β-glucans are the two priming agents used in studies on innate immune training, heterologous protection has been reported with microbial components and live or inactivated pathogens listed in **Table 6**.

Table 6: Compounds affording protection in mice against heterologous lethal infections.

Priming		Challenge		Ref
Microbial products				
Adenovirus	i.n.	*S. pneumoniae*	i.t.	[389]
β-glucan	i.p.	*C. albicans*	i.v.	[386]
β-glucan	i.p.	*S. aureus*	i.v.	[374]
Chitin from *S. cerevisiae*	i.p.	*C. albicans*	i.v.	[394]
CpG ODN	i.p.	*L. monocytogenes*	i.p.	[395]
Lipid A mimetics	i.n.	*Yersinia pestis*	i.n.	[396]
LPS (2 injections)	i.p.	GBS	i.v.	[397]
MPLA	i.v.	*S. aureus*	i.t.	[398]
Inactivated whole organisms				
BCG vaccine	i.v.	*C. albicans*	i.v.	[399]
BCG vaccine	i.p.	*Plasmodium yoelii*	i.p.	[400]
C. albicans	i.p.	GBS	i.v.	[401]
Propionibacterium acnes	i.v.	*S. enterica* Typhimurium	i.v.	[402]
Live whole organisms				
C. albicans	i.n.	*P. aeruginosa*	i.n.	[403]
C. albicans	i.v.	*S. aureus*	i.v.	[370]
Herpesvirus	i.n.	*L. monocytogenes*	i.p.	[404]
Lactobacillus plantarum	i.n.	Pneumovirus	i.n.	[405]

i.n.: intranasally, i.p.: intraperitoneally, i.v.: intravenously, MPLA: monophosphoryl lipid A

Immune responses. In humans, the most striking effect of innate immune training is observed with heterologous protection afforded by the BCG vaccine. The BCG vaccine is a live-attenuated strain of *Mycobacterium bovis* (BCG strain) showing good protection against tuberculosis when administrated to children but variable protection in adults [406]. Multiple reports associated BCG vaccination with protection against malaria, leprosy, measles and meningitis in endemic countries [371, 407, 408]. Subsequent studies in mice confirmed the protective effect of BCG against malaria [400] and candidiasis [399]. The use of heterologous pathogens and SCID-mice rules out a role for adaptive memory. In a very elegant study published in 2018, the protective effect of BCG in humans is demonstrated by successively vaccinating healthy volunteers with BCG and the live attenuated yellow fever (YF) vaccine [409]. BCG vaccination increases the reactivity of PBMCs to YF and decreases viral load in vaccinated subjects without affecting the production of adaptive memory cells against YF virus. In a controlled malaria clinical trial, BCG vaccinated volunteers show earlier activation of NK cells compared to non-vaccinated volunteers. However, parasitemia is not affected [410].

The diversity of training agents implies that training can be induced through different PRRs and signaling pathways. For example, β-glucans is sensed through dectin-1, while CpG is recognized by TLR9. *In vitro* experiments demonstrate that a first exposure to training agents (β-glucans, BCG) increases the production of IL-6, TNF and IL-1β after restimulation with bacterial ligands [374, 411, 412]. Trained macrophages have increased fungicidal and bactericidal activities with enhanced ROS production [413, 414]. Phenotypic analyses reveal specific patterns of altered gene and cell surface protein expression in trained cells. Nevertheless, the protective effect of training against infections is likely the resultant of an overall enhanced immune status involving multiple cell types. Indeed, the training agent is recognize by PRRs present on myeloid and non-immune cells, like stromal cells, activating signaling pathways leading to a trained phenotype [415], and trained cells produce cytokines, chemokines and growth factors that create locally and systemically an environment modulating immune responses.

Metabolism. Trained innate immune cells stabilize HIF-1α promoting the transcription of most of the glycolytic pathway proteins ranging from glucose transporters (GLUT1-4) to lactate dehydrogenase (LDH) [386, 416]. This shift from OXPHOS to aerobic glycolysis is observed in the activation of numerous immune cells including effector T cells and M1 macrophages. In agreement, trained cells accumulate citrate suggesting a break in the TCA cycle with increased glutaminolysis [417]. Cholesterol biosynthesis is another metabolic pathway induced upon training [418, 419]. Interestingly, not only is the energy produced through these pathways important for the functionality of trained cells, but also the intermediate metabolites. The break in TCA cycle leads to the accumulation of malate, citrate and fumarate that act as signaling molecules. Accumulation of fumarate favors histone methylation and promotes epigenetic reprogramming. The cholesterol biosynthesis pathway intermediate mevalone interacts with the insulin-like growth factor 1 (IGF-1) inducing a positive feedback loop promoting glycolysis and increasing the production of mevalonate. Single nucleotide polymorphisms (SNPs) in the autophagy genes *ATG2B* and *ATG5* impair the training effect of BCG. In line, pharmacologic or genetic inhibition of autophagy impedes epigenetic reprogramming of monocytes and training by BCG or β-glucans, suggesting that autophagy is important for the induction of a trained phenotype [420]. Finally, metformin, a drug used for treating type 2 diabetes (T2D) that activates AMPK resulting in mTOR inhibition, impairs training, comforting the assumption that targeting metabolic pathways might be used to promote training and increase immune defenses [385, 386].

Epigenetic. PBMCs from healthy volunteers vaccinated with BCG produce increased levels of inflammatory cytokines upon restimulation [387]. Despite the short lifespan of few days for blood PBMCs [421], this phenotype lasts for 3 months post-vaccination. Comparison of the epigenetic landscapes of trained and non-trained PBMCs reveals numerous differentially regulated sites [385]. Promoters and distal elements of metabolic and immune genes show increased transcription promoting marks including H3 lysine 4 mono or tri methylation (H3K4me or H3K4me3) as well as H3K27 acetylation (H3K27Ac). These modifications are not only present in mature cells, but also in progenitors of innate immune cells [413, 422]. Priming with training agents induces the production of

myelopoiesis promoting cytokines including GM-CSF. Both BCG and β-glucans increase the number of LT-HSCs, LSKs, the myeloid-based MPP3s, CMPs and GMPs. Training-induced LT-HSCs display a myeloid-biased phenotype, increasing cell division and metabolic reprogramming with a shift towards glycolysis and FAO [422]. In line, LT-HSCs increase the number of mature myeloid cells (monocytes and granulocytes) with enhanced killing capacity. Therefore, the remodeling of immune progenitors confers proliferative ability during trained immunity. However, some immune cells are tissue-resident with self-maintaining abilities and show a similar mechanism to clonal proliferation in adaptive immune cells. Lung-resident alveolar macrophages trained with adenoviral vectors appeared to maintain a trained phenotype for at least 28 days independently of BM progenitors [389].

1.4.3 Clinical implications of trained immunity

Understanding the mechanisms of trained immunity opens a broad range of therapeutic opportunities for multiple clinical conditions. In the field of vaccination, training could boost overall vaccine efficiency or the immune response in poor vaccine responders. The immunomodulatory effect of flagellin on both innate and adaptive immune cells prompted its testing as an adjuvant. Combined to pathogen antigens, flagellin acts as an adjuvant increasing antibody production and survival in mouse models [423]. These encouraging results stimulated the initiation of clinical trials of flagellin-combined vaccines against Influenza [424]. Glucan particles acting as both vaccine vector and adjuvant are in development [425].

Despite heterologous protection against infections, BCG is also a potent modulator of immune cells during cancer [426]. The FDA approved BCG for both the prevention of tuberculosis and the treatment of bladder cancer. BCG activates the immune system to produce pro-inflammatory cytokines and reprograms metabolism by promoting autophagy [420, 427]. In particular, tumor-associated macrophages (TAM) show a M2 phenotype that could be reverted to M1 by BCG. These mechanisms reverse the immunosuppressive state induced in the tumor microenvironment and promote immune function against tumor cells. Similarly, training agents may provide therapeutic

benefit during the immunoparalysis phase of sepsis [428]. In both cancer and sepsis, immune cells show a tolerant phenotypic and metabolic state with reduced TCA cycle activity. The stimulation of immunosuppressed cells with β-glucans or IFNγ reverted the metabolic state and restored cytokine production [429, 430].

Preconditioning immune responses by training may also have deleterious consequences especially in inflammatory diseases. Indeed, training worsens pathology in mouse models of Alzheimer's disease and stroke [388]. Western diet increases the level of circulating oxidized low-density lipoproteins (oxLDL) and promotes the establishment of a trained phenotype [431], in line with the M1 phenotype of macrophages in the adipose tissue of obese subjects [432]. In these conditions, reverting the training phenotype to induce tolerance might be an effective therapy. This can be achieved by targeting the immune response, the metabolic pathways or epigenetic modifications. Indeed, blocking IL-1β inhibits training in mice [422] and the inflammatory component of chronic diseases [433]. Pharmacological drugs directed against epigenetic modulators including HATs, HDACs and methyltransferases could also affect training phenotypes. However, since epigenetic modifications can be long lasting, the trained and tolerant phenotypes should be balanced to avoid shifting from one condition to another.

2 AIM AND OBJECTIVES

Our laboratory has previously shown that HDACi inhibit phagocytosis, ROS production, cytokine secretion and killing by macrophages, sensitize mice to mild infections while protecting from toxic and septic shock [139, 141]. It was also shown that cambinol, an inhibitor of SIRT1 and SIRT2, reduces inflammatory responses in macrophages and protects mice from lethal LPS shock and *K. pneumoniae* pneumonia [230]. Following these observations, the overall aim of this work was to assess the effect of HDAC and sirtuin modulation and training on innate immune cell responses and host defences. Four objectives were defined:

1. Since SCFAs act as HDACi, HDACi increase the susceptibility to infection and SCFAs are currently tested in clinical trials to treat inflammatory conditions, the **first objective** was to assess the impact of the SCFA propionate on innate immune responses *in vitro* and *in vivo* (**Section 3.1**).
2. The **second objective** was to assess the role of SIRT2, SIRT3 and SIRT5 single deletion on innate immune responses using knockout mice (**Sections 3.2, 3.3 and 3.4**).
3. Numerous sirtuin inhibitors target multiple sirtuins. Thus, the **third objective** was to study the complementarity of sirtuins during innate immune responses by developing and characterizing mouse lines with double deletions of SIRT2 and SIRT3 or SIRT3 and SIRT5 (**Sections 3.5 and 3.6**).
4. Trained immunity prompts cells to increase inflammatory responses upon restimulation, a condition opposite to the homeostatic state promoted by SIRT3. Since the magnitude of β-glucans training is not fully characterized, the **fourth objective** was to determine the range of infections against which training is protective, the cells and the functions involved in this protection and the possible role of sirtuins in this mechanism (**Sections 3.7 and 3.8**).

3 RESULTS

This part is divided into eight sections:

3.1 Impact of the microbial derived short chain fatty acid propionate on host susceptibility to bacterial and fungal infections in vivo

3.2 Sirtuin 2 Deficiency Increases Bacterial Phagocytosis by Macrophages and Protects from Chronic Staphylococcal Infection

3.3 Sirtuin 3 does not alter host defences against bacterial and fungal infections

3.4 Sirtuin 5 Deficiency Does Not Compromise Innate Immune Responses to Bacterial Infections

3.5 Dual deletion of the sirtuins SIRT2 and SIRT3 impacts on metabolism and inflammatory responses of macrophages and protects from endotoxemia

3.6 Impact of the dual deletion of the mitochondrial sirtuins SIRT3 and SIRT5 on anti-microbial host defences

3.7 Trained immunity confers broad-spectrum long-term protection against infections

3.8 Characterization of *in vivo* and *in vitro* induced trained immunity: impact on the phenotype of innate immune cell populations and hematopoiesis

3.1 Impact of the microbial derived short chain fatty acid propionate on host susceptibility to bacterial and fungal infections in vivo

Eleonora Ciarlo*[1], Tytti Heinonen*[1], Jacobus Herderschee[1], Craig Fenwick[2], Matteo Mombelli[1], Didier Le Roy[1], Thierry Roger[1]

[1]Infectious Diseases Service and Department of Medicine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Epalinges, 1066, Switzerland

[2]Division of Immunology and Allergy, Department of Medicine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Epalinges, 1066, Switzerland

*Equal contribution

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Summary

SCFAs produced by intestinal microbes by fermentation of dietary fibers mediate anti-inflammatory effects demonstrated notably at the level of Tregs. SCFAs act on their target cells through cell surface GPCRs or by diffusing into cells to act as HDACi. Since several HDACi reduce immune responses and sensitize mice to infections, we assessed the impact of propionate, one of the mostly abundant SCFAs, on immune cell responses *in vitro* and host defenses *in vivo*.

Macrophages incubated with propionate display increased acetylation of H3 and H4, supporting that propionate inhibits HDACs. Like other HDACi, propionate dose dependently reduces the production of cytokines and NO by murine macrophages, splenocytes and, to a lesser extent, dendritic cells as well as human whole blood exposed to a broad range of microbial stimuli.

Mice fed with propionate in their drinking water have increased acetylation of histones in stomach, blood and bone marrow. Propionate neither sensitizes nor protects mice against LPS-induced endotoxemia and infections induced by *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Candida albicans*. Finally, propionate slightly reduces the levels of *S. pneumoniae*-specific in infected mice, but does not impair the efficacy of passive immunization and natural immunization.

Together our data support the anti-inflammatory effect of propionate and undermines a role in susceptibility to infection. These data support the development of propionate based-therapies without putting the patient at risk of developing infections.

My contribution to this work: I performed whole blood stimulation, ELISA, Luminex and Western blots for *in vitro* experiments. I worked on the *in vivo* models of infection with *Klebsiella pneumoniae*, *Streptococcus pneumoniae* and *Candida albicans*.

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Impact of the microbial derived short chain fatty acid propionate on host susceptibility to bacterial and fungal infections *in vivo*

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Eleonora Ciarlo[1,*], Tytti Heinonen[1,*], Jacobus Herderschee[1], Craig Fenwick[2], Matteo Mombelli[1], Didier Le Roy[1] & Thierry Roger[1]

Short chain fatty acids (SCFAs) produced by intestinal microbes mediate anti-inflammatory effects, but whether they impact on antimicrobial host defenses remains largely unknown. This is of particular concern in light of the attractiveness of developing SCFA-mediated therapies and considering that SCFAs work as inhibitors of histone deacetylases which are known to interfere with host defenses. Here we show that propionate, one of the main SCFAs, dampens the response of innate immune cells to microbial stimulation, inhibiting cytokine and NO production by mouse or human monocytes/macrophages, splenocytes, whole blood and, less efficiently, dendritic cells. In proof of concept studies, propionate neither improved nor worsened morbidity and mortality parameters in models of endotoxemia and infections induced by gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*), gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*) and *Candida albicans*. Moreover, propionate did not impair the efficacy of passive immunization and natural immunization. Therefore, propionate has no significant impact on host susceptibility to infections and the establishment of protective anti-bacterial responses. These data support the safety of propionate-based therapies, either via direct supplementation or via the diet/microbiota, to treat non-infectious inflammation-related disorders, without increasing the risk of infection.

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

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

Both extracellular and intracellular SCFAs exert immunosuppressive effects. Extracellular SCFAs act through metabolite sensing G-protein coupled receptors (GPCRs) such as GPR41, GPR43 and GPR109A[7,14]. Although conflicting results have been reported[13,15], GPCRs were recently proposed to mediate the anti-inflammatory effects of SCFAs and protect form colitis, rheumatoid arthritis and airway hyper-responsiveness[16–19]. GPCRs

[1]Infectious Diseases Service and Department of Medicine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Epalinges, 1066, Switzerland. [2]Division of Immunology and Allergy, Department of Medicine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Epalinges, 1066, Switzerland. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to T.R. (email: Thierry.Roger@chuv.ch)

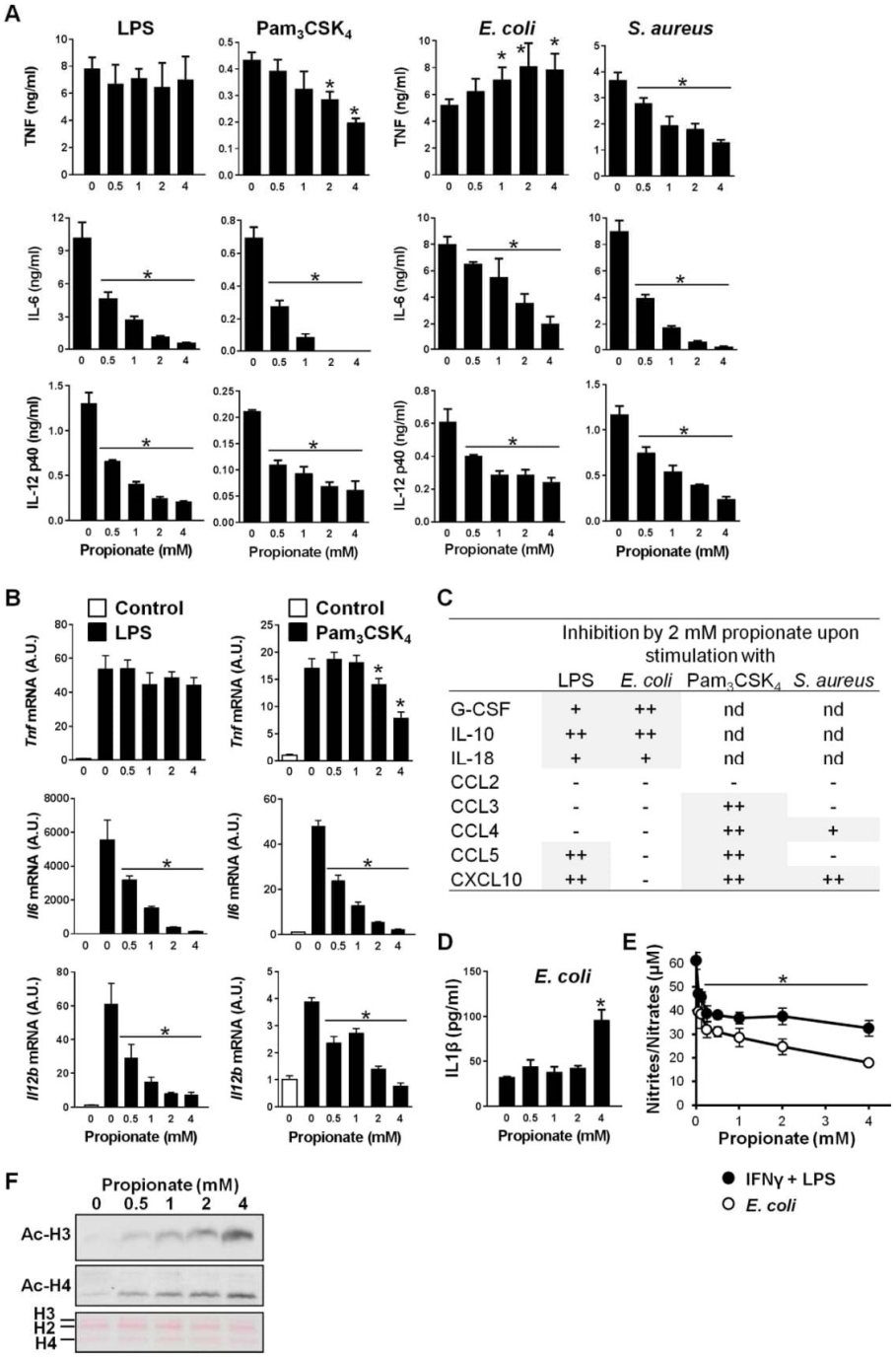


Figure 1. Impact of propionate on the response of macrophages to microbial stimulation. BMDMs were pre-incubated for 1 h with increasing concentrations (0, 0.06, 0.12, 0.25, 0.5, 1, 2 and 4 mM) of propionate before exposure for 4, 8 or 24 h to LPS (10 ng/ml), Pam_3CSK_4 (10 ng/ml), *E. coli* (10^6 CFU/ml), *S. aureus* (10^7 CFU/ml) or a combination of IFNγ (100 U/ml) plus LPS (10 ng/ml). (**A**,**B**) TNF, IL-6 and IL-12p40 concentrations in cell culture supernatants and *Tnf*, *Il6*, *Il12b* mRNA levels were quantified by ELISA (**A**, t = 8 h) and real time-PCR (**B**, t = 4 h). No cytokine was detected in the supernatants of unstimulated cells ($P < 0.001$ vs stimulus

alone). *Tnf*, *Il6* and *Il12b* mRNA levels were normalized to *Hprt* mRNA levels. Data are means ± SD of triplicate samples from one experiment performed with 4 mice and representative of 2 experiments. $^*P < 0.05$ vs stimulus without propionate. A.U.: arbitrary units. (**C**) The production of G-CSF, IL-10, IL-18, CCL2, CCL3, CCL4, CCL5 and CXCL10 was assessed by the Luminex technology (t = 8 h). Data summarize the impact of 2 mM propionate on mediators produced in response to LPS, *E. coli*, Pam_3CSK_4 and *S. aureus*: −, no inhibition; +, 1.5-2-fold inhibition; ++, >2-fold inhibition. Quantification is from one experiment performed with 4 mice. (**D**) IL-1β in cell culture supernatants. Data are means ± SD of triplicate samples from one experiment performed with 2 mice. $^*P < 0.05$ vs no propionate. (**E**) Nitrites/nitrates were quantified using the Griess reagent (t = 24 h). Data are means ± SD of quadruplicate samples from one experiment performed with 4 mice. $^*P < 0.05$ when comparing propionate at all concentrations vs no propionate. (**F**) Western blot analysis of acetylated histone 3 (Ac-H3) and Ac-H4 in BMDMs treated for 18 h with propionate. Ponceau staining of the membrane shows equal loading of total histones. Full-length blots are presented in Supplementary Figure S1.

propagate anti-inflammatory effects at least through a β2-arrestin-dependent stabilization of IκBα and inhibition of NF-κB-dependent transcription, and by promoting the generation of T regulatory cells (Tregs)[7,10].

Upon diffusion into cells, intracellular SCFAs inhibit zinc-dependent histone deacetylases (i.e. HDAC1-11)[20]. HDACs are major epigenetic erasers catalyzing the deacetylation of histones, leading to chromatin compaction and transcriptional repression[21]. HDACs also target signaling molecules and transcription factors. Inhibitors of HDAC (HDACi) directly or indirectly impair NF-κB and Foxp3 activity, mediating anticancer, anti-neurodegenerative and anti-inflammatory activities, in part by inducing Treg generation[13,21–23]. Numerous HDACi are tested in clinical trials and several have reached the clinic. Besides valproate that is used since decades as a mood stabilizer and anti-epileptic, vorinostat, romidepsin and belinostat are used for the treatment of cutaneous and/or peripheral T-cell lymphoma, and panobinostat is used to treat patients with multiple myeloma who experienced two prior therapies[23]. HDACi are also viewed as promising latency-reversing agents to purge the HIV reservoir[24]. In agreement with their powerful anti-inflammatory properties, several HDACi interfere with the development of innate immune responses, protect against lethal sepsis, and increase susceptibility to infection[25–32].

The development of SCFA-mediated therapies, either through direct supplementation with SCFAs or diet-induced modifications of the microbiota and production of endogenous SCFAs is an active area of research[16–19,33]. Considering that SCFAs carry anti-inflammatory activity and that HDACi were shown to increase susceptibility to infections in preclinical models and in patients enrolled in oncologic clinical studies[29,34–38], an important question is whether SCFA-mediated therapies are safe. Here we focused on propionate as a representative SCFA reported to modulate adaptive immune responses *in vivo*[39–43]. We analyzed the response of macrophages, dendritic cells (DCs), splenocytes and whole blood to microbial compounds. Additionally, we performed proof of concept studies using a large panel of preclinical mouse models of endotoxemia, gram-positive and gram-negative bacterial and fungal infection of diverse severity. The results show that propionate to some extent inhibits innate immune responses *in vitro,* but does not alter susceptibility to infection *in vivo* nor inhibit passive or natural immunization. These data support the safety of therapies using propionate for treating non-infectious inflammation-related disorders.

Results

Impact of propionate on the response of immune cells to microbial stimulation. To address the effects of propionate on the response of immune cells to microbial stimulation, bone marrow-derived macrophages (BMDMs) were exposed for 8 h to LPS (a TLR4 agonist), Pam_3CSK_4 (a lipopeptide triggering cells through TLR1/TLR2) and *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*), used as representative gram-negative and gram-positive bacteria. The levels of TNF, IL-6 and IL-12p40 produced by BMDMs were quantified by ELISA (Fig. 1A). Propionate (0.5–4 mM) dose-dependently inhibited TNF production induced by Pam_3CSK_4 and *S. aureus*, and IL-6 and IL-12p40 production induced by LPS, Pam_3CSK_4, *E. coli* and *S. aureus*. Similar to other HDACi[29,44,45], propionate did not inhibit TNF production induced by LPS and *E. coli*, and slightly amplified TNF response to *E. coli*. Accordingly, propionate powerfully inhibited LPS and Pam_3CSK_4-induced *Il6* and *Il12b* mRNA, to a lesser extent Pam_3CSK_4-induced *Tnf* mRNA, but not LPS-induced *Tnf* mRNA expression (Fig. 1B).

The anti-inflammatory activity of propionate was compared to that of butyrate and valproate by defining the IC_{50} of each of the SCFAs for LPS-induced IL-6 and IL-12p40 production. Similar IC_{50s} were obtained for IL-6 and IL-12p40: 0.01–0.05 mM for butyrate, 0.2–0.4 mM for valproate and 0.2–0.3 mM for propionate. Thus, propionate is as potent as valproate at inhibiting IL-6 and IL-12p40 but 8–20 fold less efficient than butyrate. The concentrations of G-CSF, IL-10, IL-18, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES and CXCL10/IP10 released by BMDMs exposed to LPS, *E. coli*, Pam_3CSK_4 and *S. aureus* were measured by Luminex (Fig. 1C). Whereas LPS and *E. coli* induced the secretion of all mediators, Pam_3CSK_4 and *S. aureus* did not induce the production of G-CSF, IL-10 and IL-18. Propionate inhibited G-CSF, IL-10 and IL-18 induced by LPS and *E. coli*, and CCL5 and CXCL10 induced by LPS. Propionate also inhibited CCL3, CCL4, CCL5 and CXCL10 induced by Pam_3CSK_4 and CCL4 and CXCL10 induced by *S. aureus*. Overall, propionate impaired more powerfully cytokine/chemokine secretion induced by Pam_3CSK_4 than LPS, like structurally unrelated HDACi[29,44,45], and more efficiently cytokine production induced by pure microbial ligands than whole bacteria triggering similar PRRs (*i.e.* LPS vs *E. coli*, and Pam_3CSK_4 vs *S. aureus*). Notably, and in line with recent reports[46,47], propionate at 4 mM increased *E. coli*-induced IL-1β secretion by BMDMs (Fig. 1D). Thus propionate impacts on inflammation

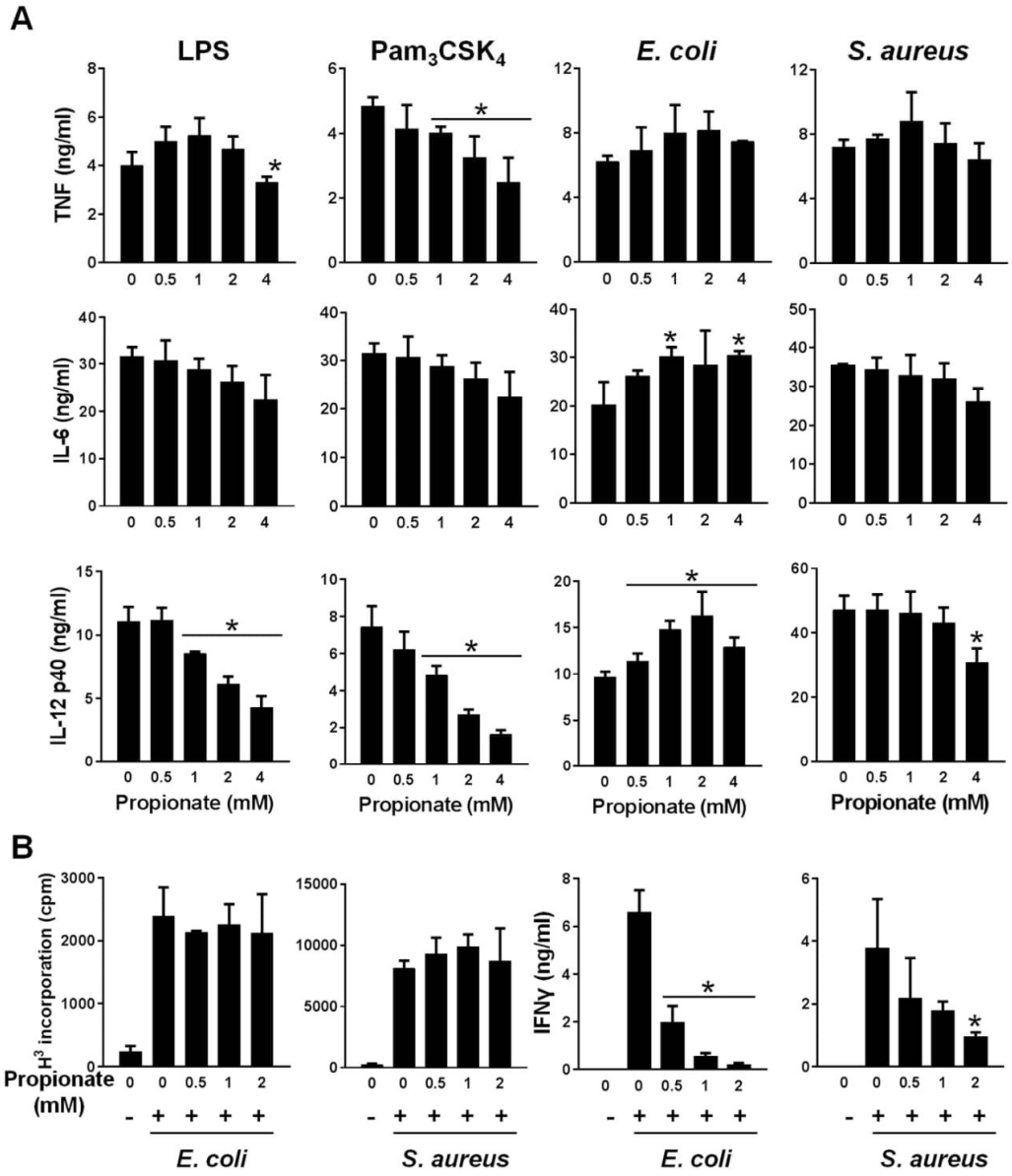
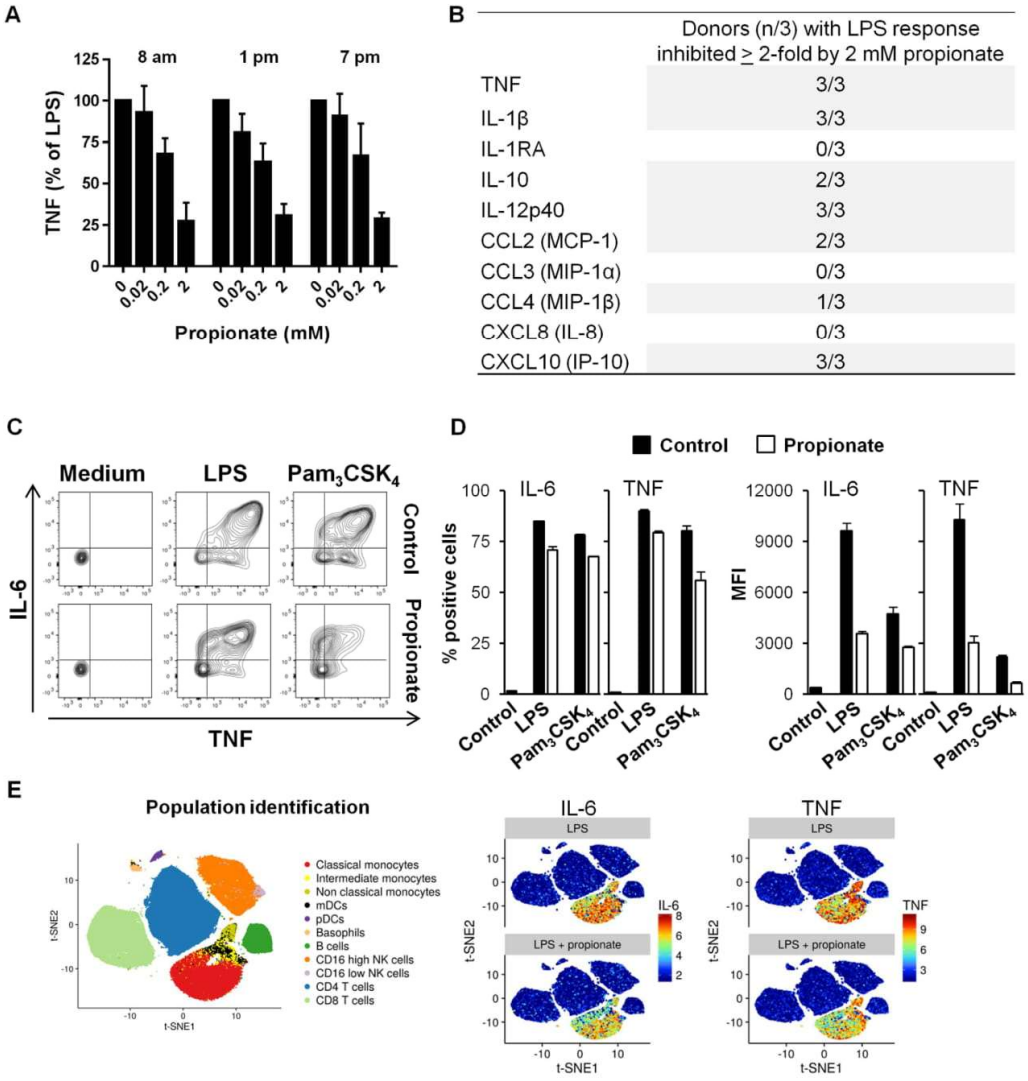


Figure 2. Impact of propionate on the response of dendritic cells and splenocytes. (**A**) BMDCs were pre-incubated for 1 h with increasing concentrations (0, 0.5, 1, 2 and 4 mM) of propionate before exposure for 8 h to LPS (10 ng/ml), Pam_3CSK_4 (10 ng/ml), *E. coli* (10^6 CFU/ml) and *S. aureus* (10^7 CFU/ml). TNF, IL-6 and IL-12p40 concentrations in cell culture supernatants were quantified by ELISA. Data are means ± SD of triplicate samples from one experiment performed with 4 mice and representative of 2 experiments. No cytokine was detected in the supernatants of unstimulated cells ($P < 0.001$ vs stimulus alone). (**B**) Mouse splenocytes were incubated for 48 h with or without propionate and *E. coli* or *S. aureus* (10^6 CFU/ml). Proliferation was measured by ^{3}H-thymidine incorporation. IFNγ concentrations in cell culture supernatants were quantified by ELISA. Data are means ± SD of triplicate samples from one experiment performed with 4 mice. *$P < 0.05$ vs stimulus without propionate.

in a cytokine dependent manner. Propionate also inhibited the production of nitric oxide (NO) induced by *E. coli* or IFNγ/LPS in BMDMs (50% inhibition using 0.6 mM and 4 mM propionate, respectively (Fig. 1E)).



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

Figure 3. Impact of propionate on the response of human whole blood and monocytes. Whole blood from 3 healthy subjects was incubated for 18 h with propionate and LPS (100 ng/ml). (**A**) TNF released by whole blood collected at 8 am, 1 pm and 7 pm was quantified by ELISA. Data are expressed as the percentage of maximal (LPS without propionate) TNF release. No TNF was detected in the absence of LPS stimulation (not shown). Data are means ± SD from 3 healthy subjects. $P < 0.005$ when comparing 0.2 and 2 mM propionate with 0 mM propionate. (**B**) TNF, IL-1β, IL-1RA, IL-10, IL-12p40, CCL2, CCL3, CCL4, CXCL8 and CXCL10 were quantified by Luminex. Results summarize the number of donors in whom propionate inhibited significantly ($P < 0.05$) and by at least 2-fold cytokine release. (**C**,**D**) PBMCs were incubated for 1 h with 2 mM propionate and stimulated for 4 h with LPS (100 ng/ml) and Pam_3CSK_4 (1 μg/ml). TNF and IL-6 expression in $CD14^+$ monocytes was analyzed by flow cytometry to calculate the percentage of positive cells (**C**) and mean fluorescence intensity (MFI) (**D**). Data are means ± SD from one experiment performed with 2 donors. (**E**) Whole blood incubated for 4 h with 2 mM propionate and 100 ng/ml LPS was fixed with Smart Tube stabilizer, and processed by CyTOF as described in *Materials and Methods*. Left: t-SNE scatter plot of non-granulocyte events. Right: t-SNE plot with arcsinh transformed signal intensity of IL-6 and TNF. Data are representative of results obtained with 3 donors.

To answer the question whether propionate acted through HDAC inhibition or via GPCRs, we first quantified mRNA levels of Hdac1-11 and free fatty acid receptor 2 (Ffar2) and Ffar3 encoding for GPR43 and GPR41. Ffar2

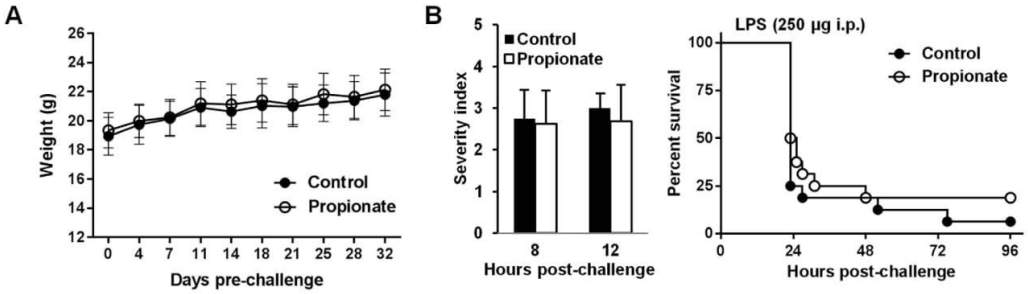


Figure 4. Propionate does not protect from lethal endotoxemia. BALB/c mice (n = 16 per group) were treated with or without 200 mM propionate in drinking water for 1 month. (**A**) Weight of animals under propionate treatment. (**B**) Severity scores ($P > 0.1$) and survival ($P = 0.3$) of mice challenged with LPS (250 µg i.p.).

and Ffar3 mRNAs were not detected in BMDMs, in line with a previous report[41]. Incubation of BMDMs with propionate (0–4 mM for 4 or 18 hours) slightly modulated Hdac1-11 expression (range: 1.2–2.5 fold increase or decrease). Yet, propionate strongly increased histone 3 (H3) and H4 acetylation in a dose-dependent manner (Fig. 1F), indicating that propionate inhibits histone deacetylase activity in BMDMs.

Bone marrow-derived dendritic cells (BMDCs) were less sensitive than BMDMs to the anti-inflammatory effects of propionate. In BMDCs, propionate only significantly inhibited Pam_3CSK_4-induced TNF and IL-12p40 production in response to LPS, Pam_3CSK_4 or *S. aureus* (Fig. 2A). Of note, propionate slightly increased *E. coli*-induced IL-6 and IL-12p40 production by BMDCs. The viability of BMDMs and BMDCs incubated for 18 h with up to 8 mM propionate was greater than 98%, suggesting that propionate's effects were not related to cytotoxicity. Along with a good tolerability of immune cells to propionate, propionate barely affected the proliferation of splenocytes exposed to *E. coli* and *S. aureus* whereas it efficiently inhibited IFNγ production (Fig. 2B).

The impact of propionate was tested on human cells. Propionate dose-dependently inhibited TNF production by whole blood exposed to LPS (Fig. 3A), albeit less efficiently than butyrate (TNF: 61 ± 6% vs 96 ± 4% and IL-6: 41 ± 7% vs 70 ± 10% inhibition using propionate vs butyrate at 2 mM, n = 3 donors collected at 8 am; $P < 0.05$). The extent of TNF inhibition by propionate was similar using blood collected at various times of the day (8 am, 1 pm and 7 pm), excluding a circadian rhythm-dependent effect. A Luminex quantification of 10 mediators produced by whole blood exposed to LPS extended to IL-1β, IL-10, IL-12p40, CCL2 and CXCL10 the spectrum of cytokines and chemokines whose expression was significantly inhibited (≥2-fold) by 2 mM propionate in at least 2 out of the 3 donors tested (Fig. 3B). In parallel experiments, butyrate inhibited more powerfully than propionate the secretion of IL-10 (in 3/3 vs 2/3 donors), CCL2 (3/3 vs 2/3) and CCL4 (2/3 vs 1/3). Butyrate also impaired the release of IL-1RA (3/3 donors) and CXCL8 (1/3). In a confirmation approach, flow cytometry analyses of intracellular cytokine expression in purified human $CD14^+$ monocytes exposed to LPS and Pam_3CSK_4 revealed that propionate reduced the percentage (11–24% reduction) and the mean fluorescence intensity (1.7–3.4 fold reduction) of TNF and IL-6 positive cells (Fig. 3C and D). Additionally, mass cytometry (CyTOF[48]) analyses on human whole blood demonstrated that propionate inhibited IL-6 and TNF production by both classical and non-classical monocytes (Fig. 3E). Altogether, propionate inhibited, in a cell and stimulus-specific manner, the response of mouse and human immune cells *in vitro*. We next investigated the impact of propionate *in vivo*.

Propionate does not protect from lethal endotoxemia and severe sepsis. Following common procedures used to study the impact of SCFAs *in vivo*[17,18,39,41–43], mice were fed with propionate at 200 mM in the drinking water for 3 weeks, unless otherwise specified, before being used in preclinical models of toxic shock and infection. Attesting of the effectiveness of the treatment, 3 weeks of propionate regimen increased the number of splenic $Foxp3^+$ Tregs (116% when compared to control mice; n = 8–11 animals per group; $P = 0.0007$) and of acetylated H4 in stomach, blood and bone marrow (3.5, 3.6 and 1.8 fold increased versus control mice, n = 2).

Overwhelming inflammatory responses are deleterious for the host, and inhibition of the release of pro-inflammatory mediators confers protection in preclinical models of sepsis[3–5]. Moreover, several HDACi were shown to protect from toxic shock[49]. Therefore, we first tested propionate in a mouse model of acute endotoxemia. One month of propionate treatment had no impact on animal weight (Fig. 4A). In mice challenged with a lethal dose of LPS, severity scores and survival rates were similar whether or not animals were treated with propionate ($P > 0.5$ and $P = 0.3$; Fig. 4B).

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

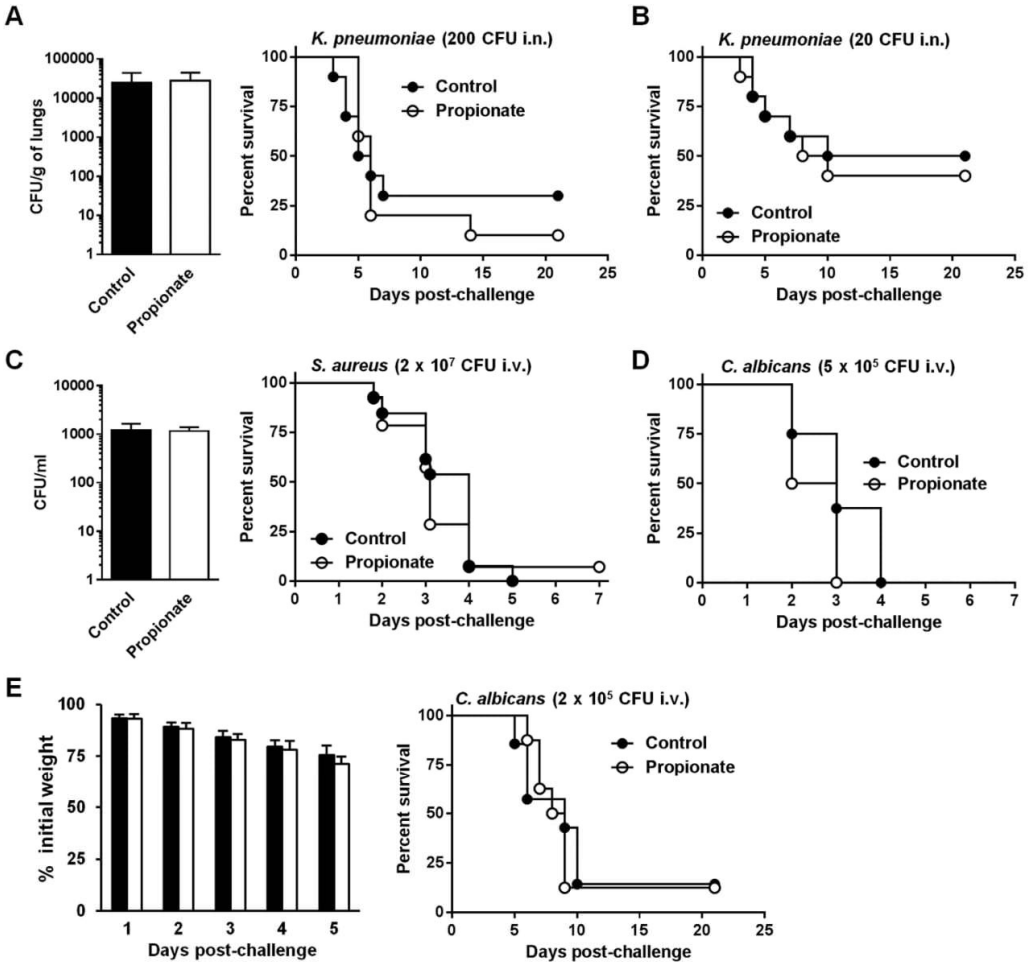


Figure 5. Propionate does not protect from lethal sepsis. BALB/c mice were treated with or without 200 mM propionate in drinking water for 3 weeks. (**A**,**B**) Bacterial counts in lungs 48 h post-infection and survival of mice (n = 10 per group) challenged i.n. with 200 CFU (**A**) or 20 CFU (**B**) of *K. pneumoniae*. $P = 0.4$, 0.8 and 0.7, respectively. (**C**) Bacterial counts in blood 24 h post-infection and survival of mice (n = 15 per group) challenged with *S. aureus* (2×10^7 CFU i.v.). $P = 0.9$ and $P = 0.6$. (**D**,**E**) Survival and body weight of mice (n = 8 per group) challenged with *C. albicans* (5×10^5 CFU i.v. in **D** and 2×10^5 CFU i.v. in **E**). $P = 0.1$, $P > 0.1$ and $P = 0.8$, respectively.

to produce a milder form of candidiasis during which mortality occurs 5 to 10 days after infection. Weight loss ($P > 0.1$) monitored during the first 5 days and survival (14.3% and 12.5%; $P = 0.8$) were comparable in untreated and propionate-treated mice (Fig. 5E).

Even though propionate was shown to impact on immune parameters of mice with a normal microbiota[17,18,39,43,45,50], propionate produced by gut bacteria may attenuate the impact of propionate supplementation in models of infection. To address this issue, mice were treated with a combination of ciprofloxacin and metronidazole (CM) to deplete the gut flora and decrease endogenous SCFAs levels[39,51]. CM-treated mice lost 17% weight during the first week of treatment and recovered initial weight after 3 weeks. CM-treated mice were more sensitive to candidiasis (median survival time: 9.5 days for CM vs 11.5 days for controls mice run in parallel; n = 10 mice/group; $P = 0.05$). Co-treatment with CM plus propionate slightly increased weight loss and impaired weight rebound of uninfected mice (Fig. 6A). CM-treated mice died in between days 6 and 15 after *C. albicans* challenge, and propionate supplementation did not protect CM-treated mice from candidiasis ($P = 0.4$; Fig. 6B). To delineate the impact of gram-positive and gram-negative bacteria, mice were treated, together with propionate, with either metronidazole to target anaerobic gram-negative bacteria or vancomycin to target gram-positive

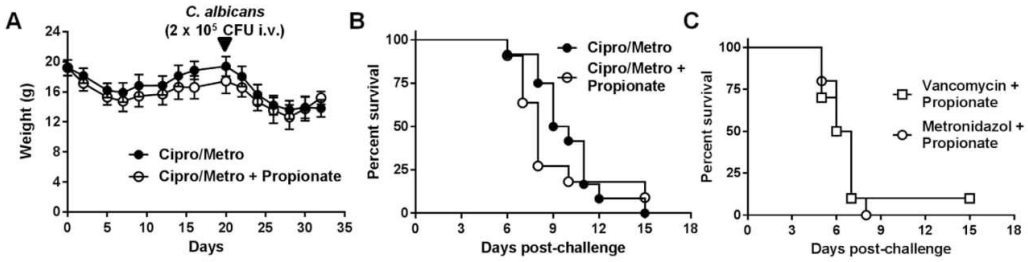


Figure 6. Propionate does not protect from candidiasis mice depleted of gut microbiota. BALB/c mice (n = 10 per group) were treated with ciprofloxacin (0.2 mg/ml) and metronidazole (1 mg/ml) or metronidazole or vancomycin (1 mg/ml) with or without 200 mM propionate in drinking water for 3 weeks and challenged with *C. albicans* (2×10^5 CFU i.v.). (**A**) Body weight. (**B**,**C**) Survival of mice. $P > 0.05$.

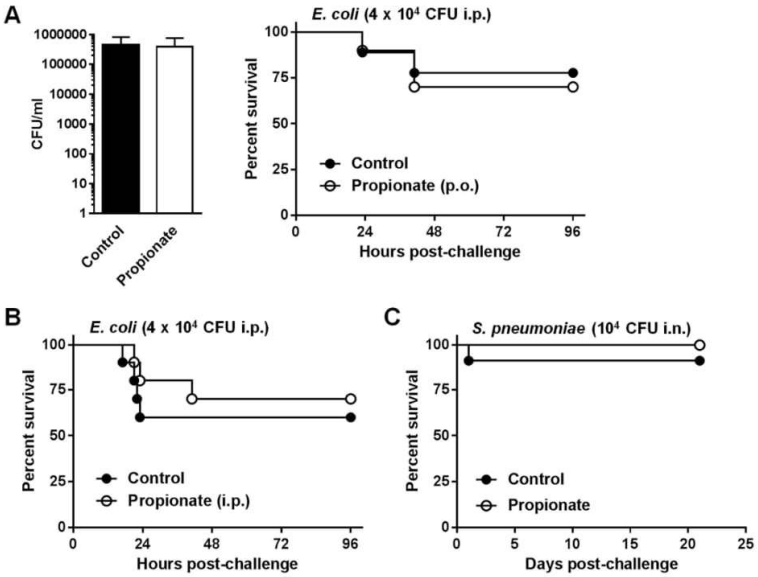


Figure 7. Propionate does not sensitize to mild infection by *E. coli* and *S. pneumoniae*. BALB/c mice were treated with or without 200 mM propionate in drinking water (**A**,**C**) or 1 g/kg propionate given i.p. every other day (**B**) for 3 weeks and challenged with *E. coli* (4×10^4 CFU i.p.; n = 10 per group; (**A**,**B**) or *S. pneumoniae* (10^4 CFU i.p.; n = 9–10; **C**). (**A**) Bacterial counts in blood 24 h post-infection and survival of mice. $P = 0.9$ and 0.7. (**B**,**C**) Survival of mice. $P = 0.6$ and 0.4.

bacteria. The two treatments resulted in identical survival profiles (Fig. 6C). Overall, propionate did not interfere with acute, lethal, bacterial and fungal infections.

Propionate does not sensitize to mild infection. Compromising innate immune responses may increase susceptibility to infection. To analyze the impact of propionate on mild infection, and to test another route of administration of propionate, propionate was given either *per os* or intraperitoneally (p.o.: 200 mM in water; i.p.: 1 g/kg i.p. every other day[43]) to mice subsequently challenged with *E. coli* titrated to cause a mild infection. Bacterial counts ($P = 0.9$) and survival rates (77% vs 70% and 60% vs 70% in control vs propionate groups upon p.o. and i.p. treatments; $P = 0.7$ and $P = 0.6$) were similar in all groups of treatment (Fig. 7A and B). Confirming that propionate does not sensitize mice to infection, 90% (9/10) of control mice and 100% (9/9) of propionate-treated mice infected i.n. with 10^4 CFU *Streptococcus pneumoniae* (*S. pneumoniae*) survived infection ($P = 0.4$; Fig. 7C). Hence, propionate did not increase susceptibility to *E. coli* peritonitis and pneumococcal pneumonia.

Propionate does not impair passive and natural immunization. We measured anti-*K. pneumoniae* and anti-*S. pneumoniae* IgG titers in mice surviving infection with 20 CFU *K. pneumoniae* (4 controls and 5

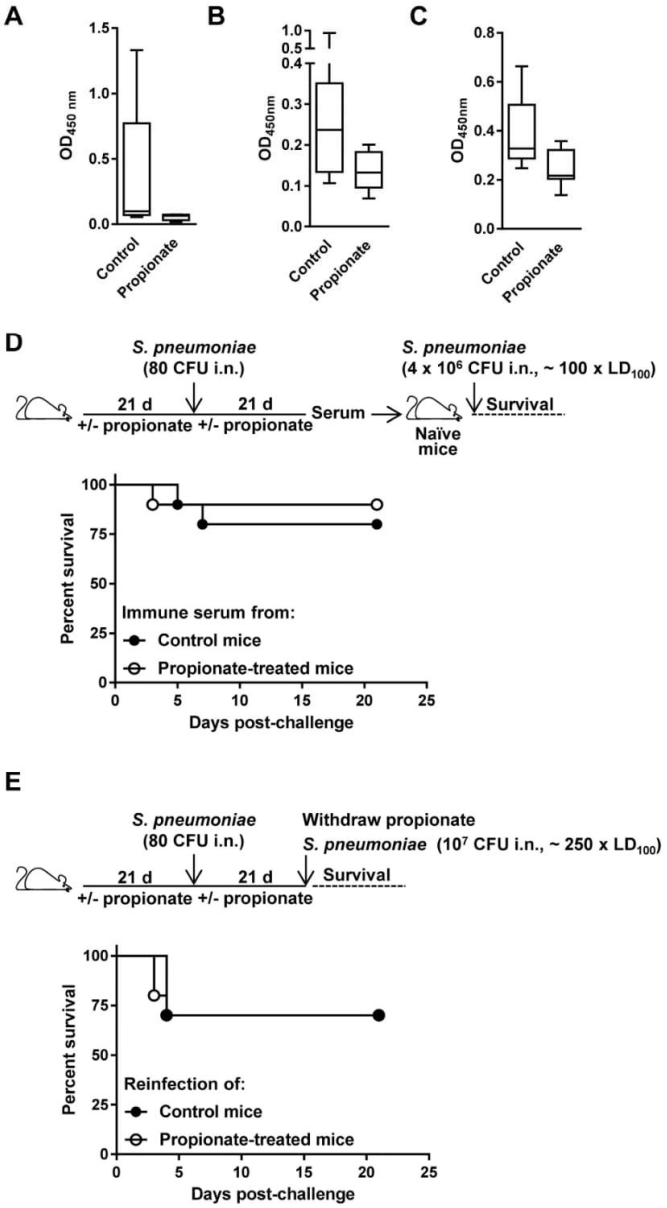


Figure 8. Propionate does not impair passive immunization and protection to secondary infection. Anti-*K. pneumoniae* (**A**), anti-*S. pneumoniae* (**B**) and anti-*C. albicans* (**C**) IgG titers in BALB/c mice surviving infection with 20 CFU *K. pneumoniae* (n = 4 control and 5 propionate-treated mice; Fig. 5B), 10^4 CFU *S. pneumoniae* (n = 9 control and 10 propionate-treated mice; Fig. 7C) and 4×10^4 CFU *C. albicans* (n = 9 control and 9 propionate-treated mice, serum was collected 3 weeks post-infection). Box and min-to-max whisker plots represent the $OD_{450\,nm}$ using plasma (diluted 1/200) collected on day 21 after infection. $P = 0.1$, 0.01 and 0.02, respectively. No signal was detected using plasma from uninfected mice. (**D**,**E**) BALB/c mice (n = 18–21 per group) were treated with or without 200 mM propionate in drinking water for 3 weeks, challenged i.n. with 80 CFU *S. pneumoniae*, and used for subsequent experimentation 3 weeks later. (**D**) Sera collected from 8 water and 11 propionate-treated mice were pooled and transferred (120 µl i.p.) into naive mice (n = 10 per group) infected 24 h later with 4×10^6 CFU *S. pneumoniae* (~100 x LD_{100}). Survival was monitored for 21 days. $P = 0.6$. (**E**) Propionate treatment was withdrawn. Mice (n = 10 per group) were infected with 10^7 CFU *S. pneumoniae* (~250 x LD_{100}). Survival was monitored for 21 days. $P = 0.8$.

propionate-treated mice; Fig. 5B) and 10^4 CFU *S. pneumoniae* (9 controls and 9 propionate-treated mice; Fig. 7C). Anti-bacterial IgG titers were reduced in propionate-treated mice ($P = 0.1$ and 0.01 for anti-*K. pneumoniae* and *S. pneumoniae* IgG titers, respectively; Fig. 8A and B). To confirm this observation, we measured IgG titers in mice infected 3 weeks earlier with a non-lethal inoculum of *C. albicans* (2×10^4 CFU i.v.). Anti-*C. albicans* IgG titers were reduced in propionate fed mice ($P = 0.02$; Fig. 8C). In addition, splenic Foxp3$^+$ Tregs were increased in propionate treated and *C. albicans* infected mice (113% when compared to control mice; n = 10 mice per group; $P = 0.006$). Therefore, although propionate did not interfere with morbidity and mortality in the models of infection presented above, it impacted to some extent on anti-microbial host responses. Two approaches were used to assess the relevance of this observation. Mice treated with or without propionate for 3 weeks were challenged with a non-lethal inoculum of *S. pneumoniae* (80 CFU). In a first setting, 3 weeks after infection, sera were collected and transferred into naive, non-treated, mice that were infected 24 h later with *S. pneumoniae* used at around 100 x LD_{100} (Fig. 8D). In a second setting, 3 weeks after infection, propionate treatment was withdrawn and mice were re-challenged with *S. pneumoniae* used at around 250 x LD_{100} (Fig. 8E). Overall, propionate treatment during the primary infection had no impact on outcome, and both transfer of immune serum and pre-exposure to a low *S. pneumoniae* inoculum protected mice from a lethal *S. pneumoniae* inoculum.

Discussion

The gut microbiota and its metabolites exert strong influences on human health. Among bacterial metabolites, SCFAs have attracted much attention because of their beneficial influence on the development of inflammation-related pathologies in combination with the fact that their production can be influenced by the diet[7,10,52,53]. Here we show that propionate has powerful yet selective anti-inflammatory activity *in vitro*, and that it does not have a major impact on host susceptibility to infection *in vivo*. This observation is particularly relevant in light of the development of diet or microbiota targeting strategies to treat immune related diseases.

Propionate impaired cytokine production by innate immune cells, albeit differently according to the cell type, the microbial trigger and the cytokine analyzed. Similar disparities have been observed with other SCFAs[29,40,45,46,54]. BMDCs were more resistant to propionate than BMDMs, human monocytes and whole blood. In human monocyte-derived DCs (moDCs) and BMDCs, propionate modestly affected IL-6 but efficiently limited IL-12p40 production induced by LPS (ref. 40 and Fig. 2). Furthermore, propionate did not inhibit MHC-II and CD86 expression but impaired CD83 expression by moDCs[40]. Disparate cell responses to propionate may reflect, at least in part, differential expression of GPCRs. In depth analyses of the pattern and the expression levels of cell-surface GPCRs by immune and non-immune cells is still missing, and could give clues about the contradictory findings reported concerning the inflammatory phenotype of GPR41 and GPR43 knockout mice[15]. Moreover, SCFA specificity of GPCRs and how redundant behave GPCRs *in vitro* and *in vivo* are largely unresolved issues. For example, mice deficient in either GPR43 or GPR109A were susceptible to gut inflammation and developed exacerbated colitis, and mice deficient in either GPR41 or GPR43 were susceptible to allergic airway inflammation[17–19,43,55]. At least in the gut, expression of both GPR43 and GPR109A by non-hematopoietic cells contributed to the protective effects of high-fiber regimen against colitis[19].

Besides acting through GPCRs, SCFAs act as inhibitors of class I and II HDACs (HDACi). HDACi impair innate and adaptive immune responses at multiple levels, including TLR and IFN signaling, cytokine production, bacterial phagocytosis and killing, leukocyte adhesion and migration, antigen presentation by DCs, cell proliferation and apoptosis, and Treg development and function[21,49,56]. In T cells, acetate, propionate and butyrate suppressed HDAC activity independently of GPR41 and GPR43[41]. Whether SCFAs mediate HDAC inhibition through GPCRs is debatable[13], but it is worth mentioning that GPCR signaling modulates kinase, redox and acetylation pathways that, in turn, influence the cellular distribution and activity of histone acetyl transferases and HDACs[57].

Like other structurally unrelated HDACi (trichostatin A and suberanilohydroxamic acid, *i.e.* vorinostat), acetate, propionate and butyrate increased acetylation of FOXP3 and potentiated the generation of peripheral Treg cells[39,42,58]. Moreover, SCFAs were recently reported to promote the generation of Th1 and Th17 cells during *Citrobacter rodentium* infection, suggesting a complex, context-dependent impact of SCFAs on immune responses[41]. Butyrate is a more potent HDACi than propionate, which is more potent than acetate. This ranking parallels the effectiveness of the anti-inflammatory activity of SCFAs[39,59,60]. Propionate failed to inhibit TNF but not IL-6 and IL-12p40 induced by LPS in BMDMs, which mirrored previous observations obtained with trichostatin A and suberanilohydroxamic acid[29,44,61–63]. Propionate strongly increased H3 and H4 acetylation in BMDMs which, like BMDCs, barely expressed *Ffar2* and *Ffar3*[41]. Thus, the effect of propionate on BMDMs is at least in part mediated by inhibition of HDACs. The fact that propionate slightly increased cytokine production under certain conditions is reminiscent of the paradoxical impact of HDACi on TNF secretion in human macrophages[46]. Further work will be required to unravel how propionate differentially affects cytokine expression induced by pure microbial ligands versus whole bacteria, for example by analyzing chromatin structure, modifications and activation of transcriptional regulators, and signaling pathways.

Acetate, propionate and butyrate are found at molar ratios of 60/20/20 in the intestinal tract and 90-55/35-5/10-4 in blood, depending on portal, hepatic and peripheral origins, where they altogether reach 50–150 mM and 0.1–1 mM, respectively[8,43]. The high plasma concentrations of propionate compared to butyrate may counterbalance its weaker anti-inflammatory activity. Further work will be required to analyze the effects of combinational treatments with SCFAs on innate immune cells. Propionate is produced primarily by Bacteroidetes via the succinate pathway and some Firmicutes through the lactate and succinate pathways, acetate by enteric bacteria and butyrate by Firmicutes[10]. SCFAs themselves modify the composition of the gut microbiota. Propionate stimulates the growth of *Bifidobacterium*[64]. Proportions of Bacteroidaceae and Bifidobacteriaceae increased in the gut of mice fed with a high-fiber diet, elevating acetate and propionate levels but decreasing butyrate concentrations in cecal content and blood[43]. Thus, changing microbiome composition affects SCFA levels both locally

and systemically. Moreover, the gut microbiota protects from pneumococcal pneumonia[65]. In the perspective of targeting the diet or the microbiota for treating inflammatory conditions[7,10,52,53], it was of prime interest to analyze the impact of propionate in preclinical models of infection.

Unlike powerful broad-spectrum HDACi[27,29,66,67], propionate had no obvious impact on morbidity and mortality parameters in models of endotoxemia and infections. This contrasts with the effectiveness of SCFAs at ameliorating the clinical outcome in chronic inflammatory diseases like rheumatoid arthritis, colitis and airway allergy[17–19,33,39,43,45,50]. The propionate regimen itself was unlikely responsible of the failure to protect septic animals since it increased H4 acetylation in organs and identical or shorter treatments had an immune impact[39,43]. Moreover, as expected[39,43,50], propionate increased the frequency of peripheral Tregs and reduced anti-microbial IgG responses, indicating that propionate influenced immune parameters during the course of infections. Multiple mechanisms may account for the reduced humoral response. Besides increasing the frequency of Tregs, SCFAs and HDACi have been shown to inhibit development and migration of DCs. Moreover, they decreased expression of costimulatory and MHC molecules, reduced production of T-cell polarizing factors and increased production of indoleamine 2,3-dioxygenase (a negative regulator of T-cell activation) by DCs. In mouse models, HDACi inhibited antigen presentation and allogeneic and syngeneic responses and decreased antibody generation[29,40,44,56].

Albeit surprising at first glance, propionate did not increase the mortality of mice subjected to sub-lethal/mild infections. Indeed, one of the possible collateral damages of administrating immunomodulatory compounds is an increased risk of infections. A well-known example is anti-TNF therapies that are associated with reactivation of latent tuberculosis and viral infections as well as an increased risk of opportunistic infections[68]. Moreover, episodes of severe infection have been reported in patients treated with HDACi[34–38]. In the present study, we tested models of systemic and local infections using the most common etiologic agents of bacterial sepsis in humans (*E. coli*, *S. aureus* and *K. pneumoniae*) to investigate the safety of propionate supplementation for clinical purposes.

The production of propionate by intestinal bacteria has been proposed to represent a mechanism through which the host response to commensals is kept under control and avoid local inflammation and tissue damage. It is however now well established that SCFAs have much broader effects on human health. Using several preclinical mouse models, we report that administration of propionate neither protects from lethal sepsis nor increases susceptibility to mild infections. These results are encouraging in the perspective of developing propionate-based therapies, e.g. direct supplementation or via the diet/microbiota, without putting patients at risk of developing infections.

Materials and Methods

Ethics statement. Animal experimentations were approved by the Service de la Consommation et des Affaires vétérinaires (SCAV) du Canton de Vaud (Epalinges, Switzerland) under authorizations n° 876.7, 876.8, 877.7 and 877.8, and performed according to our institutional guidelines and ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines). Experiments on human whole blood samples were carried out in accordance with guidelines and regulations of the Swiss Ethics Committees on research involving humans. The procedure, using anonymized human whole blood samples from healthy subjects without possibility to trace back subject identity, did not require prior ethics committee authorization. Written informed consent was obtained from blood donors at the Infectious Diseases Service, CHUV, Lausanne.

Mice, cells and reagents. Female BALB/cByJ mice (8–10 week-old; Charles River Laboratories, Saint-Germain-sur-l'Arbresle, France) were housed under specific pathogen-free conditions. Bone marrow cells were cultured for 7 days in IMDM containing 50 μM 2-ME and 30% L929 supernatant as a source of M-CSF to generate bone marrow-derived macrophages (BMDMs), or GM-CSF to generate bone marrow-derived dendritic cells (BMDCs)[69]. Splenocytes were cultured in RPMI 1640 medium containing 2 mM glutamine and 50 μM 2-ME[70]. Culture media (Invitrogen, San Diego, CA) were supplemented with 10% (v/v) heat-inactivated FCS (Sigma-Aldrich St. Louis, MO), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Propionate and butyrate were from Sigma-Aldrich, valproate from Desitin (Hamburg, Germany), *Salmonella minnesota* ultra pure lipopolysaccharide (LPS) from List Biologicals Laboratories (Campbell, CA) and Pam_3CSK_4 from EMC microcollections (Tübingen, Germany). *E. coli* O18:K1:H7 (*E. coli*), *K. pneumoniae* caroli (*K. pneumoniae*), *S. aureus* AW7 (*S. aureus*), *S. pneumoniae* 6303 (*S. pneumoniae*), and *C. albicans* were isolated from septic patients[71,72]. *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* were grown in brain heart infusion broth, *C. albicans* in yeast extract-peptone-dextrose (BD Biosciences, Erembodegem, Belgium). For *in vitro* experiments, microorganisms were heat-inactivated for 2 h at 56 °C before usage.

Cell viability assay. Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation and Viability Assay and a Synergy H1 microplate reader (BioTek, Winooski, VT)[73]. On each 96-well cell culture plate, serial quantities of cells (0.3×10^4–5×10^5) were seeded to establish a standard curve.

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

Cytokine and NO measurements and flow cytometry analyses. Cell culture supernatants and plasma were used to quantify the concentrations of TNF, IL-1β, IL-6, IL-12p40 and IFN-γ by DuoSet ELISA kits (R&D Systems, Abingdon, UK), cytokines/chemokines using mouse (G-CSF, IL-1β, IL-10, IL-12p70, IL-18, CCL2/MCP1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CXCL10/IP10) and human (TNF, IL-1β, IL-1ra, IL-10,

IL-12p40, CCL2, CCL3, CCL4, CXCL8/IL-8, CXCL10) Luminex assays (Affimetrix eBioscience, Vienna, Austria), and NO using the Griess reagent[71,74]. Intracellular cytokine staining was performed essentially as described[75]. Half a million peripheral blood mononuclear cells (PBMCs) were incubated for 1 h with propionate and then for 4 h with 1 μg/ml brefeldin A (BioLegend, San Diego, CA) with or without LPS (100 ng/ml) or Pam_3CSK_4 (1 μg/ml). PBMCs were stained with the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Molecular Probes, ThermoFisher Scientific, Zug, Switzerland), washed with Cell Stain Medium (CSM: PBS, 0.5% BSA, 0.02% NaN_3 and 2 mM EDTA), incubated with Human TruStain FcX™ (BioLegend) to block Fc receptors and stained with anti-CD3-PerCP/Cy5.5 (clone UCHT1) and anti-CD14-Pacific Blue (clone M5E2). Cells were washed with CSM, fixed using 2.4% (w/v) formaldehyde in PBS, washed with CSM-S (CSM containing 0.3% saponin, Sigma-Aldrich) and stained with anti-IL-6-allophycocyanin and anti-TNF-phycoerythrin/Dazzle™ 594 antibodies (clones MQ2-13A5 and MAB11). Single cell suspensions of splenocytes were stained with anti-CD4-APC/Cy7 (clone Gk1.5) and anti-CD25-APC (clone 3C7). Staining steps were performed at 20 °C for 20 min. All antibodies were from BioLegend. Foxp3 staining (anti-Foxp3-PE, clone FJK-16S,) was performed with the Foxp3/Transcription Factor Staining Buffer Set from eBioscience according to the manufacturer's instructions. Unstained and single stained samples were used to calculate compensation for PBMCs while UltraComp eBeads (eBioscience) were used for splenocytes. Acquisition was performed on a LSR II flow cytometer (BD Biosciences). Foxp3 and CD25 gates were determined using FMO controls. Data was analyzed using FlowJo vX (FlowJo LCC, Ashland, OR).

RNA analyses by real-time PCR. Total RNA was isolated, reverse transcribed and used for real-time PCR analyses using a QuantStudio™ 12 K Flex system (Life Technologies, Carlsbad, CA). Reactions consisted of 1.25 μl cDNA, 1.25 μl H_2O, 0.62 μl primers and 3.12 μl Fast SYBR® Green Master Mix (Life Technologies). Primer pairs for amplifying *Tnf*, *Il6*, *Il12b* and *Hprt* (*hypoxanthine guanine phosphoribosyl transferase*) cDNA were as published[69]. Samples were tested in triplicates. Gene specific expression was normalized to *Hprt* expression and expressed relative to that of untreated cells.

Western blot analyses. Histones were extracted from cells or organs (disrupted using a gentleMACS™ Octo Dissociator, Miltenyi Biotec; Bergisch Gladbach, Germany), run through SDS-PAGE and detected by Western blotting[29] using antibodies (diluted 1:1000) directed against acetylated histone 3 (H3) and H4 (06-599, 06-860) (EMD Millipore, Billerica, MA). Blots were revealed with the enhanced chemiluminescence Western blotting system (GE Healthcare, Little Chalfont, Great Britain). Images were recorded using a Fusion Fx system (Viber Lourmat, Collégien, France).

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

Mass cytometry (CyTOF) analysis. Blood was collected from healthy donors in heparin tubes and stimulated with LPS (100 ng/ml) with or without propionate (2 mM) and brefeldin A. After 4 h, EDTA (2 mM) was added and cells incubated for 15 min at room temperature (RT). Cells were fixed using a proteomic stabilizer (Smart Tube Inc., San Carlos, CA) and stored at −80 °C. Thawing and erythrocyte lysis was performed according to instructions from Smart Tube Inc. Fc receptors were blocked with Human TruStain FcX Receptor Blocker. Cells were stained using metal-conjugated antibodies according to the CyTOF manufacturer's instructions (Fluidigm, South San Franciso, CA). Briefly, individual samples were stained with unique combinations of CD45 antibodies for 30 min at RT, followed by 3 washes with CSM. Samples were pooled and stained using a cocktail of antibodies for cell surface markers, washed with CSM and PBS, fixed with 2.4% formaldehyde, washed with CSM-S, and stained for intracellular targets. Antibodies directed against CD1c (L161), CD3 (UCHT1), CD4 (RPA-T4), CD7 (CD7-6B7), CD8 (SK1), CD11c (Bu15), CD14 (M5E2), CD16 (3G8), CD20 (2H7), CD45 (HI30), CD66b (G10F5), CD123 (6H6), HLA-DR (L243), IL-6 (MQ2-13A5) and TNF (Mab11) were from BioLegend, Slan (DD1) from Miltenyi Biotec and CD56 (R19-760) and CD141 (1A4) from BD. After intracellular staining, cells were resuspended in DNA-intercalation solution (PBS, 1 μM Ir-Intercalator, 1% formaldehyde, 0.3% saponin) and stored at 4 °C until analysis. For analysis, cells were washed 3 times with MilliQ water and resuspended at 0.5×10^6 cells/ml in 0.1% EQ™ Four Element Calibration Beads solution (Fluidigm). Samples were acquired on an upgraded CyTOF 1 using a syringe pump at 45 μl/min. FCS files were concatenated and normalized using the cytobank concatenation tool and matlab normalizer respectively[76]. Data was processed and analyzed with cytobank and R using the OpenCyto and cytofkit packages[77,78]. Dimensionality reduction with t-SNE was performed on a merged dataset, consisting of a random selection of 10'000 non granulocyte events from each sample.

***In vivo* models.** Mice (n = 8–16/group) treated or not with propionate (200 mM in drinking water or 1 g/kg i.p. every other day) were challenged with LPS (250 μg i.p.), *E. coli* (4×10^4 CFU i.p.), *K. pneumoniae* (20 or 200 CFU i.n.), *S. pneumoniae* (80, 10^4, 4×10^6 or 10^7 CFU i.n.), *S. aureus* (2×10^7 CFU i.v.) or *C. albicans* (4×10^4, 2×10^5 or 5×10^5 conidia i.v.)[29,70,71,79–81]. Unless specified, propionate treatment was continued after microbial challenge. To analyze the impact of propionate on acquired immunity to bacterial infection, BALB/c mice with or without propionate treatment were infected with 80 CFU *S. pneumoniae*. Three weeks later, propionate treatment was stopped. Mice were either re-infected with 10^7 CFU *S. pneumoniae* or sacrificed to collect sera. Sera from water or propionate-treated mice were pooled and transferred (120 μl i.p.) into naïve mice that were infected 24 h later with 4×10^6 CFU *S. pneumoniae*. In selected experiments, mice were treated with ciprofloxacin (0.2 mg/ml; Fresenius, Brézins, France), metronidazole (1 mg/ml; Sintetica S.A., Couver, Switzerland) and vancomycin

(TEVA, North Wales, PA) in drinking water to deplete the gut microbiota[17,18,39,43,82]. Body weight, severity scores and survival were registered at least once daily as described previously[71].

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

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

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Author Contributions

E.C., T.H. and M.M. performed *in vitro* experiments, J.H. performed flow cytometry experiments, J.H. and C.F. designed and performed CyTOF study, E.C., T.H. and D.L.R. performed *in vivo* experiments. T.R. conceived the project, designed the experiments and wrote the paper. All authors reviewed the manuscript.

Additional Information

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3.2 Sirtuin 2 Deficiency Increases Bacterial Phagocytosis by Macrophages and Protects from Chronic Staphylococcal Infection

Eleonora Ciarlo[1], Tytti Heinonen[1], Charlotte Théroude[1], Jacobus Herderschee[1], Matteo Mombelli[1], Jérôme Lugrin[1], Marc Pfefferlé[1], Beatrice Tyrrell[1], Sarah Lensch[1], Hans Acha-Orbea[2], Didier Le Roy[1], Johan Auwerx[3], Thierry Roger[1]

[1]Infectious Diseases Service, Department of Medicine, Lausanne University Hospital, Epalinges, Switzerland

[2]Department of Biochemistry, University of Lausanne, Epalinges, Switzerland

[3]Laboratory for Integrative and Systems Physiology, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

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Summary

SIRT2 is the most expressed sirtuin in myeloid cells. Therefore, we hypothesized that SIRT2 impacts on the functions in myeloid cells and during host defenses. To address that question, we performed experiments using SIRT2 deficient mice.

The deletion of SIRT2 is not compensated by an increased expression of other sirtuins. SIRT2 deficient mice develop normally without macroscopic defects and alterations in thymic and splenic immune cell development. SIRT2 deficiency has a minor impact on intracellular signaling and the production of cytokines by murine splenocytes and macrophages. However, SIRT2 deficient macrophages phagocytose better beads and bacteria. SIRT2 deficient macrophages have increased tubulin acetylation and a higher glycolytic capacity. However, pharmacologic inhibition of glycolysis but not tubulin stabilization reduces phagocytosis by SIRT2 deficient macrophages, supporting a role for glycolysis over tubulin acetylation in SIRT2-mediated phagocytosis.

SIRT2 deficient mice are protected from chronic *S. aureus* infection, while they behave like wild type mice in models of toxic shock syndrome toxin-1, LPS or TNF-induced shock, fulminant *Escherichia coli* peritonitis, sub-lethal *Klebsiella pneumoniae* pneumonia, and chronic candidiasis.

Altogether, these data support the clinical development of SIRT2 inhibitors regarding their infection-related safety profile, and even suggest that inhibitors of SIRT2 could afford some benefit during chronic staphylococcal infection.

My contribution to this work: I worked on the mechanisms underlying the increased phagocytosis phenotype in SIRT2 deficient macrophages. I performed fluorescence imaging and Western blotting to measure tubulin acetylation. I measured cellular glycolysis with the Seahorse technology and helped with the corresponding phagocytosis experiments.

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Sirtuin 2 Deficiency Increases Bacterial Phagocytosis by Macrophages and Protects from Chronic Staphylococcal Infection

*Eleonora Ciarlo[1], Tytti Heinonen[1], Charlotte Théroude[1], Jacobus Herderschee[1], Matteo Mombelli[1], Jérôme Lugrin[1], Marc Pfefferlé[1], Beatrice Tyrrell[1], Sarah Lensch[1], Hans Acha-Orbea[2], Didier Le Roy[1], Johan Auwerx[3] and Thierry Roger[1]**

[1] Infectious Diseases Service, Department of Medicine, Lausanne University Hospital, Epalinges, Switzerland, [2] Department of Biochemistry, University of Lausanne, Epalinges, Switzerland, [3] Laboratory for Integrative and Systems Physiology, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

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***Correspondence:**
Thierry Roger
thierry.roger@chuv.ch

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Sirtuin 2 (SIRT2) is one of the seven members of the family of NAD^+-dependent histone deacetylases. Sirtuins target histones and non-histone proteins according to their subcellular localization, influencing various biological processes. SIRT2 resides mainly in the cytoplasm and regulates cytoskeleton dynamics, cell cycle, and metabolic pathways. As such, SIRT2 has been implicated in the pathogenesis of neurodegenerative, metabolic, oncologic, and chronic inflammatory disorders. This motivated the development of SIRT2-directed therapies for clinical purposes. However, the impact of SIRT2 on antimicrobial host defense is largely unknown. Here, we address this question using SIRT2 knockout mice. We show that SIRT2 is the most highly expressed sirtuin in myeloid cells, especially macrophages. SIRT2 deficiency does not affect immune cell development and marginally impacts on intracellular signaling and cytokine production by splenocytes and macrophages. However, SIRT2 deficiency enhances bacterial phagocytosis by macrophages. In line with these observations, in preclinical models, SIRT2 deficiency increases survival of mice with chronic staphylococcal infection, while having no effect on the course of toxic shock syndrome toxin-1, LPS or TNF-induced shock, fulminant *Escherichia coli* peritonitis, sub-lethal *Klebsiella pneumoniae* pneumonia, and chronic candidiasis. Altogether, these data support the safety profile of SIRT2 inhibitors under clinical development in terms of susceptibility to infections.

Keywords: sirtuin, innate immunity, cytokine, macrophage, phagocytosis, sepsis, histone deacetylase, metabolism

INTRODUCTION

Innate immune cells are at the vanguard of host defense against microbial infections. Monocytes/macrophages and dendritic cells (DCs) sense microbial or danger-associated molecular patterns (MAMPs and DAMPs released by injured or stressed cells) through pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), NOD-like receptors, C-type lectins, scavenger receptors, RIG-I-like receptors, and intra-cytosolic DNA sensors (1, 2). The interaction of MAMPs or DAMPs with PRRs triggers the mitogen-activated protein kinases (MAPKs), nuclear factor-κB (NF-κB), and interferon (IFN) response factor signaling pathways that coordinate immune gene expression. The

cellular and soluble mediators mobilized upon infection tightly regulate the development of the inflammatory response, the establishment of antimicrobial cellular and humoral responses, and the restoration of homeostasis once pathogens have been contained or eradicated.

The superfamily of histone deacetylases (HDACs) comprises eleven Zn-dependent HDACs (HDAC1-11) and seven NAD^+-dependent sirtuins (SIRT1-7). HDACs are epigenetic erasers catalyzing histone deacetylation, chromatin compaction, and transcriptional repression. In addition, HDACs target thousands of non-histone proteins affecting many biological processes (3). The subfamily of sirtuins attracted much interest when sirtuins were proposed to promote longevity and represent attractive therapeutic targets for age-related pathologies, such as type 2 diabetes, as well as neurodegenerative, cardiovascular, and oncologic diseases (4–6). Our knowledge about the impact of sirtuins on innate immune responses is limited. Most studies have focused on SIRT1 and SIRT6, resulting in an overall complex picture attributing both proinflammatory and anti-inflammatory properties to sirtuins (7).

Sirtuin 2 (SIRT2) was originally reported to colocalize with the microtubule network and to deacetylate α-tubulin (8). Indeed, SIRT2 is mainly cytoplasmic, although it can translocate during the G2/M transition phase of cell cycle into the nucleus where it deacetylates histone H4 lysine 16 (9). Of note, SIRT2 possesses a proficient demyristoylation activity, the physiological relevance of which remains to be established (10). By targeting numerous proteins besides histones, SIRT2 regulates cytoskeleton dynamics, cell cycle, and metabolic pathways including inhibition of adipogenesis and promotion of lipolysis and gluconeogenesis. Additionally, SIRT2 can act as a tumor suppressor gene, and is involved in myelogenesis and other brain functions. Accordingly, SIRT2 has been implicated in tumorigenesis and neurodegeneration, and likely plays a role in metabolic disorders, such as obesity and type 2 diabetes (11–13).

With respect to inflammatory processes, SIRT2 was first shown to deacetylate NF-κB p65, resulting in the expression of a subset of p65-dependent genes in mouse embryonic fibroblasts (14). SIRT2 deficiency sustained brain inflammation in a model of traumatic brain injury and increased the severity of collagen-induced arthritis and colitis (15–17). However, SIRT2 deficiency was also reported to decrease NF-κB p65-mediated inflammatory response, renal tubular inflammation, and ischemia reperfusion-induced hepatocellular inflammation (18–20). Finally, in an experimental stroke model, SIRT2 deficiency preserved neurological functions without affecting inflammatory parameters (21). Overall, the function of SIRT2 in sterile and chronic inflammatory disorders appears to be context dependent.

Because sirtuins are pleiotropic and in consideration of the development of sirtuin-targeting drugs for clinical conditions, we sought to delineate the role of SIRT2 in the innate immune response. To this end, we used SIRT2 knockout mice to investigate the response of immune cells to immunological and microbial stimuli using *in vitro* experiments and *in vivo* preclinical models. Preclinical models included models of shock as well as Gram-negative and Gram-positive bacterial infections and fungal infection. Overall, SIRT2 deficiency does not modulate cytokine production by innate immune cells, but enhances bacterial phagocytosis by macrophages. SIRT2 deficiency protects from chronic staphylococcal infection, while having no impact on toxic shock, endotoxemia, fulminant peritonitis, non-lethal pneumonia, and chronic candidiasis. These data largely support the safety, in terms of susceptibility to infections, of SIRT2 inhibitors developed for clinical applications.

MATERIALS AND METHODS

Mice, Cells, and Reagents

8- to 12-week-old female BALB/cByJ mice, C57BL/6J mice (Charles River Laboratories, Saint-Germain-sur-l'Arbresle, France), and SIRT2 knockout mice backcrossed 12 times on a C57BL/6J background (15) were used. Mice were housed under specific pathogen-free conditions and free of mouse norovirus. Splenocytes were cultured in RPMI 1640 medium containing 2 mM glutamine, 50 µM 2-ME, 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen, San Diego, CA) and 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO) (22). Bone marrow (BM) cells were cultured in IMDM (Invitrogen) containing 50 µM 2-ME, penicillin, streptomycin, and 10% FCS. Medium was supplemented with 20 ng/ml M-CSF, 20 ng/ml GM-CSF plus 20 ng/ml IL-4 (ProSpec, East Brunswick, NJ) or 200 ng/ml FMS-like tyrosine kinase 3 ligand (Flt3L, Shenandoah biotechnology, Warwick, PA) to generate BM-derived macrophages (BMDMs), BM-derived dendritic cells (BMDCs), or Flt3L-DCs, respectively. BMDCs were collected after 6 days. BMDMs and Flt3L-DCs were collected after 7 days of culture. Cells (1, 5, and 20×10^5) were seeded in 96-well, 24-well or 6-well plates in complete medium without growth factors and antibiotics unless specified.

Salmonella minnesota ultra pure lipopolysaccharide (LPS) was from List Biologicals Laboratories (Campbell, CA), Pam_3CSK_4 from EMC microcollections (Tübingen, Germany), CpG ODN 1826 (CpG) from InvivoGen (San Diego, CA, USA), toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxin B (SEB) from Toxin Technology (Sarasota, FL, USA), concanavalin A and phytohemagglutinin (PHA) from Sigma-Aldrich, and anti-CD3ε and anti-CD28 antibodies (clones 145-2C11 and 37.51) from eBioscience (San Diego, CA, USA). Clinical strains of *Escherichia coli* (*E. coli*) O18, *E. coli* J5, *E. coli* O111, *Salmonella enterica* serovar Typhimurium C5 (*Salmonella* Typhimurium), *Klebsiella pneumoniae* caroli (*K. pneumoniae*), *Neisseria meningitis*, *Streptococcus pneumoniae*, *Staphylococcus aureus* AW7 (*S. aureus*), and Group B *Streptococcus* (GBS) were grown in brain heart infusion broth (BD Biosciences, Erembodegem, Belgium) (23–27). *Candida albicans* 5102 (*C. albicans*) (22) was cultured in yeast extract–peptone–dextrose (BD Biosciences). Microorganisms were washed in PBS and adjusted at 10^{10} CFU/ml. For *in vitro* stimulation, bacteria were heat-inactivated for 2 h at 56°C. Nocodazole and 2-deoxyglucose were from Sigma-Aldrich, cytochalasin D from Millipore (Billerica, MA, USA).

RNA Analyses

Total RNA was isolated, reverse transcribed (RNeasy and QuantiTect reverse transcription kits, Qiagen, Hilden, Germany),

and used in real-time PCRs conducted with a QuantStudio™ 12K Flex system (Life Technologies, Carlsbad, CA, USA). Reactions consisted of 1.25 µl cDNA, 1.25 µl H_2O, 0.62 µl 10 nM primers [Table S1 in Supplementary Material and Ref. (28, 29)], and 3.12 µl Fast SYBR® Green Master Mix (Life Technologies) and were tested in triplicate. Gene specific expression was normalized to hypoxanthine guanine phosphoribosyl transferase expression. Sirt2 expression levels in organs were extracted from the BioGPS resource (http://biogps.org).

Western Blot Analyses

Nuclear and total protein extracts were submitted to PAGE and transferred onto nitrocellulose membranes (30, 31). Membranes were incubated with antibodies directed against SIRT2, acetylated α-tubulin, total α-tubulin, total and phosphorylated ERK1/2, p38, JNK, and NF-κB p65 and TATA-box binding protein (used as a control of nuclear extracts) (see antibody description in Table S2 in Supplementary Material), then with a secondary horseradish peroxidase-conjugated antibody (Sigma-Aldrich) (32). Blots were imaged with the enhanced chemiluminescence Western blotting system (GE Healthcare, Little Chalfont, Royaume-Uni). Images were recorded using a Fusion Fx system (Viber Lourmat, Collégien, France).

Flow Cytometry

Single cell suspensions from thymus and spleen, or BMDMs were enumerated and incubated with 2.4G2 monoclonal antibody (mAb). Immune cell subpopulations were tracked by staining performed using mAbs described in Table S2 in Supplementary Material. Splenic $CD4^+$ $CD25^+$ $Foxp3^+$ cells were detected using The Mouse Regulatory T Cell Staining Kit (eBioscience). Data were acquired using a LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo Version 10.2 software (FlowJo LLC, Ashland, OR, USA) (33).

Proliferation Assay

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

Cytokine Measurements

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

In Vivo Models

8- to 12-week-old female $SIRT2^{+/+}$ and $SIRT2^{-/-}$ mice (6–13 mice per group) matched for age were used. To analyze the response to TSST-1, mice were challenged intraperitoneally (i.p.) with TSST-1 (0.5 mg/kg). Models of endotoxic shock were performed by challenging mice i.p. with LPS (10 and 25 mg/kg). To induce TNF shock, mice were sensitized with D-galactosamine (30 mg/kg i.p., Sigma-Aldrich) just before being challenged with TNF (25 mg/kg i.p., Preprotech, Rocky Hill, NJ, USA). Bacterial sepsis was induced by challenging mice i.p. with 10^5 CFU *E. coli* O18, intravenously (i.v.) with 10^7 CFU *S. aureus* or 10^5 CFU *C. albicans* or intranasally (i.n.) with 30 CFU *K. pneumoniae*. Blood and spleen were collected 0, 1, 6, 8, 24, or 48 h post-challenge to quantify cytokines and bacteria (28). Body weight loss, severity score, and survival were registered at least once daily. The severity score was graded from 1 to 5 (36). Animals were euthanized when they met a severity score of 4. Two to three operators performed animal follow-up.

Phagocytosis Assays

Fluoresbrite® Yellow Green Microspheres (Polysciences Inc, Warrington, PA, USA) or FITC-labeled bacteria were added to cells at a ratio of 10 beads or bacteria/cell. After 1 h, cells were washed, incubated for 1 min with trypan blue (0.25 mg/ml) and analyzed by flow cytometry. When specified, beads were opsonized with serum for 30 min at 37°C. To assess phagocytosis of live bacteria, BMDMs (in quadruplicates or sextuplates) were incubated for 1 h with *E. coli* O18, *S. aureus*, and GBS (10 bacteria/cell). Non-adherent and extracellular bacteria were removed by washing and killed by a 30-min exposure to 100 µg/ml gentamicin (Essex Chemie, Luzern, Switzerland; for *E. coli* and GBS) or 10 µg/ml ciprofloxacin (Fresenius Kabi, Oberdorf, Switzerland; for *S. aureus*). Serial dilutions of cell lysates were plated on agar plates. Colonies were enumerated to calculate the number of phagocytosed bacteria.

Glycolytic Activity

The glycolytic activity of BMDMs was analyzed using a 96-well format Seahorse XFe® system and the Seahorse XF Glycolysis Stress Test Kit (Agilent Technologies, Santa Clara, CA, USA). Briefly, 4×10^4 BMDMs were plated in 96-well plates in IMDM medium. The next day, cells were incubated with or without 5×10^7 CFU/ml heat-killed *S. aureus* and rested 1 h in Seahorse medium without glucose. The glycolytic capacity was assessed by measuring the extracellular acidification rate following the sequentially addition of 10 mM glucose, 1 µM oligomycin, and 50 mM 2-deoxy-glucose (2-DG) according to manufacturer's instructions.

Statistical Analyses

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

RESULTS

SIRT2 Is Highly Expressed by Myeloid Cells

SIRT1-7 mRNA expression was quantified in BM, BMDMs, and DCs (conventional BMDCs and Flt3L-derived DCs) (**Figure 1A**). SIRT2 was the most highly expressed sirtuin in all populations. SIRT2 was also the predominantly expressed sirtuin in RAW 264.7

macrophages and in the spleen, liver, and kidneys (**Figure 1B** and data not shown). Western blot analyses confirmed SIRT2 protein expression in BMDMs (see below). Primary osteoblasts, osteoclasts, macrophages, and mast cells expressed 2.6-fold to 7.1-fold higher levels of SIRT2 mRNA than granulocytes, NK cells, T cells, B cells, DCs, and thymocytes (**Figure 1C**). Overall, SIRT2 was highly expressed by myeloid-derived cells, suggesting that it could play a role in the control of immune responses. To address this question, SIRT2-deficient mice were used [**Figures 1D,E** and Ref. (15)].

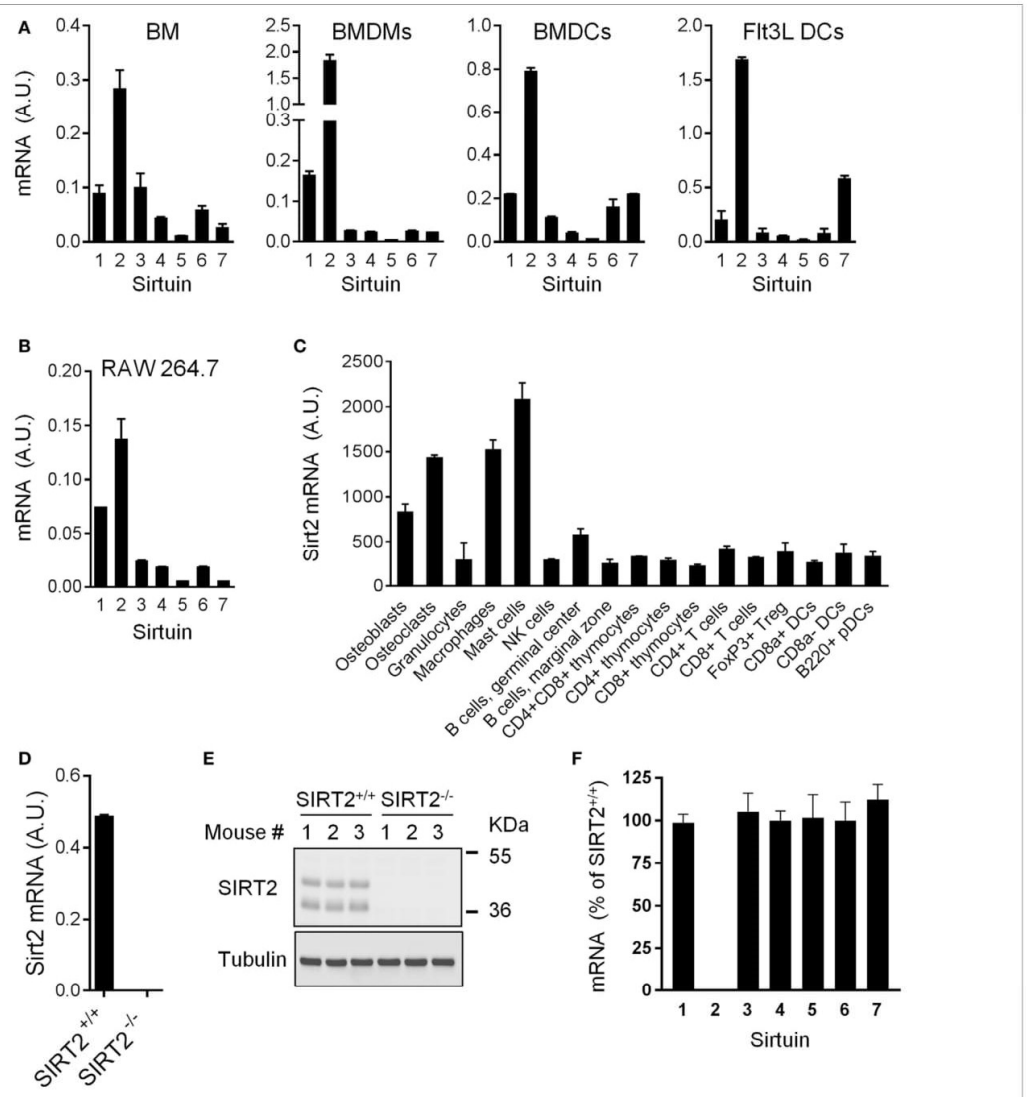

FIGURE 1 | Sirt2 is strongly expressed in myeloid cells. **(A,B)** Sirt1-7 mRNA levels in bone marrow (BM), BM-derived macrophages (BMDMs) and dendritic cells (BMDCs), and Flt3L-derived DCs **(A)** and RAW 264.7 macrophages **(B)** analyzed by RT-PCR and normalized to Hprt mRNA levels. Data are means ± SD of triplicate samples from one experiment performed with four BALB/c mice **(A)** or three preparations of RAW 264.7 macrophages **(B)**. **(C)** Sirt2 mRNA expression levels in a panel of cells (extracted from http://biogps.org). **(D,E)** Sirt2 mRNA **(D)** and Sirtuin 2 (SIRT2) protein **(E)** expression in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ BMDMs assessed by RT-PCR and western blotting, respectively. Data are means ± SD from one experiment performed with three mice **(D)**. **(F)** Sirt1-7 mRNA expression levels in SIRT2$^{-/-}$ BMDMs, expressed relative to the mRNA levels in SIRT2$^{+/+}$ BMDMs set at 100%. Data are means ± SD from one experiment performed with three mice. A.U., arbitrary units. Full-length blots of panel **(E)** are presented in Figure S1 in Supplementary Material.

SIRT2 Deficiency Has No Major Impact on the Development of Immune Cells and Host Response to TSST-1

SIRT2$^{-/-}$ mice were described previously (15). These mice were born at the expected Mendelian ratio and developed without abnormalities. SIRT2 mRNA and protein were undetectable in SIRT2$^{-/-}$ BMDMs (**Figures 1D,E**). Expression levels of Sirt1 and Sirt3-7 mRNA were unaltered in SIRT2$^{-/-}$ BMDMs (**Figure 1F**), suggesting that the lack of SIRT2 was not compensated by an increase in expression of other sirtuins.

Compared to SIRT2$^{+/+}$ mice, SIRT2$^{-/-}$ mice expressed normal proportions and absolute numbers of CD4/CD8 double negative (DN1–4), double positive, and single positive (SP) thymocytes (**Table 1**). Additionally, SIRT2$^{-/-}$ mice had normal populations of splenic T cells (DN, SP, naïve, and memory), B cells (immature and mature B cells), DCs (B220$^-$ CD11c$^+$ cDCs and B220$^+$ CD11c$^+$ pDCs), and Foxp3$^+$ regulatory T cells (**Table 2**). Therefore, SIRT2 had no major impact on immune cell development.

TABLE 1 | Thymic cell subsets in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice.

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

Data are means ± SD of four animals per group expressed as the percentage of total cells (CD4$^+$ CD8$^+$, CD4$^-$ CD8$^-$, CD4$^+$ CD8$^-$, and CD4$^-$ CD8$^+$) or percentage of CD4$^-$ CD8$^-$ parental cells (CD25$^+$ CD44$^+$, CD25$^-$ CD44$^+$, CD25$^+$ CD44$^-$ and CD25$^-$ CD44$^-$). Total cell numbers were 49.2 ± 15.4 and 55.2 ± 5.7 millions per thymus in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice, respectively. No statistically significant differences in subset percentages or absolute numbers were detected.

TABLE 2 | Splenic cell subsets in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice.

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

Data are means ± SD of four animals per group expressed as the percentage of CD3$^+$, B220$^+$, CD11c$^+$, and CD4$^+$ Foxp3$^+$ splenic cells or the percentage of the CD3$^+$, B220$^+$, and CD11c$^+$ parental populations expressing CD4, CD8, CD44, CD62L, IgD, and CD23. Total cell numbers were 74.2 ± 5.6 and 67.4 ± 8.7 millions per spleen in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice, respectively. No statistically significant differences in subset percentages or absolute numbers were detected.

As a first approach to evaluate whether SIRT2 influenced immune responses, SIRT2$^{+/+}$ and SIRT2$^{-/-}$ splenocytes were exposed to microbial and immunological stimuli: LPS, CpG, concanavalin A, anti-CD3/CD28, PHA, TSST-1, and SEB. The proliferation and production of IL-2 and IFNγ (measured by ELISA) by splenocytes were not affected by SIRT2 deficiency (**Figures 2A,B**). In agreement, blood concentrations of IFNγ were similar in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice injected with TSST-1 (**Figure 2C**), a staphylococcal superantigen responsible of toxic shock syndrome. A Luminex assay was then used to quantify TNF, IL-6, IL-10, IL-12p70, CCL3/Mip1α, CCL4/Mip1β, and CCL5/Rantes secretion by splenocytes exposed to LPS, CpG, concanavalin A, anti-CD3/CD28, PHA, TSST-1, and SEB (**Figure 2D**). No differences were observed between SIRT2$^{+/+}$ and SIRT2$^{-/-}$ splenocytes apart from a 20–28% reduction of LPS-induced IL-6, IL-10, and CCL5 and anti-CD3/CD28-induced CCL4, while the secretion of CCL5 was increased in response to anti-CD3/CD28.

SIRT2 Deficiency Increases Phagocytosis by Macrophages

Macrophages are professional phagocytic cells that play a major role in antimicrobial host defenses. Therefore, we tested whether SIRT2 deficiency had an effect on phagocytosis by BMDMs. SIRT2$^{+/+}$ and SIRT2$^{-/-}$ BMDMs were incubated with fluorescent beads and analyzed by flow cytometry (**Figures 3A–C**). A higher percentage of SIRT2$^{-/-}$ than SIRT2$^{+/+}$ BMDMs phagocytosed beads (32.4 ± 1.9 vs 24.5 ± 1.2 percent positive cells, $P = 0.002$; **Figures 3A,B**), regardless of opsonization (**Figure 3C**). SIRT2$^{-/-}$ BMDMs also exhibited higher phagocytosis using a panel of fluorescently labeled heat-inactivated bacteria (% of SIRT2$^{-/-}$ vs SIRT2$^{+/+}$ BMDMs ingesting bacteria: *E. coli* J5: 53.6 vs 43.6%, *E. coli* O111: 31.6 vs 23.0%, *Salmonella* Typhimurium: 24.1 vs 17.6%, *Neisseria meningitis*: 46.1 vs 37.2%, *S. pneumoniae*: 49.6 vs 34.7%). BMDMs were additionally exposed to live *E. coli*, *S. aureus* and GBS for 1 h before measuring the number of intracellular bacteria by plating cell lysates and enumerating colonies. Confirming the results obtained using inert beads and fluorescent bacteria, the numbers of phagocytosed *E. coli*, *S. aureus* and GBS were 1.3-fold to 1.6-fold higher using SIRT2$^{-/-}$ BMDMs (**Figure 3D**).

Macrophages express phagocytic receptors, including macrophage scavenger receptor 1 (Msr1/SR-AI/CD204), CD14, CD36, C-type lectins such as dectin-1 (encoded by Clec7a), and members of the integrin superfamily (integrin α5/Itga5/CD49e, integrin αM/Itgam/CD11b, integrin αX/Itgax/CD11c, integrin β2/Itgb2/CD18). SIRT2$^{-/-}$ and SIRT2$^{+/+}$ BMDMs expressed comparable mRNA levels of Itga5, Itga6, Itgal, Itgam, Itgax, Itgb1, Itgb2, Cd14, Cd36, Msr1, and Clec7a (**Figure 3E**). Moreover, SIRT2$^{-/-}$ and SIRT2$^{+/+}$ BMDMs expressed similar levels of membrane-bound CD11b, CD11c, CD14, and Msr1 (**Figure 3F**). Hence, SIRT2 deficiency likely improved phagocytosis by BMDMs in a phagocytic receptor independent fashion.

Stabilization of microtubules and high glycolytic activity have been associated with efficient phagocytosis by macrophages (37–40). Since SIRT2 impacts on microtubules stabilization and glucose metabolism (8, 13, 41), we questioned whether these processes influenced phagocytosis by SIRT2$^{-/-}$ BMDMs. In

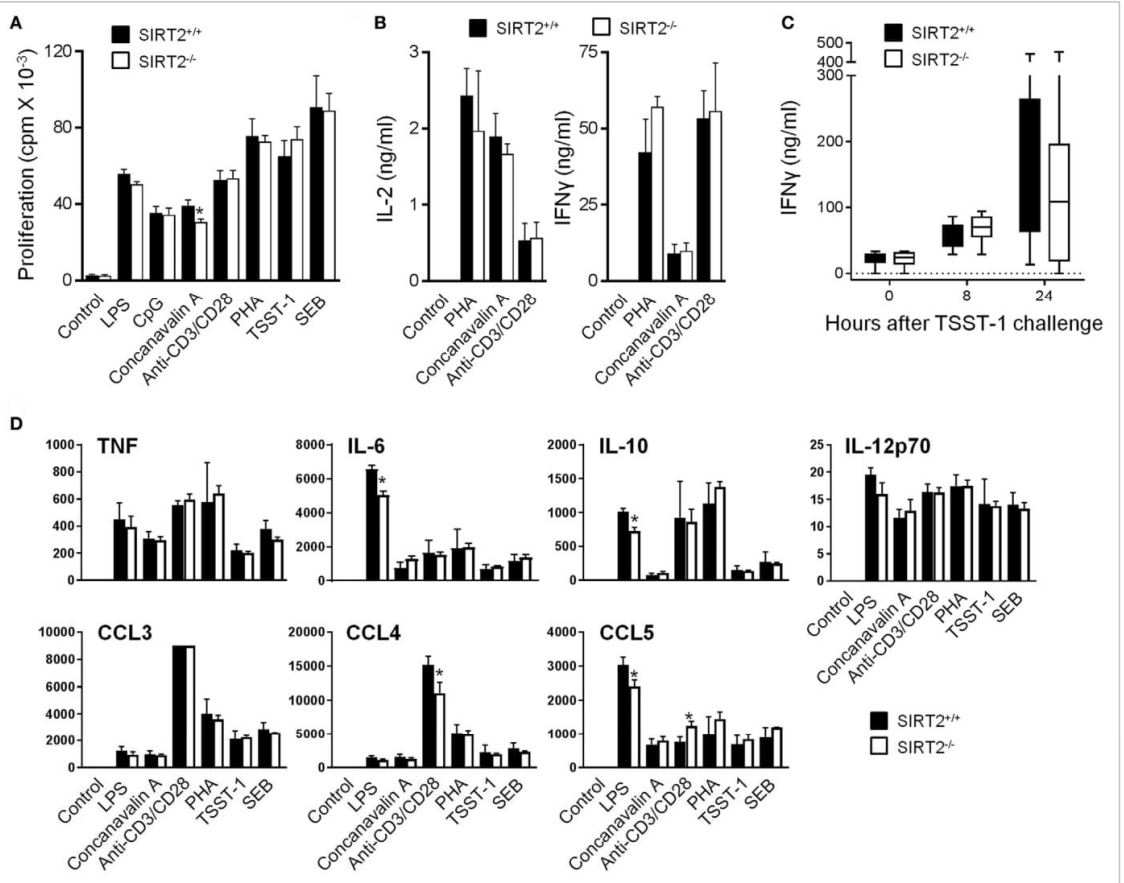

FIGURE 2 | Sirtuin 2 (SIRT2) deficiency does not affect proliferation and cytokine response of splenocytes and IFNγ production in mice challenged with toxic shock syndrome toxin-1 (TSST-1). **(A,B)** SIRT2$^{+/+}$ and SIRT2$^{-/-}$ splenocytes were incubated for 48 h with lipopolysaccharide (LPS) (5 μg/ml), CpG (2 μg/ml), concanavalin A (5 μg/ml), anti-CD3/CD28 antibodies (1 μg/ml), phytohemagglutinin (PHA) (10 μg/ml), TSST-1 (2 μg/ml), and staphylococcal enterotoxin B (SEB) (5 μg/ml). Proliferation was measured by ^{3}H-thymidine incorporation **(A)** while IL-2 and IFNγ concentrations in cell culture supernatants were quantified by ELISA **(B)**. Data are means ± SD of triplicate samples from one experiment performed with four mice and are representative of two experiments (*$P < 0.05$). **(C)** SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice ($n = 8$ per group) were injected with TSST-1 (0.5 mg/kg i.p.). Blood was collected after 0, 8, and 24 h to quantify IFNγ concentrations. Data are means ± SD. $P > 0.5$ for all time points. **(D)** SIRT2$^{+/+}$ and SIRT2$^{-/-}$ splenocytes were incubated for 48 h with LPS, CpG, concanavalin A, anti-CD3/CD28 antibodies, PHA, TSST-1, and SEB. TNF, IL-6, IL-10, IL-12p70, CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) were quantified by Luminex. Data (in pg/ml) are means ± SD of one experiment performed with three mice. CCL3 values in response to anti-CD3/CD8 were over the upper limit of detection of the assay (*$P < 0.05$).

BMDMs, SIRT2 deficiency increased 1.5-fold tubulin acetylation (**Figure 3G**), a hallmark of microtubule stabilization. However, the microtubule destabilizer nocodazole did not impair the phagocytosis of *S. aureus* by BMDMs (**Figure 3H**), while the actin depolymerization agent cytochalasin D efficiently inhibited phagocytosis. Interestingly, the glycolytic activity was higher in SIRT2$^{-/-}$ than SIRT2$^{+/+}$ BMDMs exposed to *S. aureus* (**Figure 3I**). Moreover, 2-DG, which inhibits glycolysis, significantly reduced the phagocytosis of *S. aureus* by BMDMs (**Figure 3J**). Albeit preliminary, these results suggested that differences in the metabolic capacity of SIRT2$^{+/+}$ and SIRT2$^{-/-}$ BMDMs may provide a mechanism by which SIRT2 impedes phagocytosis.

SIRT2 Deficiency Does Not Affect Cytokine Response of BMDMs Exposed to Microbial Ligands and Sensitivity of Mice to Endotoxemia

Sensing of microbial ligands through TLRs initiates MAPK and NF-κB signaling involved in the control of cytokine gene expression by innate immune cells (2). To address whether SIRT2 impacted intracellular signaling, the phosphorylation of ERK1/2, p38, and JNK MAPKs in BMDMs exposed to LPS for 0, 10, 30, and 60 min was analyzed by western blotting. The rate of LPS-induced phosphorylation of ERK1/2, p38 and JNK was very similar in SIRT2$^{+/+}$

and SIRT2$^{-/-}$ BMDMs, with only a slight and late reduction of ERK1/2 phosphorylation in SIRT2$^{-/-}$ BMDMs (30% reduction at 1 hour) (**Figure 4A**). The nuclear translocation of phosphorylated NF-κB p65 was not different in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ BMDMs exposed to LPS for 0, 10, 30, and 60 min ($P > 0.5$ for all time points).

SIRT2$^{+/+}$ and SIRT2$^{-/-}$ BMDMs exposed to LPS, Pam_3CSK_4, CpG (i.e., TLR4, TLR1/2 and TLR9 ligands, respectively), and *E. coli* upregulated Tnf and Il6 mRNA levels and secreted TNF and IL-6 to the same extend (**Figure 4B**). Additionally, Tlr1, Tlr2, Tlr4, and Tlr9 mRNA were modulated likewise in SIRT2$^{+/+}$

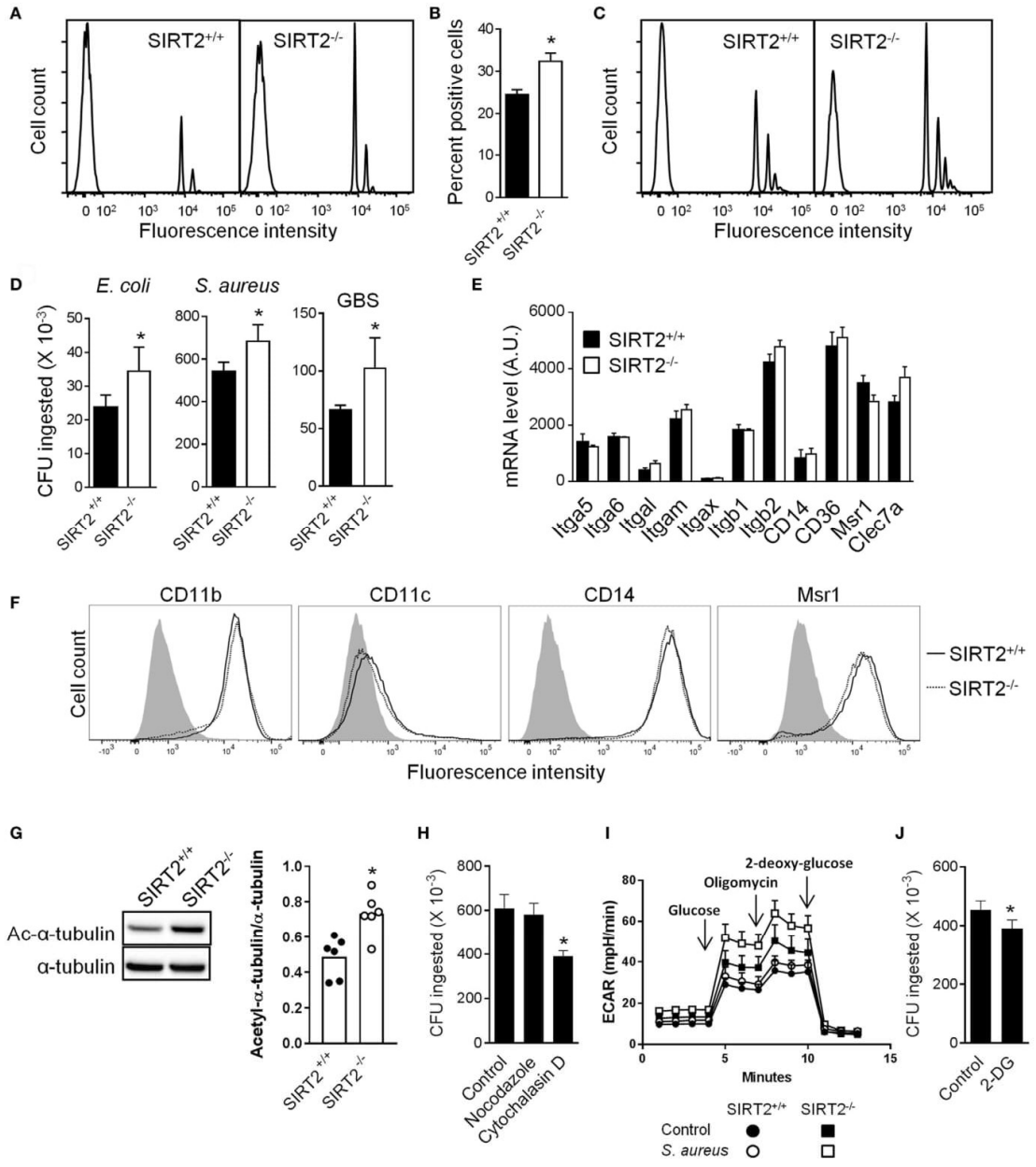

FIGURE 3 | Continued

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Sirtuin 2 (SIRT2) deficiency increases bacterial phagocytosis by macrophages. **(A–C)** SIRT2$^{+/+}$ and SIRT2$^{-/-}$ BM-derived macrophages (BMDMs) were incubated with 10 fluorescent beads [opsonized in **(C)**] per cell. After 1 h, cells were analyzed by flow cytometry. Representative histograms are depicted in **(A,C)**. The percentage of fluorescent cells was calculated **(B)**. Data are means ± SD from an experiment performed with four mice (*P = 0.002). **(D)** SIRT2$^{+/+}$ and SIRT2$^{-/-}$ BMDMs were incubated with live *Escherchia coli*, *Staphylococcus aureus*, and Group B *Streptococcus* (GBS) (10 bacteria/cell). Phagocytosis was assessed after 1 h. Data are means ± SD from one experiment performed with four (*E. coli* and GBS) or eight (*S. aureus*) mice. *P = 0.03, 0.006, and 0.03 for *E. coli*, *S. aureus*, and GBS, respectively. **(E)** Itga5, Itga6, Itgal, Itgam, Itgax, Itgb1, Itgb2, Cd14, Cd36, Msr1, and Clec7a mRNA expression levels in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ BMDMs. Data are means ± SD of one experiment performed with three mice. **(F)** CD11b, CD11c, CD14, and Msr1 expression levels were analyzed by flow cytometry. The gray histogram represents staining with an isotype control antibody. **(G)** Expression levels of acetylated (Ac) and total tubulin in BMDMs were analyzed by western blotting and quantified by imaging. Data are means ± SD from six mice (right panel) (*P = 0.015). Full-length blots are presented in Figure S2 in Supplementary Material. **(H)** Phagocytosis of *S. aureus* [performed as in **(D)**] by SIRT2$^{-/-}$ BMDMs preincubated for 1 h with or without nocodazole (10 μM) and cytochalasin D (10 μM). Data are means ± SD from one experiment performed with four mice (*P = 0.008). **(I)** Glycolytic capacity of BMDMs assessed by measuring the extracellular acidification rate (ECAR) using the Seahorse XF Glycolysis Stress Test Kit as described in Section "Materials and Methods." Data are means ± SD from one experiment performed with three mice. **(J)** Phagocytosis of *S. aureus* [performed as in **(D)**] by SIRT2$^{-/-}$ BMDMs preincubated for 1 h with or without 25 mM 2-deoxy-glucose (2-DG). Data are means ± SD from one experiment performed with four mice (*P = 0.017).

and SIRT2$^{-/-}$ BMDMs (Figure S3 in Supplementary Material). SIRT2$^{+/+}$ and SIRT2$^{-/-}$ BMDMs expressed also comparable mRNA levels of Il1a, Il1b, Il10, Il12b, Il15, Il18, Il27, Ccl2/Mcp1, Ccl3/Mip1a, Ccl4/Mip1b, Ccl5/Rantes, Ccl8/Mcp2, Ccl12/Mcp5, Cxcl10/Ip10, and Cxcl11/Itac at baseline and following LPS stimulation (**Figure 4C**), and secreted comparable levels of IL-10, IL-12p70, IL-18, CXCL10, CCL2, CCL3, CCL4, and CCL5 upon exposure to LPS, Pam_3CSK_4, CpG, *E. coli*, and *S. aureus* (**Figure 4D**). Altogether, these results argued against an important role of SIRT2 in controlling proinflammatory and anti-inflammatory cytokine response by macrophages exposed to TLR ligands.

To assess the relevance of these observations *in vivo*, we developed models of endotoxemia of different severity (**Figures 5A–D**). In a mild model of endotoxemia (induced by an i.p. challenge with 10 mg/kg LPS), TNF and IL-12p40 concentrations in blood and mortality rates (83 vs 100%, $P = 0.3$) were comparable in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice (**Figures 5A,B**). In a severe model of endotoxemia (induced by 25 mg/kg LPS), TNF, IL-6, and IL-12p40 concentrations in blood and mortality rates (88% in both groups, $P = 0.69$) were strongly increased, but remained similar in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice (**Figures 5C,D**). Furthermore, SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice were equally sensitive to fulminant shock induced by TNF (25 mg/kg i.p. in D-galactosamine sensitized mice), the main driver of the lethal effect of endotoxemia ($P = 0.6$; **Figure 5E**). Overall, SIRT2 did not interfere with endotoxemia.

SIRT2 Deficiency Protects from Chronic Staphylococcal Infection

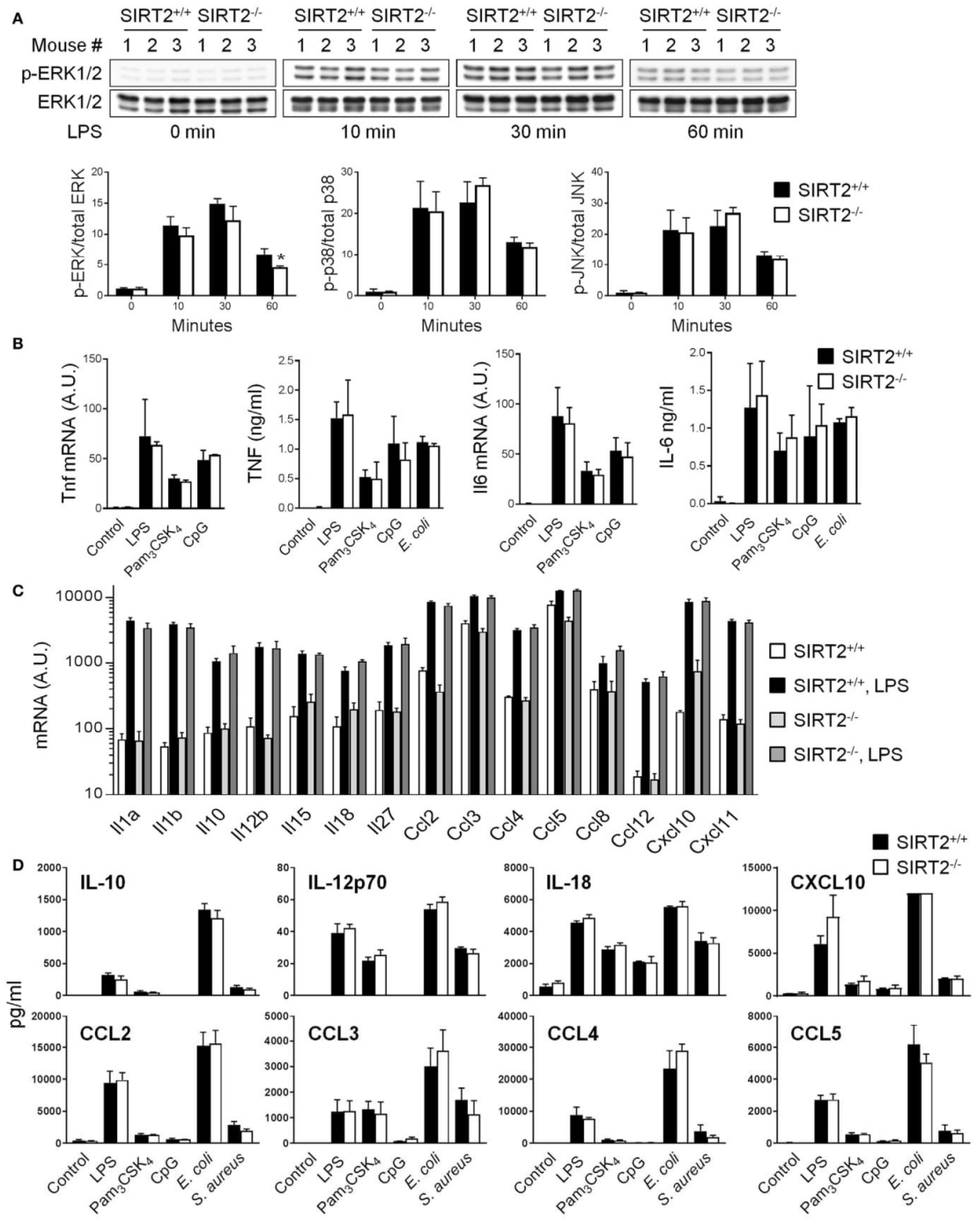
Considering that SIRT2 impacted phagocytosis but not cytokine expression, we hypothesized that SIRT2 deficiency should provide some benefit during chronic lethal infection but not fulminant sepsis, and should not sensitize to benign infection. Therefore, we compared the impact of SIRT2 deficiency during rapidly lethal, sub-lethal, and chronic bacterial infections induced by *E. coli*, *K. pneumonia*, and *S. aureus*, three of the most frequent causes of bacterial sepsis in humans (42).

Supporting our working hypothesis, in a model of chronic infection by *S. aureus* in which mortality occurred 3 to 16 days post i.v. challenge with the bacteria, severity score, body weight loss, and survival (SIRT2$^{+/+}$ vs SIRT2$^{-/-}$: 33.3 vs 69.2%; $P = 0.04$) were all markedly improved in SIRT2$^{-/-}$ mice (**Figure 6A**). In agreement with these findings, 48 h postinfection, only 37.5% (3/8) of SIRT2$^{-/-}$ mice were bacteremic while 71.4% (5/7) of SIRT2$^{+/+}$ mice were bacteremic (**Figure 6B**). Moreover, bacterial burden in the spleen was much lower in SIRT2$^{-/-}$ than in SIRT2$^{+/+}$ mice (1.9×10^2 vs 3.3×10^3 mean CFU/organ; $P = 0.04$). TNF was not detected in blood, while IL-6 and IL-12p40 levels were not different between SIRT2$^{-/-}$ and SIRT2$^{+/+}$ mice, although there was a trend toward lower IL-12p40 levels in SIRT2$^{-/-}$ mice (**Figure 6C**). In a model of fulminant, rapidly lethal peritonitis induced by *E. coli*, body weight loss, bacterial dissemination into the blood, and survival rate (12.5% in both groups, $P = 0.7$) were comparable in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice (**Figure 6D**). In a non-severe model of *K. pneumoniae* pneumonia, body weight loss and survival (85.7% in both groups, $P = 0.9$) were not affected by SIRT2 deficiency (**Figure 6E**). Finally, we questioned whether SIRT2 influenced host susceptibility to a non-bacterial chronic infection. Candidiasis was induced by i.v. inoculation of 10^5 CFU/ml *C. albicans* into SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice ($n = 14$ and 16). Mice died 9–40 days postinfection, without survival differences between the SIRT2$^{+/+}$ and SIRT2$^{-/-}$ groups (71 vs 56%; $P = 0.4$), suggesting that SIRT2 deficiency did not compromise host defenses to *Candida* infection.

DISCUSSION

In the present study, we identified a unique role for SIRT2 in host–pathogen interactions. SIRT2 deficiency promoted bacterial phagocytosis by macrophages but not cytokine production. In agreement with these findings, SIRT2 deficiency protected from chronic staphylococcal infection while having no influence on the course of endotoxemia, TNF-induced shock, fulminant bacterial peritonitis, non-severe bacterial pneumonia, and chronic candidiasis. These observations are particularly relevant in light of the development of pharmacological inhibitors of SIRT2 for clinical applications (43), as they suggest that their usage would not increase susceptibility to bacterial and candidal infections.

SIRT2 was the most highly expressed sirtuin in myeloid cells. Macrophages expressed elevated levels of SIRT2, in accordance previous reports describing SIRT2 expression in microglial cells *in vivo* (16,17). Interestingly, SIRT2 deficiency had no major impact on LPS-induced MAPK activation, NF-κB nuclear translocation,



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Sirtuin 2 (SIRT2) deficiency does not affect the response of macrophages to microbial stimulation. SIRT2$^{+/+}$ and SIRT2$^{-/-}$ BM-derived macrophages were exposed to lipopolysaccharide (LPS) (10 ng/ml), Pam_3CSK_4 (10 ng/ml), CpG (2 μg/ml), *Escherichia coli* (10^6 CFU/ml), and *Staphylococcus aureus* (10^6 CFU/ml). **(A)** Expression levels of phosphorylated (p) and total ERK1/2 (upper panel), p38 and JNK were analyzed by western blotting and quantified by imaging. Data are means ± SD from one experiment performed with three mice (lower panel) (*P = 0.02). **(B)** Tnf and Il6 mRNA levels and TNF and IL-6 concentrations in cell culture supernatants 1 and 8 h after stimulation, respectively. **(C)** Il1a, Il1b, Il10, Il12b, Il15, Il18, Il27, Ccl2/Mcp1, Ccl3/Mip1a, Ccl4/Mip1b, Ccl5/Rantes, Ccl8/Mcp2, Ccl12/Mcp5, Cxcl10/Ip10, Cxcl11/Itac mRNA levels after 8 h of culture with or without LPS. **(D)** IL-10, IL-12p70, IL-18, CXCL10, CCL2, CCL3, CCL4, and CCL5 concentrations in cell culture supernatants 8 h after stimulation measured by Luminex. Data are means ± SD of triplicate samples from one experiment performed with three mice (mRNA analyses) or six mice (TNF and IL-6 secretion), or means ± SD of single measurements from one experiment performed with three mice (Luminex). CXCL10 values in response to *E. coli* were over the upper limit of detection of the assay. No statistically significant differences were detected in **(B–D)**. A.U., arbitrary units. Full-length blots of panel **(A)** are presented in Figure S4 in Supplementary Material.

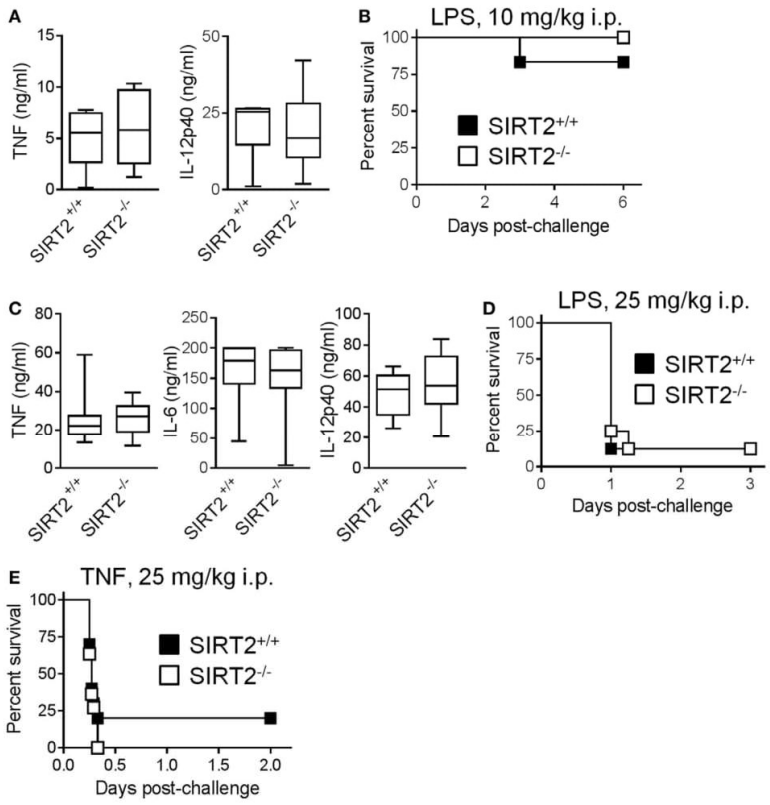

FIGURE 5 | Sirtuin 2 (SIRT2) deficiency does not affect endotoxemia and TNF-induced shock. SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice were injected i.p. with 10 mg/kg lipopolysaccharide (LPS) [**(A,B)**, n = 6 per group] and 25 mg/kg LPS (**C,D**, n = 8 per group). **(A,C)** TNF, IL-6, and IL-12p40 concentrations in blood collected 1 h (TNF) and 6 h (IL-6 and IL-12p40) after LPS challenge. $P > 0.5$ for all conditions. **(B,D)** Survival of mice. P = 0.3 and 0.9. **(E)** Survival of SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice (n = 11 per group) sensitized with 30 mg/kg D-galactosamine and challenged i.p. with 25 mg/kg TNF. P = 0.6.

and cytokine production in macrophages. Moreover, cytokine levels in blood were similar in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ endotoxemic mice. Likewise, inflammatory parameters were comparable in SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice with experimental stroke and *Mycobacterium tuberculosis* infection (21, 44). However, contradictory findings have been reported in the literature. While SIRT2 deficiency promoted NF-κB p65 acetylation and p65-dependent gene expression, it was also reported to reduce NF-κB and p38 and JNK MAPKs activation through an increased stability of IκB and activity of MAPK phosphatase-1, respectively (14–16, 18, 20, 45). In these studies, SIRT2 deficiency sustained brain inflammation, colitis, and collagen-induced arthritis, but protected from renal and liver inflammation (15, 16, 18, 20, 45).

The discrepancy of the effects of SIRT2 on inflammatory responses mirrors conflicting results observed for other sirtuins. For example, SIRT1 protected from experimental autoimmune

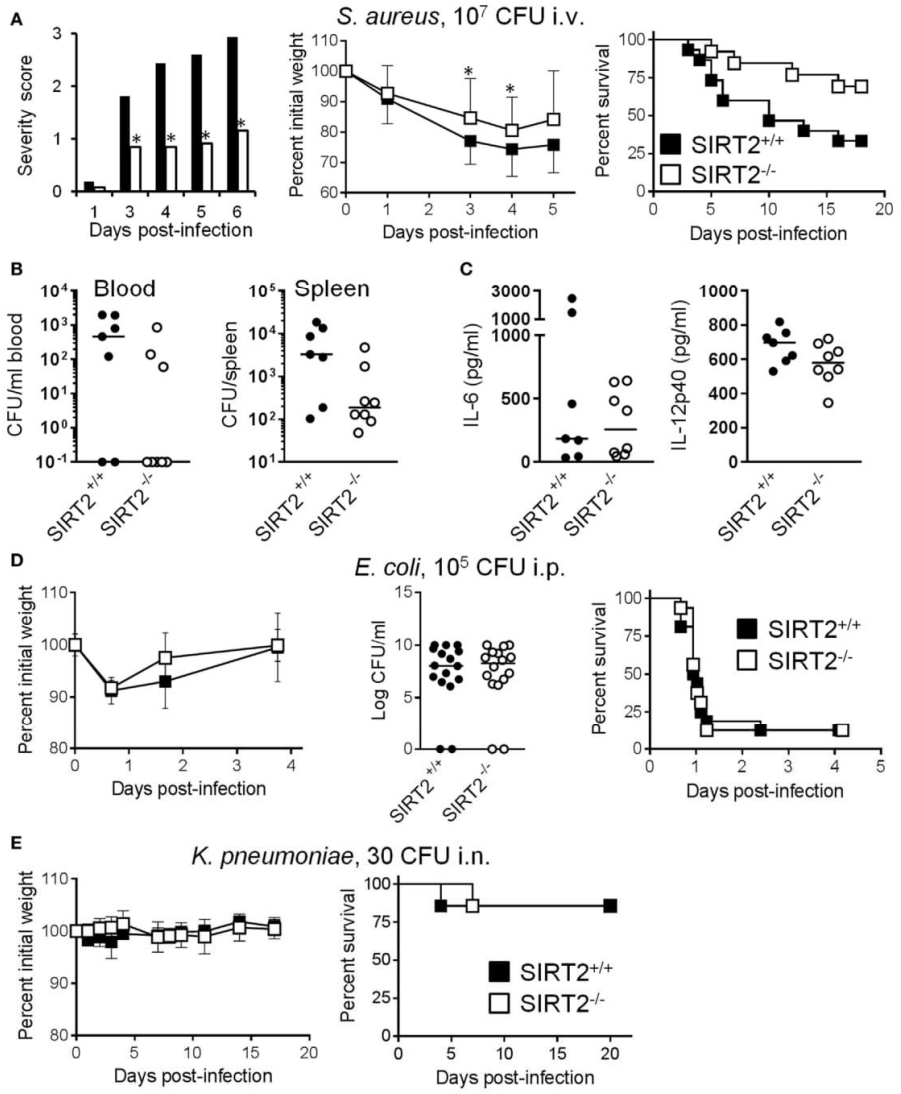

FIGURE 6 | SIRT2 deficiency protects from chronic *Staphytococcus aureus* infection. **(A)** Severity score (*$P \leq 0.01$), body weight (*$P = 0.03$ and 0.04) and survival ($P = 0.04$) of SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice challenged i.v. with 10^7 CFU *S. aureus* ($n = 13$ and 9). **(B,C)** Blood and spleen were collected from SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice ($n = 7$ and 8) 48 h after *S. aureus* challenge. Bacteria were quantified in blood and spleen **(B)** while IL-6 and IL-12p40 concentrations were quantified in blood **(C)**. Horizontal bars represent the medians. $P = 0.07$ and 0.04 in **(B)**, and $P = 0.95$ and 0.12 in **(C)**. **(D)** Body weight, bacteria in blood 18 h postinfection ($P = 0.9$), and survival ($P = 0.8$) of SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice challenged i.p. with 10^5 CFU *E. coli* ($n = 16$ per group). **(E)** Body weight and survival ($P = 0.9$) of SIRT2$^{+/+}$ and SIRT2$^{-/-}$ mice challenged i.n. with 30 CFU *K. pneumoniae* ($n = 7$ per group).

encephalomyelitis, arthritis, lung inflammation, hepatic steatosis, and insulin resistance, but promoted lupus, arthritis, allergic airway disease, and allograft rejection (46–54). SIRT6 has not only been reported to protect against liver fibrosis, atherosclerosis, osteoarthritis, and arthritis but has also been associated with increased TNF production and the development of autoimmune encephalomyelitis and cerebral ischemia (55–62). Different experimental conditions might explain these apparent conflicting results, for example qualitative and quantitative differences in caloric input or subtle variations of NAD^+ availability influencing sirtuin activity indirectly. Moreover, SIRT1, SIRT3, and SIRT6 modulate circadian function and are affected by circadian

oscillation in the abundance of NAD^+ (63, 64). Furthermore, sirtuins might primarily play a role in the development of long-lasting, chronic metabolic, and/or inflammation-related disorders while having a modest impact during acute infectious processes. For instance, SIRT3 deficiency has been reported to increase insulin resistance, diabetic cardiac dysfunction, allograft graft injury, and lung fibrosis, but had no impact on innate immune responses and susceptibility to endotoxemia or bacterial and fungal sepsis (65–73).

Two prior studies examined SIRT2 in the context of infection by intracellular bacteria. SIRT2 deletion in the myeloid compartment had no noticeable impact on host defenses against *M. tuberculosis* infection as attested by cellular infiltrates, cytokine expression, and long-term bacterial burden in lungs (44). *Listeria monocytogenes* promoted SIRT2-dependent histone H3 deacetylation and redirected host gene expression to favor infection (74). Whether other microorganisms subvert SIRT2 or other sirtuins at their own benefit is unknown.

We analyzed host responses to extracellular bacteria most frequently isolated from septic patients. Strikingly, SIRT2 deficiency enhanced the engulfment of Gram-positive and Gram-negative bacteria by macrophages, an effect apparently unrelated with a differential expression of phagocytic receptors or microtubule polymerization. This observation was surprising considering that on the one hand SIRT2 deacetylates α-tubulin and destabilizes the microtubule network (8, 41) and on the other end microtubule depolymerizating agents were reported to inhibit phagocytosis (75, 76). Yet, the effects of microtubule depolymerizating agents were tested using an immortalized macrophage-like mouse cell line and human neutrophils that may behave differently than primary BMDMs. As a positive control (77), actin depolymerization efficiently inhibited phagocytosis by BMDMs. Increased glycolysis has been associated with efficient phagocytosis by macrophages (39, 40). SIRT2 deficiency reduced HIF-1α deacetylation and destablization (78), and augmented glycolysis in human fibroblasts (79). We observed that SIRT2 deficiency increased glycolysis and that glycolysis inhibition reduced phagocytosis in BMBMs. Albeit preliminary, these results suggest that SIRT2 may influence phagocytosis through metabolic constraints.

The improved control of bacterial burden during chronic staphylococcal infection might be related to improved phagocytosis but also to enhanced autophagy in $SIRT2^{-/-}$ mice. Autophagy facilitates the clearance of cytoplasmic bacteria (80) and has been involved in host defenses and tolerance to *S. aureus* infection (81, 82). Hyperacetylation of tubulin stimulated autophagy upon nutrient deprivation, and SIRT2 deficiency increased autophagy in a colorectal cancer cell line (83, 84). Thus, by regulating tubulin acetylation and metabolic activity, SIRT2 may contribute to modulate phagocytic and autophagy defense mechanisms, though the latter has not been formally demonstrated.

From a translational perspective, it was important to define the impact of SIRT2 in preclinical models of infection. A main observation of this study is that SIRT2 deficiency protected mice from chronic staphylococcal infection, while it neither protected nor sensitized mice to TNF-induced shock, endotoxemia, rapidly lethal *E. coli* peritonitis and mild *K. pneumoniae* pneumonia. Additionally, SIRT2 deficiency did not influence the development of chronic candidiasis as it did for chronic staphylococcal infection. This may not be surprising considering differences in host–pathogen interactions during fungal and bacterial infections. Whether SIRT2 protects from other chronic bacterial infections should be tested in the future. Nonetheless, these results support the clinical development of SIRT2 inhibitors regarding their infection-related safety profile. This contrasts with inhibitors of HDAC1-11 that impaired innate immune defenses, increased susceptibility to infection in preclinical mouse models and have been associated with severe infections in patients (29, 85–89).

Overall, SIRT2 has a subtle impact on host defense responses to bacterial infections. Considering that sirtuins are intricately linked with metabolism, age-associated dysfunctions and lifespan, it will be important to analyze the impact of SIRT2 on host defenses under metabolic stress conditions and according to age. To conclude, our results are encouraging with respect to developing inhibitors of SIRT2, which are safe in terms of susceptibility to infections, for treating metabolic and neurodegenerative diseases, such as Parkinson's disease and Huntington's disease (90, 91).

ETHICS STATEMENT

Animal experimentation was approved by the Service de la Consommation et des Affaires Vétérinaires (SCAV) du Canton de Vaud (Epalinges, Switzerland) under authorizations no. 876.8 and 877.8, and performed according to Swiss and ARRIVE guidelines.

AUTHOR CONTRIBUTIONS

EC, TH, CT, JH, MM, JL, MP, BT, and SL performed *in vitro* experiments. EC, TH, CT, and DLR performed *in vivo* experiments. HA-O and JA contributed to reagents. TR conceived the project, designed the experiments, and wrote the paper. All the authors revised the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01037/full#supplementary-material.

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3.3 Sirtuin 3 does not alter host defences against bacterial and fungal infections

Eleonora Ciarlo[1], Tytti Heinonen[1], Jérôme Lugrin[1], Hans Acha-Orbea[2], Didier Le Roy[1], Johan Auwerx[3], Thierry Roger[1]

[1]Infectious Diseases Service, Department of Medicine, Lausanne University Hospital, CH-1066, Epalinges, Switzerland

[2]Department of Biochemistry, University of Lausanne, CH-1066, Epalinges, Switzerland

[3]Laboratory for Integrative and Systems Physiology, Ecole Polytechnique Fédérale de Lausanne, CH-1015, Lausanne, Switzerland

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Summary

SIRT3 is the main mitochondrial deacetylase and mitochondrial activity is linked to immune cell activation. Thus, we used SIRT3 deficient mice to investigate whether SIRT3 plays a role in innate immune responses.

SIRT3 deficient mice develop normally with no macroscopic abnormalities and no alterations in splenic and thymic immune cell populations. SIRT3 deletion has no effect on the activation of TLR-induced signaling pathways (ERK, JNK, p38) and on cytokine production by splenocytes, macrophages and dendritic cells. SIRT3 deficiency does not affect cytokine production, bacterial burden and survival of mice subjected to endotoxemia, *Escherichia coli* peritonitis, *Klebsiella pneumoniae* pneumonia, listeriosis and candidiasis.

Overall, these data support the ongoing development of SIRT3 inhibitors to treat age-related diseases.

My contribution to this work: I performed Western blotting and Luminex. I helped with the experiments required for the revision.

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Sirtuin 3 deficiency does not alter host defenses against bacterial and fungal infections

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Eleonora Ciarlo[1], Tytti Heinonen[1], Jérôme Lugrin[1], Hans Acha-Orbea[2], Didier Le Roy[1], Johan Auwerx[3] & Thierry Roger[1]

Sirtuin 3 (SIRT3) is the main mitochondrial deacetylase. SIRT3 regulates cell metabolism and redox homeostasis, and protects from aging and age-associated pathologies. SIRT3 may drive both oncogenic and tumor-suppressive effects. SIRT3 deficiency has been reported to promote chronic inflammation-related disorders, but whether SIRT3 impacts on innate immune responses and host defenses against infections remains essentially unknown. This aspect is of primary importance considering the great interest in developing SIRT3-targeted therapies. Using SIRT3 knockout mice, we show that SIRT3 deficiency does not affect immune cell development and microbial ligand-induced proliferation and cytokine production by splenocytes, macrophages and dendritic cells. Going well along with these observations, SIRT3 deficiency has no major impact on cytokine production, bacterial burden and survival of mice subjected to endotoxemia, *Escherichia coli* peritonitis, *Klebsiella pneumoniae* pneumonia, listeriosis and candidiasis of diverse severity. These data suggest that SIRT3 is not critical to fight infections and support the safety of SIRT3-directed therapies based on SIRT3 activators or inhibitors for treating metabolic, oncologic and neurodegenerative diseases without putting patients at risk of infection.

The innate immune system provides the first line of defense against microbial infections. Innate immune cells such as macrophages and dendritic cells (DCs) detect invading microorganisms through pattern recognition receptors (PRRs). The best-characterized family of PRRs is constituted by Toll-like receptors (TLRs), which mediate the sensing of a broad range of microbial structures[1]. The interaction between PRRs and microbial ligands activates intracellular signaling pathways that coordinate the expression of immune-regulatory genes among which cytokines/chemokines, and the development of humoral and cellular responses required to neutralize or eliminate pathogens and restore homeostasis.

Sirtuins (SIRT1–7) belong to the NAD^+-dependent class III subfamily of histone deacetylases (HDACs)[2]. Besides histones, sirtuins target thousands of non-histone proteins, among which chromatin modifiers, transcription regulators, signal transduction molecules, metabolic enzymes and structural cell components[3]. SIRT1–7 localize in the cytosol, nucleus and/or mitochondria, which dictates their accessibility to substrates and effector functions.

SIRT3 is the main mitochondrial deacetylase[4,5]. SIRT3 concentrates primarily to the matrix of the mitochondria but may also localize into the nucleus[6,7]. SIRT3 deacetylase activity is intrinsically linked to cell metabolism[8]. SIRT3 promotes fatty acid β-oxidation, tricarboxylic acid cycle, ketogenesis, urea cycle and brown adipose tissue thermogenesis[9–15]. SIRT3 also regulates the activity of the electron transport chain and dampens oxidative stress by targeting superoxide dismutase 2 and the glutathione system[16]. As a regulator of metabolism and oxidative stress homeostasis, SIRT3 protects from aging and age-associated dysfunctions, and genetic studies identified *SIRT3* polymorphisms associated with increased longevity[17–20].

SIRT3 protects from stress-induced cardiovascular diseases and impacts on the development of neurodegenerative and oncologic diseases[21–28]. SIRT3 deficiency increases allograft graft injury, diabetic cardiac dysfunction, insulin resistance, acute kidney injury and lung fibrosis[29–38], suggesting that SIRT3 may counteract the development of chronic metabolic and inflammation-related disorders. SIRT3 has been reported to drive oncogenic and

[1]Infectious Diseases Service, Department of Medicine, Lausanne University Hospital, CH-1066, Epalinges, Switzerland. [2]Department of Biochemistry, University of Lausanne, CH-1066, Epalinges, Switzerland. [3]Laboratory for Integrative and Systems Physiology, Ecole Polytechnique Fédérale de Lausanne, CH-1015, Lausanne, Switzerland. Correspondence and requests for materials should be addressed to T.R. (email: Thierry.Roger@chuv.ch)

tumor-suppressive effects[39]. All these observations stimulated the development of both activators and inhibitors of SIRT3 for clinical purposes[40]. Within this context, it is important to ascertain that SIRT3 targeting would not negatively impact on host resistance to infection, an aspect of SIRT3 biology that is so far poorly characterized[41,42].

In the present study, we used SIRT3 knockout mice to investigate whether SIRT3 deficiency altered the response of immune cells to microbial ligands *in vitro*. We then analyzed the impact of SIRT3 deficiency in a panel of severe and non-severe models of endotoxemia, peritonitis, pneumonia, listeriosis and candidiasis. Overall, our results suggest that SIRT3 deficiency has no major impact on host defenses against infections, supporting the safety of SIRT3-oriented therapies currently under development.

Materials and Methods

Ethics statement. Animal experimentations were approved by the Service de la Consommation et des Affaires Vétérinaires (SCAV) du Canton de Vaud (Epalinges, Switzerland) under authorizations n° 876.8, and 877.8, and performed according to Swiss guidelines and ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines).

Mice, cells and reagents. *Sirt3* floxed (*Sirt3*$^{L2/L2}$) mice were generated as described[43] and crossed with mice expressing the CMV-Cre deleter in the male germline to create full knockouts. SIRT3$^{-/-}$ mice were back-crossed nine times on a C57BL6/J background. Mice were housed under specific pathogen-free conditions and were exempt of mouse hepatitis virus and murine norovirus infections. Splenocytes were cultured in RPMI 1640 medium containing 2 mM glutamine, 50 μM 2-ME, 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen, San Diego, CA) and 10% heat-inactivated fetal calf serum (FCS, Sigma-Aldrich St. Louis, MO)[44]. Bone marrow (BM) cells were cultured seven days in IMDM (Invitrogen) containing 2-ME, penicillin, streptomycin, 10% FCS and 50 U/ml macrophage colony-stimulating factor or 250 U/ml granulocyte macrophage colony-stimulating factor (Immunotools, Friesoythe, Germany) to generate BM-derived macrophages (BMDMs) and dendritic cells (BMDCs)[45]. For experiments, 10^5, 5×10^5 or 2×10^6 cells were seeded in 96, 24 or 6-well plates in complete medium without growth factor and antibiotics. The stimuli used were: *Salmonella minnesota* ultra pure LPS (List Biologicals Laboratories, Campbell, CA), Pam_3CSK_4 (EMC microcollections, Tübingen, Germany), CpG ODN 1826 (CpG, InvivoGen, San Diego, CA), concanavalin A (Sigma-Aldrich), anti-CD3ε and CD28 antibodies (clones 145-2C11 and 37.51, eBioscience, San Diego, CA) and toxic shock syndrome toxin-1 (TSST-1, Toxin Technology, Sarasota, FL). Clinical strains of *Escherichia coli* O18 (*E. coli*), *Klebsiella pneumoniae* caroli (*K. pneumoniae*), Group B Streptococcus (GBS)[46–49] and *Listeria monocytogenes* 10403 s (*L. monocytogenes*, a gift from D. Zehn, Lausanne University Hospital, Switzerland) were grown in brain heart infusion broth (BD Biosciences, Erembodegem, Belgium). *Candida albicans* 5102 (*C. albicans*)[44] was cultured in yeast extract-peptone-dextrose (BD Biosciences). Bacteria were heat-inactivated for *in vitro* experiments[50].

RNA analyses. RNA was isolated and reverse transcribed using the RNeasy and QuantiTect reverse transcription kits (Qiagen, Hilden, Germany). Real-time PCR was conducted using the Fast SYBR® Green Master Mix and a QuantStudio™ 12 K Flex system (Life Technologies, Carlsbad, CA)[44,45]. Primers have been described[45,51]. Sirt3 expression was normalized to actin expression. Sirt3 mRNA expression levels in organs were extracted from the BioGPS resource (http://biogps.org).

Western blot analyses. Proteins were extracted from liver or BMDMs using RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% Triton-X-100, 0.1% NP-40, 1 mM PMSF) or an in house cell lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5% NP-40, 1 mM PMSF, 1 mM Na-orthovanadate, 10 mM NaF) containing protease and phosphatase inhibitors (Merck)[52,53] and electrophoresed through SDS-PAGE[54]. Membranes were incubated with antibodies directed against SIRT3 and total and phosphorylated ERK1/2, p38 and JNK (Cell Signaling Technology), then with a secondary horseradish peroxidase-conjugated antibody (Sigma-Aldrich). Blots were revealed with the enhanced chemiluminescence Western blotting system (GE Healthcare, Little Chalfont, Royaume-Uni). Images were recorded using a Fusion Fx system (Viber Lourmat, Collégien, France).

Flow cytometry. Single cell suspensions from thymus and spleen were incubated with 2.4G2 monoclonal antibody (mAb) and stained using mAbs listed in Supplementary Table S1 as described previously[55]. Data were acquired using a LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo Version 8.5.3 software (FlowJo LLC, Ashland OR).

Proliferation assay. Splenocytes were cultured in 96-well plates for 48 hours with different stimuli and proliferation quantified by measuring ^{3}H-thymidine incorporation over 18 hours[56].

Cytokine measurements. Cytokines were quantified using DuoSet ELISA kits (R&D Systems, Abingdon, UK)[57].

***In vivo* models.** Eight to twelve-week-old SIRT3$^{+/+}$ and SIRT3$^{-/-}$ C57BL/6 J mice matched for age were used. Endotoxemia was performed by challenging mice intraperitoneally (i.p.) with 400 μg LPS (20 mg/kg). Peritonitis, pneumonia, listeriosis and candidiasis were induced by injecting i.p. $1–3 \times 10^4$ CFU *E. coli*, intranasally (i.n.) 30 CFU *K. pneumoniae*, intravenously (i.v.) 10^5 CFU *L. monocytogenes* and i.v. 10^5, 3×10^5 or 10^6 CFU *C. albicans*, respectively. Blood, spleen and liver were collected 1–48 hours post-challenge to quantify cytokines and bacteria. Survival and severity scores graded from 1 to 5 were registered at least once daily[58]. Animals were euthanized when they met a severity score of 4. Animal follow-up was performed by two operators.

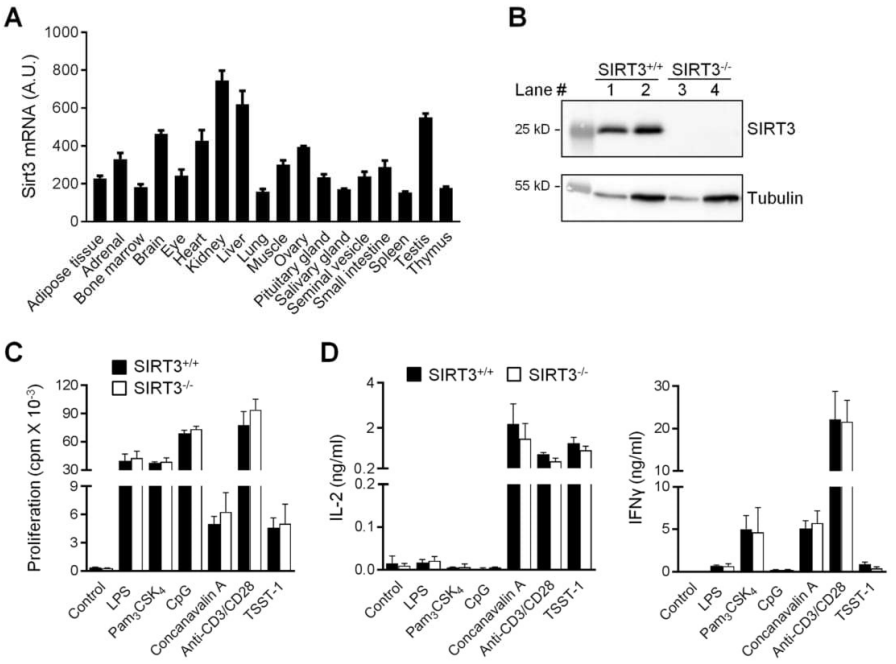

Figure 1. SIRT3 deficiency does not influence proliferation and cytokine response of splenocytes. (**A**) Sirt3 mRNA expression levels in a panel of organs (from the BioGPS resource). A.U.: arbitrary units. (**B**) SIRT3 protein expression in SIRT3$^{+/+}$ and SIRT3$^{-/-}$ liver (protein extracts from lanes 1 and 3 and lanes 2 and 4 were obtained using RIPA lysis buffer and in house cell lysis buffer, respectively) assessed by Western blotting. Full-length blots are presented in Supplementary Figure S1. The first lane of each blot corresponds to a molecular weight marker. (**C**,**D**) SIRT3$^{+/+}$ and SIRT3$^{-/-}$ splenocytes were incubated for 48 hours with LPS (5 μg/ml), Pam_3CSK_4 (10 μg/ml), CpG (2 μg/ml), concanavalin A (5 μg/ml), anti-CD3/CD28 antibodies (1 μg/ml) and TSST-1 (2 μg/ml). (**C**) Proliferation was measured by ^{3}H-thymidine incorporation. Data are means ± SD of triplicate samples from one experiment performed with 3 mice and are representative of 2 experiments. (**D**) IL-2 and IFNγ concentrations in cell culture supernatants were quantified by ELISA. Data are means ± SD from two experiments each performed with 3 mice.

Statistical analyses. Comparisons between groups were performed using the ANOVA F-test followed by two-tailed unpaired Student's t-test. Survival curves were built using the Kaplan-Meier method and differences were analyzed by the log-rank sum test. All analyses were performed using PRISM (GraphPad, San Diego, CA). *P* values were two-sided and significance level was set at 0.05.

Results

SIRT3 deficiency has no major impact on the composition of thymic and splenic immune cell subsets and on the response of splenocytes to immune stimuli. SIRT3 is expressed ubiquitously, including in immune organs (Fig. 1A). SIRT3$^{-/-}$ mice used in this study were backcrossed nine times on a C57BL6/J background. SIRT3$^{-/-}$ mice developed without macroscopic abnormalities and expressed no detectable levels of SIRT3 protein (Fig. 1B). The absolute number of cells and the proportions of the major immune cell subsets in the thymus and the spleen were similar in SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice, including CD4/CD8 double negative (DN1-4), double positive (DP) and single positive (SP) thymocytes and splenic CD3$^+$ T cells (DN and SP, naïve and memory), B220$^+$ B cells (immature and mature) and CD11c$^+$ DCs (Table 1 and Table 2). SIRT3$^{+/+}$ and SIRT3$^{-/-}$ splenocytes were cultured for 48 hours with LPS (TLR4 ligand), Pam_3CSK_4 (TLR1/TLR2 ligand), CpG (TLR9 ligand), concanavalin A, anti-CD3/CD28 and TSST-1 before measuring cell proliferation and IL-2 and IFNγ production. SIRT3$^{+/+}$ and SIRT3$^{-/-}$ splenocytes reacted similarly to all stimuli (Fig. 1C and D). Hence, SIRT3 deficiency had no apparent impact on immune cell development and splenocyte response to stimulation.

SIRT3 deficiency does not alter macrophage and dendritic cell response to microbial products. TLR triggering activates the MAPK pathway involved in the control of cytokine production[1]. To test whether SIRT3 had an impact on the response of macrophages to TLR ligands, the phosphorylation of ERK1/2, p38 and JNK MAPKs in BMDMs exposed for 0, 10, 30 and 60 minutes to LPS was analyzed by Western blotting (Fig. 2A). ERK1/2, p38 and JNK phosphorylation was reduced in SIRT3$^{-/-}$ BMDMs 10 minutes post-stimulation (Fig. 2A). No differences were observed 30 and 60 minutes post-stimulation. Nonetheless, SIRT3$^{+/+}$ and

	SIRT3$^{+/+}$	SIRT3$^{-/-}$
CD4$^+$ CD8$^+$	82.3 ± 3.1	84.3 ± 1.0
CD4$^-$ CD8$^-$	2.0 ± 0.6	1.6 ± 0.1
CD25$^-$ CD44$^+$	1.4 ± 0.6	1.2 ± 0.2
CD25$^+$ CD44$^+$	0.1 ± 0.01	0.1 ± 0.03
CD25$^+$ CD44$^-$	1.8 ± 0.6	1.8 ± 0.3
CD25$^-$ CD44$^-$	96.6 ± 1.2	96.8 ± 0.42
CD4$^+$ CD8$^-$	12.0 ± 2.3	10.6 ± 0.7
CD4$^-$ CD8$^+$	3.6 ± 0.3	3.5 ± 0.2

Table 1. Thymic cell subsets in SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice. Data are means ± SD of 4 animals per group and expressed as the percentage of total cells (CD4$^+$ CD8$^+$, CD4$^-$ CD8$^-$, CD4$^+$ CD8$^-$ and CD4$^-$ CD8$^+$) or percentage of CD4$^-$ CD8$^-$ parental cells (CD25$^-$ CD44$^+$, CD25$^+$ CD44$^+$, CD25$^+$ CD44$^-$ and CD25$^-$ CD44$^-$). Total cell numbers were 49.2 ± 15.4 and 47.4 ± 6.9 millions per thymus in SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice, respectively. No statistically significant differences in subsets' percentages and absolute numbers were detected.

	SIRT3$^{+/+}$	SIRT3$^{-/-}$
CD3$^+$ T cells (%)	27.3 ± 4.6	31.1 ± 1.0
CD4$^+$	62.3 ± 2.7	61.2 ± 4.2
CD4$^+$ CD44^{low} CD62L^{high} (naive)	46.0 ± 4.2	48.9 ± 6.8
CD4$^+$ CD44^{high} CD62L^{low} (memory)	16.2 ± 2.6	12.0 ± 2.8
CD8$^+$	31.5 ± 2.0	32.5 ± 3.3
CD8$^+$ CD44^{low} CD62L^{high} (naive)	23.3 ± 1.0	28.6 ± 1.7
CD8$^+$ CD44^{high} CD62L^{low} (memory)	8.2 ± 0.5	6.6 ± 0.3
CD4$^-$ CD8$^-$	1.3 ± 0.1	1.5 ± 0.3
B220$^+$ B cells (%)	50.5 ± 7.6	53.3 ± 3.1
B220$^+$ IgD$^+$ CD23$^+$ (mature)	45.7 ± 2.6	48.8 ± 2.7
B220$^+$, non-IgD$^+$/CD23$^+$ (immature)	6.5 ± 0.5	6.4 ± 0.5
CD11c$^+$ DCs (%)	6.2 ± 0.5	6.5 ± 0.6
B220$^-$	62.8 ± 2.5	59.4 ± 2.6
B220$^+$	37.2 ± 2.5	40.6 ± 2.6

Table 2. Splenic cell subsets in SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice. Data are means ± SD of 4 animals per group and expressed as the percentage of CD3$^+$, B220$^+$, and CD11c$^+$ splenic cells or the percentage of the CD3$^+$, B220$^+$ and CD11c$^+$ parental populations expressing CD4, CD8, CD44, CD62L, IgD and CD23. Total cell numbers were 75.1 ± 7.5 and 75.4 ± 21.1 millions per spleen in SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice, respectively. No statistically significant differences in subset's percentages and absolute numbers were detected.

SIRT3$^{-/-}$ BMDMs secreted comparable levels of TNF and IL-6 (t = 8 hours) in response to stimulation with LPS, Pam_3CSK_4, CpG, *E. coli* and GBS (Fig. 2B). SIRT3$^{+/+}$ and SIRT3$^{-/-}$ BMDCs also produced identical levels of TNF and IL-6 when exposed to the same panel of stimuli (Fig. 2C). In agreement with these observations, SIRT3$^{+/+}$ and SIRT3$^{-/-}$ BMDMs expressed similar mRNA levels of a selection of PRRs (Tlr1, Tlr2, Tlr6, Tlr9, Cd14, Md2, Msr1) and cytokines/chemokines (Il1a, Il1b, Il10, Il12b, Il15, Il27, Ccl2/Mcp1, Ccl3/Mip1a, Ccl4/Mip1b, Cxcl1/Groa, Cxcl10/Ip10, Cxcl11/Itac) at baseline and 2 hours following stimulation with LPS (Supplementary Figure S3). Altogether, these results suggested that SIRT3 deficiency marginally influenced MAPK signaling and cytokine production by macrophages and DCs exposed to TLR ligands and whole bacteria.

SIRT3 deficiency does not affect the course of endotoxemia and bacterial and fungal infections. To address the relevance of our *in vitro* findings, we developed a model of endotoxemia by challenging mice i.p. with 20 mg/kg LPS (Fig. 3A and B). TNF and IL-12p40 concentrations in blood collected from SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice 1 hour (TNF) and 6 hours (IL-6) post-challenge were comparable (Fig. 3A). In line with these results, the overall survival of SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice was similar (75% *vs* 89%, P = 0.4, Fig. 3B).

We next examined the contribution of SIRT3 to host defenses against bacterial pneumonia and peritonitis, listeriosis and candidiasis. Infection models of diverse severity were used considering that SIRT3-mediated hypo-inflammatory response would jeopardize survival to otherwise non-lethal infection, while SIRT3-mediated hyper-inflammatory response would worsen outcome during severe infection[47,51]. SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice survived equivalently to non-severe pneumonia induced by *K. pneumoniae* (82.5% *vs* 93%, P = 0.4, Fig. 3C). In a model of fulminant *E. coli* peritonitis, where all deaths occurred within 3 days, bacterial loads in blood collected 18 hours post-infection (median: 4.7×10^6 CFU/ml *vs* 5.2×10^6 CFU/ml; P = 0.4) and survival (27% *vs* 27%) were identical in SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice (Fig. 3D and E). Listeriosis was induced by i.v. challenge with the intracellular bacterium *L. monocytogenes*. Two days post-infection, bacteremia was low but slightly higher

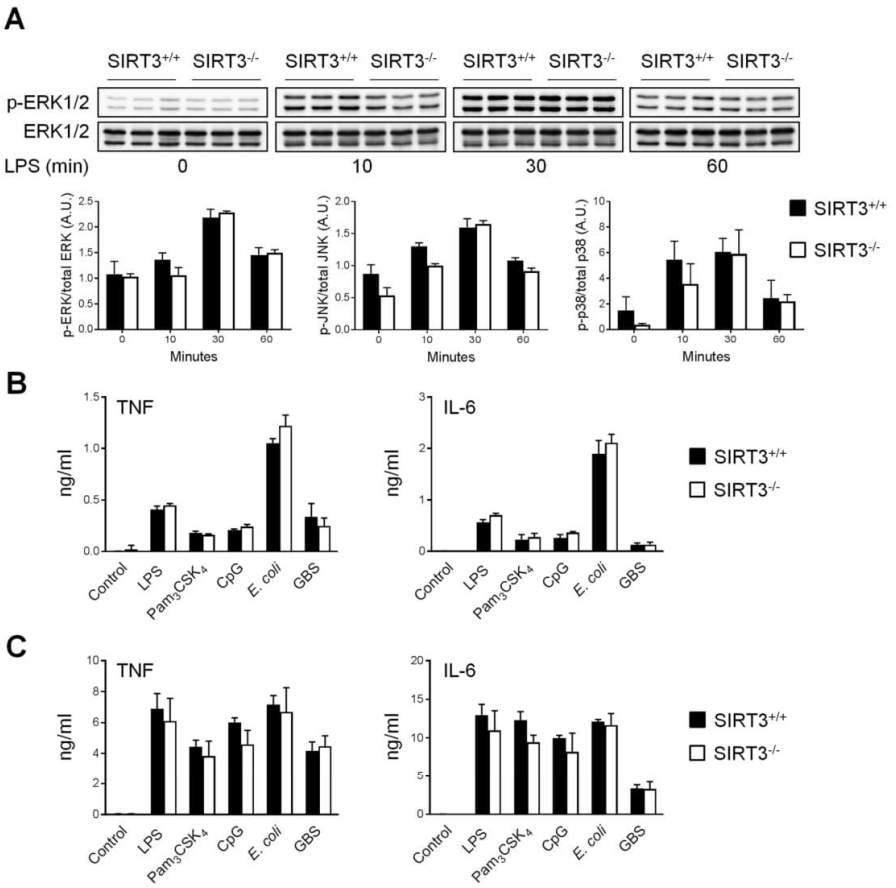

Figure 2. SIRT3 does not affect TNF and IL-6 production by BMDMs and BMDCs exposed to microbial stimuli. SIRT3$^{+/+}$ and SIRT3$^{-/-}$ BMDMs (**A**,**B**) and BMDCs (**C**) were exposed to LPS (10 ng/ml), Pam_3CSK_4 (10 ng/ml), CpG (2 μg/ml), *E. coli* (10^6 CFU/ml) and GBS (2.5×10^6 CFU/ml). (**A**) Expression levels of phosphorylated (p) and total ERK1/2, JNK and p38 were analyzed by Western blotting and quantified by imaging. Data are means ± SD obtained with 3 mice. Full-length blots are presented in Supplementary Figure S2. (**B**,**C**) TNF and IL-6 concentrations in cell culture supernatants collected 8 hours after stimulation. Data are means ± SD of triplicate samples from one experiment performed with 3 mice and are representative of 2 experiments.

in SIRT3$^{-/-}$ mice (median: 7.5×10^2 CFU/ml *vs* 1.1×10^3 CFU/ml; P = 0.02). *L. monocytogenes* burden in spleen and liver was massive but not significantly different between SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice (3.6×10^6 CFU/mg *vs* 4.9×10^6 CFU/mg, P = 0.8 and 1.3×10^5 CFU/mg *vs* 1.8×10^5 CFU/mg, P = 0.1; Fig. 4A). Death occurred between days 2 and 7, and overall survival was not influenced by SIRT3 deficiency (0% *vs* 7%, P = 0.5, Fig. 4B). Finally, candidiasis was induced by inoculating 10^5, 3×10^5 or 10^6 CFU *C. albicans* to produce a mild/chronic infection inducing animal death over a period of 5 weeks or a severe infection leading to animal death within 3 days. In the three models, the survival rates of SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice were comparable (90% *vs* 70%, P = 0.3; 56% *vs* 56%, P = 0.8 and 0% *vs* 0%, P = 0.8) (Fig. 5A–C).

Discussion

We report that SIRT3 deficiency has no major impact on immune cell development and host defenses against bacterial and fungal infections. These observations are particularly topical considering the promises of SIRT3-targeting strategies to treat age-related disorders.

SIRT3 deficiency did not alter the composition of the main lymphoid and DC subsets in thymus and spleen, in line with a previous study showing normal thymic, splenic and lymph node CD4$^+$ and CD8$^+$ T-cell subpopulations in SIRT3$^{-/-}$ mice, including CD4$^+$ Foxp3$^+$ T regulatory cells (Tregs)[29]. Immune cells exposed to microbial products or cytokines undergo metabolic reprogramming characterized by a switch from oxidative

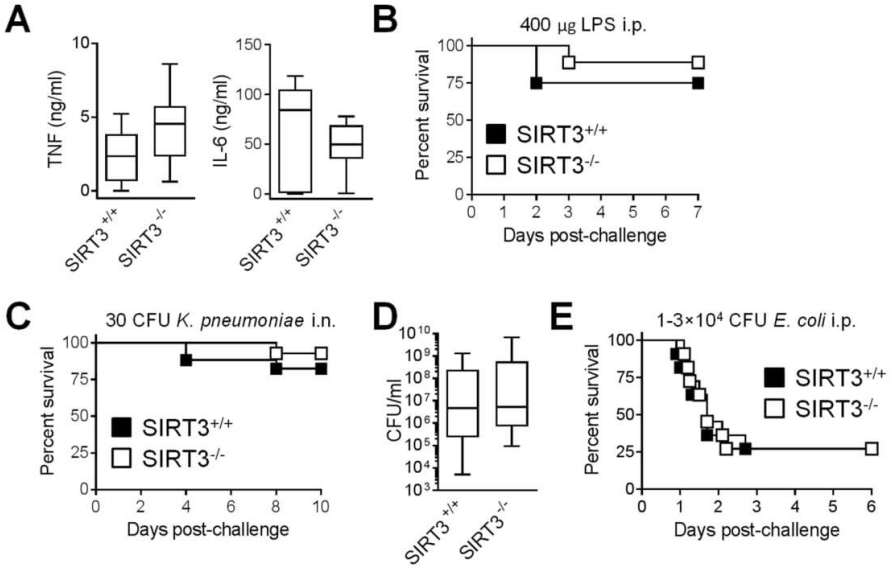

Figure 3. SIRT3 deficiency does not affect the course of endotoxemia and bacterial infection. SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice were injected i.p. with 400 µg LPS ((**A**,**B**) n = 8–9 per group), i.n. with 30 CFU *K. pneumoniae* ((**C**) n = 17 SIRT3$^{+/+}$ and 14 SIRT3$^{-/-}$ mice) and i.p. with 1–3 × 10^4 CFU *E. coli* ((**D**,**E**) n = 11 per group). (**A**) TNF and IL-6 concentrations in blood collected 1 hour (TNF) and 6 hours (IL-6) after LPS challenge. P = 0.1 and 0.2. (**B**,**C** and **E**) Survival of mice. P = 0.4, 0.4 and 1.0 (**D**) CFU counts in blood collected 18 hours after *E. coli* challenge. P = 0.4.

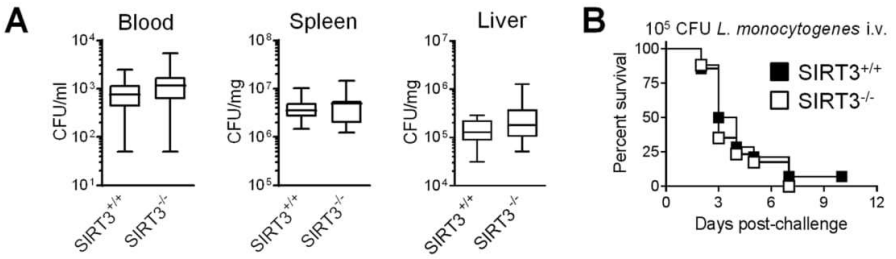

Figure 4. SIRT3 deficiency does not impact on organ colonization and survival of mice infected with *L. monocytogenes*. SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice (**A**) n = 14–15 per group; (**B**) n = 14–16 per group) were injected i.v. with 10^5 CFU *L. monocytogenes*. ((**A**) Blood, spleen and liver were collected 48 hours after challenge to quantify bacterial loads. P = 0.02, 0.8 and 0.1. (**B**) Survival of mice. P = 0.5.

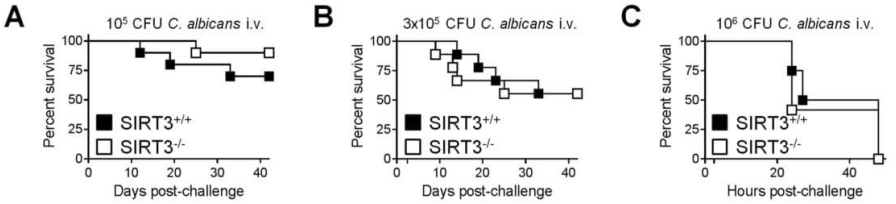

Figure 5. SIRT3 deficiency does not impact on survival of mice infected with *C. albicans*. SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice were injected i.v. with 10^5 (**A**) n = 10 per group), 3 × 10^5 (**B**) n = 9 per group) and 10^6 CFU *C. albicans* (**C**) n = 12 per group). (**A**–**C**) Survival of mice. P = 0.3, 0.8 and 0.5.

phosphorylation to glycolysis associated with the development of inflammatory and antimicrobial functions[59]. Considering that SIRT3 impacts on mitochondrial biogenesis and functions[60], we expected SIRT3 deficiency to enhance immune cell response to microbial stimulation. MAPK phosphorylation was reduced to some extend early on after LPS stimulation in SIRT3$^{-/-}$ BMDMs. However, proliferation and cytokine production by splenocytes, macrophages and DCs were largely unaffected by SIRT3 deficiency. In contrast, shRNA-mediated SIRT3 silencing increased baseline TNF mRNA levels in RAW 264.7 macrophages[61], and adenovirus-mediated SIRT3 overexpression inhibited MAPK phosphorylation in phenylephrine-treated myocytes and palmitate-stimulated pancreatic beta-cells[62,63]. Thus, SIRT3 seems to have cell and possibly context-dependent effects. Supporting this assumption, SIRT3 deficiency impaired *in vitro* the suppressive function of Tregs, which are particularly dependent on SIRT3-mediated mitochondrial activity and oxidative phosphorylation to develop optimal functions. However, SIRT3$^{-/-}$ Tregs retained their suppressive functions in an adoptive transfer model of cardiac allograft rejection[29]. SIRT3 deficiency also affected endothelial function in mice fed with a high cholesterol diet but not a normal diet[64], and organ-specific SIRT3 deficiency increased mitochondrial protein acetylation but did not induce mitochondrial dysfunction and did not impact on overall metabolic homeostasis as observed in germline SIRT3 knockouts[43,65].

To assess the safety of SIRT3-targeting therapies, it was most important to analyze the contribution of SIRT3 in preclinical models of infection. As a first approach, we tested a model of endotoxemia, which revealed that cytokine response and survival rates were not different in SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice. Although SIRT3$^{-/-}$ mice survived slightly better than SIRT3$^{+/+}$ mice, whether this was a genuine effect would require large groups of animals (>40 mice per genotype) according to power calculation. Albeit very unlikely, we also cannot totally rule out that some differences in the genetic background of SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice play a role. To address that question, SIRT3$^{-/-}$ mice with additional backcrosses should be tested. SIRT3$^{+/+}$ mice had a minor yet significant survival advantage (10% *vs* 0% survival in SIRT3$^{+/+}$ *vs* SIRT3$^{-/-}$ mice) in a highly stringent model of endotoxemia[38], suggesting that SIRT3 may provide benefits in sterile, deep inflammatory, processes. Unfortunately, the cytokine response was not reported. Endotoxemia does not recapitulate the complexity to host defense mechanisms generated to fight against living microorganisms. Moreover, immunomodulatory compounds may interfere with innate immune responses and compromise host defenses, as well documented for anti-TNF and anti-IL-1 agents[66,67]. Thus, we elected to test models of sepsis induced by *E. coli* and *K. pneumoniae*, two of the most frequent etiologic agents of human sepsis. SIRT3 deficiency did not impact on the development of sub-acute pneumoniae and acute peritonitis, going well along with normal *in vitro* responses to bacterial stimulation of immune cells. In line with our observations, the survival rates of SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice were not significantly different following cecal ligation and puncture sepsis[22]. Additionally, SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice behaved roughly identically following systemic infection with *L. monocytogenes* and *C. albicans* given to produce chronic/mild and acute candidiasis. Interestingly, *Listeria* loads were slightly increased in the blood of SIRT3$^{-/-}$ mice, which might feature transient alteration of mitochondrial dynamics during *L. monocytogenes* infection[68]. However, bacteremia was very low when compared to liver and spleen bacterial burdens, which were not different between SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice. The absence of patent phenotype in a panel of sepsis models suggests that infection-induced phagocyte recruitment and/or activity was not impaired in SIRT3$^{-/-}$ mice. Indeed, SIRT3 deficiency did not affect endothelial activation, plaque macrophage and T cell infiltration and atherosclerosis in low-density lipoprotein receptor knockout mice[64]. Our observations are somehow reminiscent of that obtained analyzing SIRT1. While SIRT1 was globally shown to inhibit inflammation[69], it had little impact on macrophage and neutrophil antimicrobial functions, and myeloid deficiency in SIRT1 did not influence the outcome of endotoxemia and Gram-positive sepsis[70].

SIRT3 activity is strongly associated with metabolism, and there is a tight relationship between metabolism and immune functions[59]. Thus, work will be required to address whether SIRT3 impacts on host defenses under metabolic stress. It is also possible that sirtuins have complementary or redundant effects, as suggested by protein interaction studies[5]. Therefore, future studies should analyze the impact of targeting multiple sirtuins on innate immune responses. Supporting this strategy, dual inhibitors of SIRT1/2 and pan-classical HDAC inhibitors affected host defenses against infections[46,51,56,71]. Considering that SIRT3 has been associated with age-related dysfunctions and that immune functions are decreased in elderly[72], one should analyze the impact of SIRT3 in populations of different ages. Finally, a limitation of this study is that preclinical mouse models were performed with female mice. In a preliminary experiment using a limited number of males (seven animals), the survival of SIRT3$^{+/+}$ and SIRT3$^{-/-}$ mice to *Klebsiella*-induced pneumonia was not different. However, larger groups of mice and additional models should be tested to settle whether there is or not a sex-dependent impact of SIRT3 deficiency on susceptibility to infection.

Overall, our data support the assumption that SIRT3 has no major impact on innate immune functions and host defenses against bacterial and fungal infections, at least in healthy immunocompetent hosts. The present data largely support the safety of SIRT3-oriented therapies, in terms of susceptibility to infections, for treating metabolic, oncologic and neurodegenerative diseases.

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Author Contributions

E.C., J.L. and T.H. performed *in vitro* experiments. E.C., T.H. and D.L.R. performed *in vivo* experiments. H.A.O. and J.A. contributed to reagents. T.R. conceived the project, designed the experiments and wrote the paper. All the authors revised the paper.

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3.4 Sirtuin 5 Deficiency Does Not Compromise Innate Immune Responses to Bacterial Infections

Tytti Heinonen*, Eleonora Ciarlo*, Charlotte Théroude, Aimilia Pelekanou, Jacobus Herderschee, Didier Le Roy, Thierry Roger

Infectious Diseases Service, Department of Medicine, Lausanne University Hospital, Epalinges, Switzerland

*Equal contribution

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Summary

SIRT5 is a mitochondrial sirtuin with weak deacetylase but potent desuccinylase activity. SIRT5 targets numerous proteins involved in metabolic and ROS detoxifying pathways. We therefore questioned whether SIRT5 influences innate immune responses *in vitro* and *in vivo* using SIRT5 deficient mice.

SIRT5 deficient mice develop normally with no defect in immune cell development. SIRT5 deficiency has no impact on the expression of PRRs by macrophages and on cytokine production and proliferation by macrophages and splenocytes exposed to microbial ligands and immunological stimuli. In line, SIRT5 deficient mice are susceptible like wild-type mice to endotoxemia and infections with *K. pneumoniae, S. pneumoniae*, *E. coli, L. monocytogenes* and *S. aureus*.

Altogether, these data support the development of SIRT5 inhibitors for the treatment of oncologic conditions.

My contribution to this work: I performed Western blotting, measured metabolic parameters and performed *in vivo* models of infection.

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Sirtuin 5 Deficiency Does Not Compromise Innate Immune Responses to Bacterial Infections

*Tytti Heinonen†, Eleonora Ciarlo†, Charlotte Théroude, Aimilia Pelekanou, Jacobus Herderschee, Didier Le Roy and Thierry Roger**

Infectious Diseases Service, Department of Medicine, Lausanne University Hospital, Epalinges, Switzerland

Sirtuin 5 (SIRT5) is a member of the family of NAD^+-dependent lysine/histone deacetylases. SIRT5 resides mainly in the mitochondria where it catalyzes deacetylation, demalonylation, desuccinylation, and deglutarylation of lysine to regulate metabolic and oxidative stress response pathways. Pharmacologic inhibitors of SIRT5 are under development for oncologic conditions, but nothing is known about the impact of SIRT5 on antimicrobial innate immune defenses. Using SIRT5 knockout mice, we show that SIRT5 deficiency does not affect immune cell development, cytokine production and proliferation by macrophages and splenocytes exposed to microbial and immunological stimuli. Moreover, preclinical models suggest that SIRT5 deficiency does not worsen endotoxemia, *Klebsiella pneumoniae* and *Streptococcus pneumoniae* pneumonia, *Escherichia coli* peritonitis, listeriosis, and staphylococcal infection. Altogether, these data support the safety profile in terms of susceptibility to infections of SIRT5 inhibitors under development.

Keywords: sirtuin, innate immunity, cytokine, macrophage, endotoxemia, sepsis, histone deacetylase, metabolism

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Marc Le Bert, UMR7355 Immunologie et Neurogénétique Expérimentales et Moléculaires (INEM), France

****Correspondence:***
Thierry Roger
thierry.roger@chuv.ch
orcid.org/0000-0002-9358-0109

†These authors have contributed equally to this work

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INTRODUCTION

Innate immune cells such as monocytes/macrophages, neutrophils and dendritic cells (DCs) express pattern recognition receptors (PRRs) that mediate the sensing of microbial associated molecular patterns (such as endotoxin, lipoproteins, peptidoglycans, glucans, mannans, and nucleic acids) and danger-associated molecular patterns released by injured or infected cells. PRRs encompass Toll-like receptors (TLRs), C-type lectin receptors, NOD-like receptors, RIG-I-like receptors, and cytosolic DNA sensors (1, 2). Upon ligand sensing, PRRs initiate intracellular signaling cascades remodeling host transcriptome to promote cytokine/chemokine production and the development of antimicrobial effector mechanisms. Innate immune responses have to be tightly regulated to avoid imbalanced life-threatening immune responses.

Sirtuins belong to the highly conserved family of NAD^+-dependent lysine/histone deacetylases (HDACs). The seven mammalian sirtuins (SIRT1-7) are characterized by their domain organization, enzymatic activity and subcellular nuclear, nucleolar, cytoplasmic or mitochondrial localization. Sirtuins catalyze enzymatic reactions beyond deacetylation, and can function as ADP-ribosyltranferase, demyristolase, decrotonylase, desuccinylase, deglutarylase, demalonylase, deformylase, and demyristolase (3–7). Proteome analyses identified thousands of targets of sirtuins, and sirtuins have been involved in the regulation of many biological functions and pathological processes. Sirtuins are promising therapeutic targets for metabolic, cardiovascular, neurodegenerative, and oncologic diseases (3–7).

SIRT5 is one of the least characterized sirtuins. SIRT5 belongs, together with SIRT3 and SIRT4, to the so-called mitochondrial sirtuins. SIRT5 also localizes into the cytoplasm (8). SIRT5 was initially shown to deacetylate carbamoyl phosphate synthase (CPS1) to promote urea cycle (9). SIRT5 is a weak deacetylase and recent data suggest that SIRT5 primarily performs lysine demalonylation, desuccinylation, and deglutarylation (10, 11). SIRT5 desuccinylates and deglutarylates CSP1 to increase ammonia detoxification and desuccinylates 3-hydroxy-3-methylglutaryl-CoA synthase 2 to increase ketogenesis (10–12). SIRT5 desuccinylates succinate dehydrogenase and pyruvate dehydrogenase to repress cellular respiration and activates superoxide dismutase 1 and isocitrate dehydrogenase 2 through desuccinylation and glucose-6-phosphate dehydrogenase through deglutarylation. In this way, SIRT5 regulates NADPH homeostasis, scavenges reactive oxygen species (ROS), and increases resistance to oxidative stress (8, 13, 14). A malonylome analysis in liver identified gluconeogenesis and glycolysis as the most enriched pathways regulated by SIRT5 (15), while succinylome analyses of heart and liver identified fatty acid oxidation (FAO), amino acid metabolism and TCA cycle (8, 12, 16). Overall, SIRT5 is emerging as a key regulator of metabolism. SIRT5 protects from cardiac dysfunctions and dextran sulfate sodium-induced colitis and promotes or restricts cancer growth depending of the context (16–18). Thus, SIRT5 is a potential therapeutic target for several pathological conditions. Efforts are currently devoted to the generation of SIRT5 inhibitors such as thiosuccinyl peptides, cyclic pentapeptide harboring a central N(ε)-carboxyethyl-thiocarbamoyl-lysine residue and 3-arylthiosuccinylated and 3-benzylthiosuccinylated peptide derivatives (19–21) for specific cancer types (18, 22).

The impact of SIRT5 on antimicrobial host defenses is unknown; which is an important missing piece considering the clinical development of SIRT5 inhibitors. Using SIRT5 knockout mice, we show that SIRT5 deficiency has no major impact on immune cell development and on the response of macrophages and splenocytes to microbial stimulation. Going well along with these observations, preclinical models revealed that SIRT5 knockout mice are not particularly sensitive to endotoxemia, *Klebsiella pneumoniae* and *Streptococcus pneumoniae* pneumonia, *Escherichia coli* peritonitis, listeriosis and staphylococcal infection. Up to now, these data support the assumption that SIRT5 inhibitors should not increase patients' susceptibility to infections.

MATERIALS AND METHODS

Ethics Statement

Animal experimentation was approved by the *Service de la Consommation et des Affaires Vétérinaires* of *Canton de Vaud* (Epalinges, Switzerland) under authorizations n°VD 3287, 876.8, 876.9, 877.8, and 877.9 and performed according to Swiss and ARRIVE guidelines.

Mice, Cells and Reagents

Experiments were performed using 8 to 12-week-old C57BL/6J mice (Charles River Laboratories, Saint-Germain-sur-l'Arbresle, France) and SIRT5 knockout mice (kindly provided by Prof Johan Auwerx, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) backcrossed 7 times on a C57BL/6J background (23). Mice were housed (12 h light/dark cycle, 22°C, 70% humidity) under specific pathogen-free conditions in the animal facility of the Centre des Laboratoires d'Epalinges (CLE, Epalinges, Switzerland, license number VD-H04). Colonies were free of mouse norovirus and mouse hepatitis virus infections. Mice were fed with γ-irradiated food (Global Rodent XP 18, Provimi Kliba AG, Kaiseraugst, Switzerland) and water *ad libitum*. Mice were transferred in a BSL2 unit to perform *in vivo* models of infection.

Bone marrow-derived macrophages (BMDMs) and splenocytes were obtained and cultured as described (24, 25). For experiments, cells were seeded in complete medium without growth factors and antibiotics (1 or 20 × 10^5 cells in 96 or 6-well plates). Stimuli were *Salmonella minnesota* ultra pure LPS (InvivoGen, San Diego, CA), Pam_3CSK_4 (EMC microcollections, Tübingen, Germany), CpG ODN 1826 (CpG, InvivoGen), toxic shock syndrome toxin-1 (TSST-1, Toxin Technology, Sarasota, FL), concanavalin A (Sigma-Aldrich, St. Louis, MI), anti-CD3ε, and anti-CD28 antibodies (clones 145-2C11 and 37.51, eBioscience, San Diego, CA) and phorbol-12-myristate-13-acetate (PMA) plus ionomycin (Sigma-Aldrich) or bacteria. Clinical strains of *E. coli* O18, *S. aureus* AW7, *K. pneumoniae*, *S. pneumoniae,* and *L. monocytogenes* 10403s were grown in brain heart infusion broth (BD Biosciences, Erembodegem, Belgium), washed in 0.9% NaCl and adjusted at 10^9-10^{10} CFU/ml (26–29). Bacteria were heat-inactivated for 2 h at 56°C for *in vitro* use.

Flow Cytometry Analyses

Single cell suspensions from thymus and spleen were enumerated and incubated with 2.4G2 monoclonal antibody (mAb) (30). Cells were stained using mAbs listed in **Table S1**. Data were acquired using a LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo Version 10.2 software (FlowJo LLC, Ashland OR) (31).

Western Blot Analyses

Protein extracts were submitted to PAGE and transferred onto nitrocellulose membranes (32–34). Membranes were incubated with antibodies directed against SIRT5 (8782, 1:1,000, Cell Signaling Technology, Danvers, MA) or β-actin (4967S, 1:1,000, Cell Signaling Technology) and then with a secondary HRP-conjugated antibody (31460, 1:10,000, Thermo Fisher, Waltham, MA) (35). Blots were imaged with the ECL Western blotting system (GE Healthcare, Little Chalfont, UK). Images were recorded using a Fusion Fx system (Viber Lourmat, Collégien, France) (36).

Metabolic Activity Measurements

The oxygen consumption rate (OCR, in pmole O_2/minute) and the extracellular acidification rate (ECAR, in mpH/minute) were analyzed using a 96-well format Seahorse XFe® system, the Seahorse XF Cell Mito Stress Test Kit and the Seahorse XF Glycolysis Stress Test Kit (Agilent Technologies, Santa Clara, CA). Four × 10^4 BMDMs were plated in 96-well

plates in complete IMDM medium. The next day, cells were rested one hour in Seahorse medium with or without glucose. Mitochondrial respiration was assessed by measuring OCR following the addition of 1 μM oligomycin (OM), 1 μM FCCP and 2 μM antimycinA/1 μM rotenone (AA/Rot). Glycolytic function was assessed by measuring ECAR following the addition of 10 mM glucose, 1 μM oligomycin and 50 mM 2-deoxy-glucose (2-DG).

RNA Analyses

Total RNA was isolated, reverse transcribed (RNeasy and QuantiTect reverse transcription kits, Qiagen, Hilden, Germany) and used in real-time PCR using Fast SYBR® Green Master Mix and a QuantStudio™ 12K Flex system (Life Technologies, Carlsbad, CA) as reported (24, 37). Samples were tested in triplicate. Gene specific expression was normalized to actin expression. Primers are listed in **Table S2**. Sirt5 mRNA expression levels in organs were extracted from the BioGPS resource (http://biogps.org).

Proliferation and Cytokine Measurements

The proliferation of splenocytes cultured for 48 h in 96-well plates was quantified by measuring ^{3}H-thymidine incorporation over 18 h (38). Cytokine concentrations were quantified using DuoSet ELISA kits (R&D Systems, Abingdon, UK) (39). The viability, assessed using the MTT assay (40), of resting and stimulated SIRT5$^{+/+}$ and SIRT5$^{-/-}$ BMDMs was not different.

In vivo Models

Mice were challenged intraperitoneally (i.p.) with 20 mg/kg LPS or 4×10^2 or 3×10^4 CFU *E. coli* O18, intranasally (i.n.) with 30 CFU *K. pneumoniae* or 1×10^6 CFU *S. pneumoniae* and intravenously (i.v.) with 1.2×10^3 or 9×10^4 CFU *L. monocytogenes* or 3×10^4 or 2×10^7 CFU *S. aureus*. Blood was collected to quantify cytokines and bacteria (24). At least once daily, body weight, severity score (graded from one to five) and survival were registered (41). Animals were euthanized when they met a severity score of four. Two operators performed animal follow-up.

Statistical Analyses

Comparisons between different groups were performed by analysis of variance followed by two-tailed unpaired Student's *t*-test. *In vivo* bacteria and cytokine data were analyzed using the Mann-Whitney test. Survival curves were built using the Kaplan-Meier method and differences were analyzed by the log-rank sum test. All analyses were performed using PRISM (GraphPad Software). *P* values were two-sided, and $P < 0.05$ was considered to indicate statistical significance.

RESULTS

SIRT5 Deficiency Has No Major Impact on the Development of Immune Cells

SIRT5 mRNA was ubiquitously expressed in organs, including immune organs (bone marrow, lymph nodes, spleen, and thymus). Highest levels were observed in brown adipose tissue, heart and liver (**Figure 1A**). Germline *Sirt5* knockout mice [described in (23)] were used to address whether SIRT5-deficiency affected immune cell development by analyzing thymus and spleen cell contents. The absolute numbers of cells in the thymus and the spleen of SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice were similar (**Figure 1B**). When compared to SIRT5$^{+/+}$ mice, SIRT5$^{-/-}$ mice expressed normal proportions and absolute numbers of CD4/CD8 double negative (DN), double positive (DP), and single positive (SP) thymocytes (**Figure 1C**), such as of DN1-DN4 subpopulations (CD25^{+}CD44^{+}, CD25^{-}CD44^{+}, CD25^{+}CD44^{-}, and CD25^{-}CD44^{-}). SIRT5$^{-/-}$ mice expressed normal proportions and absolute numbers of splenic CD3^{+} T cells (SP, DN as well as CD4^{+} and CD8^{+} CD44^{low}CD62L^{high} naïve and CD44^{high}CD62L^{low} memory T cells), B cells (non-IgD^{+}/CD23^{+} immature B cells and IgD^{+}CD23^{+} mature B cells) and DCs (B220^{-}CD11c^{+} conventional DCs and B220^{+}CD11c^{+} plasmacytoid DCs) (**Figure 1D**). Overall, SIRT5 deficiency had no impact on the development of the main T-cell, B-cell and DC populations.

SIRT5 Deficiency Does Not Affect the Response of Macrophages and Splenocytes to Immune Stimulation

Macrophages are highly proficient at sensing microbial products through TLRs and play a central role in anti-microbial host defenses by orchestrating innate and adaptive immune responses through the production of cytokines. Bone marrow derived macrophages (BMDMs) expressed SIRT5 protein, albeit less than liver. SIRT5 was undetectable in SIRT5$^{-/-}$ BMDMs (**Figure 2A**). SIRT5$^{-/-}$ BMDMs showed a slight increased oxygen consumption rate (OCR, readout of mitochondrial activity, **Figure 2B**) and decreased acidification rate (ECAR, readout of glycolytic activity, **Figure 2C**).

BMDMs were exposed to LPS, Pam_3CSK_4, CpG [i.e., TLR4, TLR1/TLR2, and TLR9 agonists (1, 2)] and heat killed bacteria before measuring cytokine response. SIRT5$^{+/+}$ and SIRT5$^{-/-}$ BMDMs up-regulated likewise Tnf and Il6 mRNAs (**Figures 2D,E**). Moreover, SIRT5$^{+/+}$ and SIRT5$^{-/-}$ BMDMs secreted similar levels of TNF and IL-6 in response to LPS, Pam_3CSK_4, CpG, *E. coli* and *S. aureus* (**Figures 2F,G**). In accordance with these results, SIRT5$^{+/+}$ and SIRT5$^{-/-}$ BMDMs expressed similar levels of Tlr1, Tlr2, Tlr4, and Tlr9 mRNA at baseline and upon exposure to LPS, Pam_3CSK_4, and CpG (**Figure 3**, upper panels). Finally, SIRT5$^{+/+}$ and SIRT5$^{-/-}$ BMDMs cultured with medium, LPS, Pam_3CSK_4, and CpG expressed comparable gene expression levels of CXCL1 (KC/GROα) and CXCL10 (IP10) chemokines, CD36 scavenger receptor and CD40 costimulatory molecule (**Figure 3**, lower panels).

To address further whether SIRT5 deficiency affected the response of immune cells, the proliferation of SIRT5$^{+/+}$ and SIRT5$^{-/-}$ splenocytes exposed to LPS, CpG, Pam_3CSK_4, TSST-1, and anti-CD3/CD28 was assessed by ^{3}H-thymidine incorporation, (**Figure 4A**), while the production of IL-2 by SIRT5$^{+/+}$ and SIRT5$^{-/-}$ splenocytes exposed to TSST-1, anti-CD3/CD28 and PMA plus ionomycin was measured by ELISA

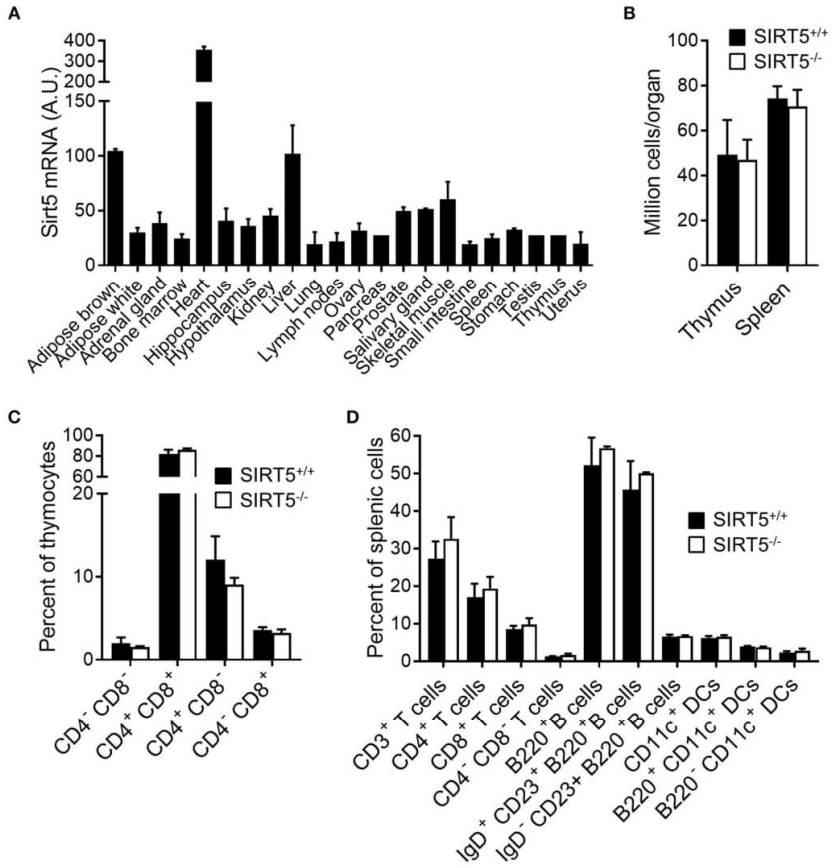

FIGURE 1 | SIRT5 deficiency does not affect immune cell development in thymus and spleen. **(A)** Sirt5 mRNA expression levels in a panel of mouse organs (extracted from the BioGPS resource). **(B)** Total cell numbers per thymus and spleen of SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice. **(C,D)** Percentages of T cell subpopulations in the thymus **(C)** and of T cells, B cells and DCs subpopulations in the spleen **(D)** of SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice. Data are means ± SD from one experiment performed with three mice and are representative of two experiments. $P > 0.5$ for all conditions **(B–D)**.

(**Figure 4B**). Neither proliferation nor IL-2 production was modified by SIRT5 deficiency. Altogether, the results argued against an important role of SIRT5 in controlling cytokine production by macrophages exposed to TLR ligands and the response of splenocytes to microbial and immune stimuli.

SIRT5 Deficiency Does not Affect Endotoxemia and Does Not Worsen Bacterial Infections

SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice were subjected to endotoxemia induced by an i.p. challenge with 20 mg/kg LPS. Consistent with the results observed *in vitro*, TNF, and IL-6 concentrations in blood collected 1 h (TNF) and 6 h (IL-6) after LPS challenge were similar in SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice ($P = 0.2$ and 0.4; **Figure 5A**). Accordingly, the mortality rates of SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice were not significantly different (75% vs. 90%; $P = 0.4$; **Figure 5B**). To mimic clinical situations, we then explored the impact of SIRT5-deficiency on host defenses in models of infections induced by challenging mice with *K. pneumoniae* and *S. pneumoniae* i.n., *E. coli* i.p. and *L. monocytogenes* and *S. aureus* i.v.

In a non-severe model of *K. pneumoniae*-induced pneumonia, body weight loss was similar in the SIRT5$^{+/+}$ and SIRT5$^{-/-}$ groups (**Figure 6A**, left panel). Moreover, mouse survival was not impaired by SIRT5 deficiency (SIRT5$^{+/+}$ vs. SIRT5$^{-/-}$: 77% vs. 100% survival; $P = 0.1$, **Figure 6A**, right panel). SIRT5 deficiency did not worsen the outcome of mice in a quickly lethal model of *S. pneumoniae*-induced pneumonia (**Figure 6B**). Two days post-infection, the proportions of bacteremic mice (6/10 vs. 5/10) and blood *S. pneumoniae* loads (SIRT5$^{+/+}$ vs. SIRT5$^{-/-}$: $4.1 \pm 2.2 \times 10^3$ CFU/ml vs. $4.8 \pm 3.6 \times 10^3$ CFU/ml; mean ± SEM; $P = 0.9$)

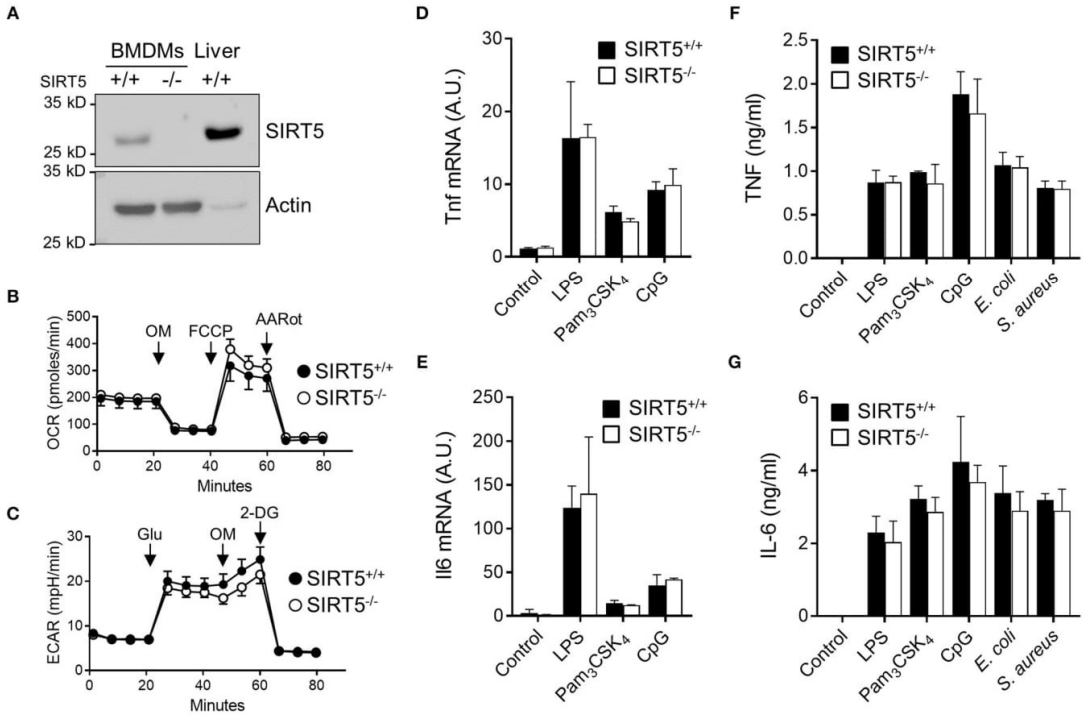

FIGURE 2 | SIRT5 deficiency does not affect TNF and IL-6 production by macrophages exposed to microbial stimuli. **(A)** SIRT5 protein expression in SIRT5$^{+/+}$ and SIRT5$^{-/-}$ BMDMs and SIRT5$^{+/+}$ liver assessed by Western blotting. Full-length blots are presented in **Figure S1**. **(B,C)** Oxygen consumption rate (OCR, **B**) and extracellular acidification rate (ECAR, **C**) of SIRT5$^{+/+}$ and SIRT5$^{-/-}$ BMDMs. Data are means ± SD from one experiment performed with two mice. **(D–G)** SIRT5$^{+/+}$ and SIRT5$^{-/-}$ BMDMs were exposed to LPS (10 ng/ml), Pam_3CSK_4 (10 ng/ml), CpG (2 μg/ml), *E. coli*, and *S. aureus* (10^6 CFU/ml). Gene expression levels were quantified by RT-PCR, normalized to actin levels, and expressed relative to SIRT5$^{+/+}$ control set at one [**(D)**: 1 h, **(E)**: 4 h]. The concentrations of TNF (4 h) and IL-6 (18 h) in cell culture supernatants were quantified by ELISA **(F,G)**. Data are means ± SD of triplicate samples from one experiment performed with three mice and are representative of two-three experiments **(A, D–G)**. $P > 0.05$ for all conditions.

were equivalent in the two groups. Accordingly, mortality rate was not significantly different (100 vs. 89% mortality; $P = 0.08$).

In a model of acute peritonitis induced by *E. coli*, bacterial dissemination into the blood (SIRT5$^{+/+}$ vs. SIRT5$^{-/-}$: 2.3 ± 1.3 × 10^8 CFU/ml vs. 3.8 ± 2.0 × 10^3 CFU/ml; mean ± SEM; $P = 0.4$) and mortality rate (73 vs. 91%, $P = 0.6$) were comparable using SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice (**Figure 6C**, plain lines). Upon challenge with a low inoculum of *E. coli*, all SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice survived (**Figure 6C**, dashed lines). During acute listeriosis, SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice displayed similar bacteremia (SIRT5$^{+/+}$ vs. SIRT5$^{-/-}$: 4.1 ± 2.2 × 10^3 CFU/ml vs. 4.8 ± 3.6 × 10^3 CFU/ml; mean ± SEM, $P = 0.8$) and survival rate ($P = 0.9$) (**Figure 7A**, plain lines). In a model of sublethal listeriosis, the mortality rate of SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice was not statistically different (SIRT5$^{+/+}$ vs. SIRT5$^{-/-}$: 100% vs. 75% survival; $P = 0.14$, **Figure 7A**, dashed lines). In a model of severe, systemic staphylococcal infection (**Figure 7B**, plain lines), there was no difference in severity score, body weight loss and survival (0 vs. 0%; $P = 0.7$) between SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice. Analogous to what observed upon challenge with low inocula of *K. pneumoniae*, *E. coli* and *L. monocytogenes* (**Figures 6A,C**, **7B**), SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice were similarly resistant to sublethal staphylococcal infection (**Figure 7B**, dashed lines) suggesting that SIRT5-deficient mice are not particularly susceptible to bacterial infections.

DISCUSSION

This is the first report of the impact of SIRT5 on antimicrobial host defenses. *In vitro* studies using macrophages and splenocytes and preclinical models of endotoxemia and Gram-positive and Gram-negative bacteria infections suggest that SIRT5 deficiency has no major impact on antibacterial defenses. These observations are particularly relevant in light of the development of pharmacological inhibitors of SIRT5 for clinical applications (42).

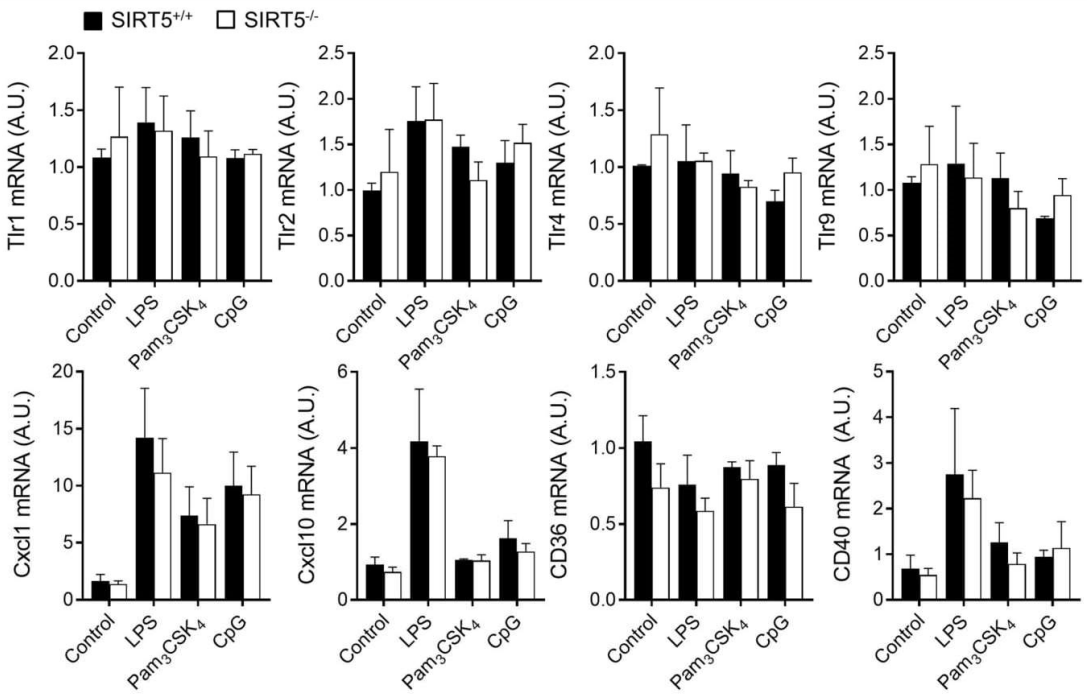

FIGURE 3 | SIRT5 deficiency does not affect macrophage response to microbial stimulation. $SIRT5^{+/+}$ and $SIRT5^{-/-}$ BMDMs were exposed for 1 h to LPS (10 ng/ml), Pam_3CSK_4 (10 ng/ml) and CpG (2 μg/ml). Gene expression levels were quantified by RT-PCR, normalized to actin levels, and expressed relative to $SIRT5^{+/+}$ control set at one. Data are means ± SD of triplicate samples from one experiment performed with three mice. $P > 0.05$ for all conditions.

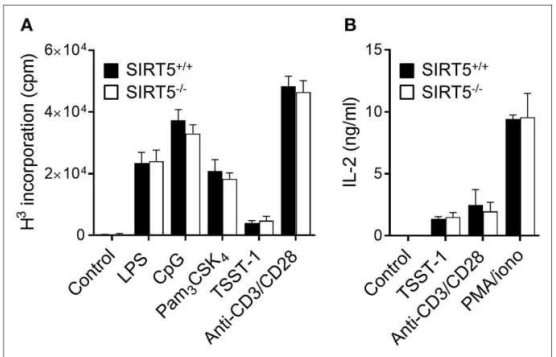

FIGURE 4 | SIRT5 deficiency does not affect proliferation of and IL-2 production by splenocytes. $SIRT5^{+/+}$ and $SIRT5^{-/-}$ splenocytes were incubated for 48 h with LPS (5 μg/ml), CpG (2 μg/ml), Pam_3CSK_4 (5 μg/ml), TSST-1 (2 μg/ml), anti-CD3/CD28 antibodies (1 μg/ml) and PMA + ionomycin (PMA/iono, 10 ng/ml/100 ng/ml). **(A)** Proliferation was measured by ^{3}H-thymidine incorporation. **(B)** IL-2 concentrations in cell culture supernatants were quantified by ELISA. Data are means ± SD of one experiment performed with three mice and are representative of two experiments. $P > 0.05$ for all conditions.

SIRT5 deficiency neither affects the development of the major T cells, B cells and DCs subsets in thymus and spleen nor the proliferation and the production of IL-2 by splenocytes. Similar observations were obtained using $SIRT2^{-/-}$ and $SIRT3^{-/-}$ mice (43, 44). In SIRT1-deficient mice, $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ thymic subpopulations were normal but highly sensitive to DNA-damaging ionizing radiation (45). Circulating T cell, B cell and monocyte counts were normal in 5 months old $SIRT7^{-/-}$ mice that developed inflammatory cardiomyopathy (46). SIRT6-deficent mice developed, after 2 weeks of life, a progeroid syndrome associated with decreased lymphocyte counts in thymus and spleen. However, lymphocyte flaw was not cell-intrinsic but linked to systemic defects (47). Overall sirtuins do not seem to affect the development of adaptive immune cells. Yet, SIRT1 was reported to influence T-helper (Th) 2, Th9, Th17 and T-regulatory (Treg) responses and SIRT3 to sustain the suppressive function of Tregs (48–52). Thus, it would be interesting to define whether SIRT5 shapes T cell responses.

SIRT5 influences diverse metabolic pathways in cardiac and hepatic cells, including urea cycle, amino acid metabolism, the TCA cycle, FAO, glycolysis and oxidative stress response (7–16). $SIRT5^{-/-}$ BMDMs showed a modest increased mitochondrial activity and decreased glycolytic

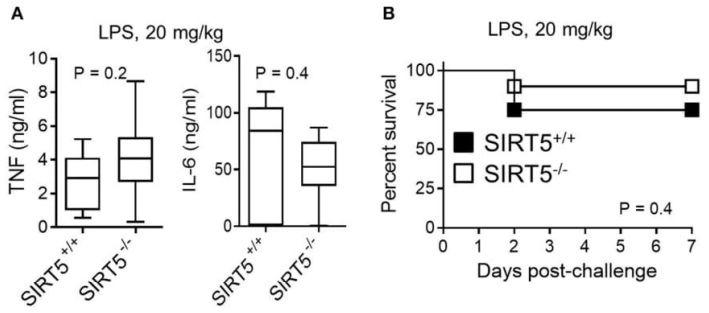

FIGURE 5 | SIRT5 deficiency does not affect endotoxemia. SIRT5$^{+/+}$ and SIRT5$^{-/-}$ female mice were injected i.p. with 20 mg/kg LPS ($n = 8$ and 10 mice per group). **(A)** TNF and IL-6 concentrations in blood collected 1 h (TNF) and 6 h (IL-6) after LPS challenge. $P = 0.2$ and 0.4. **(B)** Survival of mice. $P = 0.4$.

activity, suggesting that SIRT5 may be less influential in macrophages than in heart and liver (8, 15). Correlatively, SIRT5 mRNA and protein were expressed at much lower levels in immune organs [our data and (9)] and in primary macrophages than in liver and heart (5 and 10-fold less SIRT5 mRNA in BMDMs than in liver and heart, respectively).

SIRT5 deficiency had no major impact on LPS-induced cytokine production by macrophages and circulating TNF and IL-6 levels were similar in SIRT5$^{+/+}$ and SIRT5$^{-/-}$ endotoxemic mice. SIRT5-deficient mice under high fat diet, a condition inducing inflammation and oxidative stress, showed normal metabolic parameters and signs of inflammation attested by *Tnf*, *Cd68* (a monocyte/macrophage marker) and *Cd36* (a scavenger receptor) gene expression in the liver (23). Two recent studies analyzed the impact of SIRT5 deficiency on mouse macrophage response to LPS, leading to opposite conclusions. SIRT5$^{-/-}$ peritoneal macrophages produced reduced levels of TNF, IL-6, and MCP-1 (monocyte chemoattractant protein-1/CCL2). SIRT5 competed with SIRT2 to interact with NF-κB p65. Since SIRT2 deacetylates p65 to inhibit its transduction activity, SIRT5 indirectly promoted p65 acetylation and activity (53). In sharp contrast, LPS-stimulated SIRT5$^{-/-}$ BMDMs expressed increased levels of Tnf, Il1b, and Il6 mRNA but not Il10 mRNA. SIRT5 desuccinylated PKM2 (pyruvate kinase M2), promoting tetramer-to-dimer transition and inhibiting pyruvate kinase activity of PKM2. In that study, SIRT5 deficiency protected from DSS-induced colitis. The inconsistency of the impact of SIRT5 on inflammatory responses echoes those reported for SIRT1, SIRT2, SIRT3, and SIRT6 *in vitro* and *in vivo* [discussed in (43, 44)]. Differences in experimental conditions (BMDMs vs. peritoneal macrophages, germline vs. cell-type specific gene knockout, use of si/shRNA and pharmacological modulators of sirtuins) and subtle variations in qualitative and quantitative caloric input and NAD^+ availability may explain these differences. Additionally, the length of stimulation and the doses of stimulus [10 ng/ml of ultra-pure LPS here vs. 100 ng/ml of crude LPS in (17) and (53)] may have affected the results. It should also be stressed that SIRT5 deficiency was obtained by disruption of exon 4 in the case of the mice used in this study (23), while exons 2–5 were deleted in the SIRT5 knockout mice available from the Jackson Laboratory used in other studies (17, 53). Nonetheless, even in these studies, the background of the animals may have differed substantially considering that commercial knockout mice are of 85% 129 and 15% C57BL/6 backgrounds and that mice were backcrossed 10 times on a BL/6J background in one study (17) while SIRT5$^{+/+}$ and SIRT5$^{-/-}$ littermates were derived from the SIRT5$^{+/-}$ heterozygote mice in the other study (53). Of note, all broad screening proteomic analyses identified metabolic pathways as the most targeted pathways by SIRT5, while pathways commonly associated with immune/inflammatory responses (such as NF-kB, interferon-response, cytokine, cell migration and inflammation pathways) were not evidenced (8, 11, 12, 15, 16).

Endotoxemia reflects pathological situations such as fulminant meningococcemia characterized by high blood loads of endotoxin, but does not reproduce the complex host-pathogen interactions generally taking place during bacterial infections. Therefore, we sought to define the impact of SIRT5 in preclinical models of infections mimicking common clinical situations. SIRT5 deficiency did not sensitize mice to severe *S. pneumoniae* pneumonia, rapidly lethal *E. coli* peritonitis, listeriosis and staphylococcal infection. In the most stringent models, SIRT5 deficiency did not protect from lethal infection, as foreseen if SIRT5 would amplify cytokine response. SIRT5 deficiency also did not render mice particularly susceptible to bacterial infections as suggested by the results obtained using models of sub-lethal/mild infection with *K. pneumoniae*, *E. coli*, *L. monocytogenes*, and *S. aureus*. Considering the diversity of the agents (Gram-positive and Gram-negative and intracellular and extracellular bacteria) and of the routes of infection tested (i.n., i.p. and i.v.), these results so far support the assumption that SIRT5 has no dramatic influence on host defenses against bacterial infections and the clinical development of SIRT5 inhibitors for

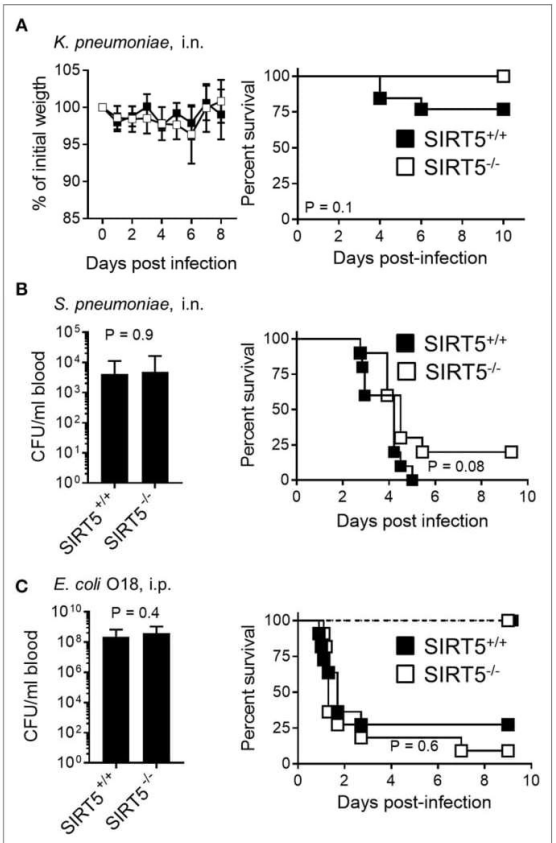

FIGURE 6 | SIRT5 deficiency does not worsen *Klebsiella pneumoniae* and *Streptococcus pneumoniae* pneumonia and *E. coli* peritonitis. SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice were injected i.n. with 30 CFU *K. pneumoniae* (**A**, $n = 11$ females + 2 males and 7 females + 3 males) or 1.6×10^6 CFU *S. pneumoniae* (**B**, $n = 10$ males per genotype) and i.p. with 4×10^2 CFU *E. coli* [**(C)**, $n = 7$ males per genotype, dashed lines] or 3×10^4 CFU *E. coli* [**C**, $n = 11$ females per genotype, plain lines]. Body weight is expressed in percentage of initial weight (**A**, left panel). Blood was collected 18 h post-infection to quantify bacteria [**(B,C)**, left panels; **(C)**: following infection with 3×10^4 CFU *E. coli*. $P = 0.9$ and 0.4]. [**(A–C)**, right panels] Survival: $P > 0.05$ for all models.

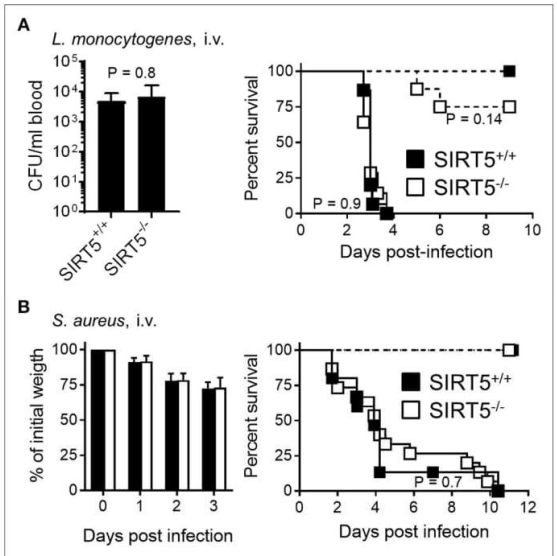

FIGURE 7 | SIRT5 deficiency does not worsen listeriosis and staphylococcal infection. SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice were injected i.v. with 1.2×10^3 CFU *L. monocytogenes* [**(A)**, $n = 5$ females + 3 males per genotype, dashed lines], 9×10^4 CFU *L. monocytogenes* [**A**, $n = 15$ and 14 females, plain lines], 3×10^4 CFU *S. aureus* (**B**, $n = 6$ females per genotype, dashed lines) or 2×10^7 CFU *S. aureus* (**B**, $n = 9$ females + 6 males per genotype, plain lines, 2 experiments). Blood was collected 48 h post-infection with 9×10^4 CFU *L. monocytogenes* to quantify bacteria (**A**, left panel, $P = 0.8$). Body weight following infection with $2–10 \times 10^7$ CFU *S. aureus* is expressed in percentage of initial weight (**B**, left panel). Survival: $P = 0.14$ when comparing SIRT5$^{+/+}$ and SIRT5$^{-/-}$ mice challenged with 1.2×10^3 CFU *L. monocytogenes* and $P > 0.5$ for all other models.

oncologic purposes (18, 22). This contrasts with inhibitors of HDAC1-11 which impaired innate immune defenses against infections in mouse models and have been associated with episodes of severe infection when infused into cancer patients (37, 54–58). Further work will be required to test the efficacy of potential SIRT5 inhibitors (19–21) in models of cancer (18, 22) and of infections and sepsis, then to define whether these inhibitors may predispose to infections in the setting of comorbidities, e.g., in elderly patients and patients with chronic inflammatory disorders like for example colitis and diabetes mellitus.

Overall, SIRT5 does not worsen host defenses to bacterial infections under the conditions tested here. Since sirtuins are linked to metabolism, age-associated dysfunctions and lifespan, it would be of interest to investigate the role of SIRT5 under metabolic stress conditions and in older mice. To conclude, our results support the development of SIRT5 inhibitors for clinical purposes, as they suggest that these drugs would not increase patients' susceptibility to infections.

AUTHOR CONTRIBUTIONS

TH, EC, CT, AP, and DLR performed *in vitro* experiments. JH participated to flow cytometry analyses. TH, EC, CT, and DLR performed *in vivo* experiments. TR conceived the project, designed the experiments and wrote the paper. All the authors revised the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02675/full#supplementary-material

Figure S1 | Full-length Western blots of SIRT5 and actin expression in protein extracts obtained from SIRT5$^{+/+}$ and SIRT5$^{-/-}$ BMDMs and SIRT5$^{+/+}$ liver.

Table S1 | Antibodies used for flow cytometry analyses.

Table S2 | Oligonucleotides used in RT-PCR analyses.

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3.5 Dual deletion of the sirtuins SIRT2 and SIRT3 impacts on metabolism and inflammatory responses of macrophages and protects from endotoxemia

Tytti Heinonen, Eleonora Ciarlo, Ersilia Rigoni, Jean Regina, Didier Le Roy, Thierry Roger

Infectious Diseases Service, Department of Medicine, Lausanne University Hospital, Epalinges, Switzerland

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Summary

SIRT2 and SIRT3 are respectively found in the nucleus and cytoplasm and in the mitochondria, where they regulate diverse biologic processes. SIRT2 and SIRT3 are linked to metabolic, oncologic and chronic inflammatory diseases. However, the deficiency in either sirtuin had little effect on the innate immune responses and on the survival of mice in preclinical infection models. Since SIRT2 and SIRT3 share targets, we hypothesized that the two sirtuins could compensate each other.

SIRT2 and SIRT3 deficient mice were crossed to obtained SIRT2/3 double deficient mice. These mice had minor alterations of thymic, splenic, peritoneal and bone marrow immune subpopulations. Mainly, the peritoneal cavity of SIRT2/3 double deficient mice contained less NK cells and more anti-inflammatory B-1 cells. SIRT2/3 double deficient macrophages showed reduced glycolysis and increased inflammatory cytokine production. In line, SIRT2/3 double deficient mice were protected against endotoxemia.

Our results suggest that sirtuins may compensate each other or act in concert during innate immune responses.

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Dual Deletion of the Sirtuins SIRT2 and SIRT3 Impacts on Metabolism and Inflammatory Responses of Macrophages and Protects From Endotoxemia

Tytti Heinonen, Eleonora Ciarlo, Ersilia Rigoni, Jean Regina, Didier Le Roy and Thierry Roger[*†]

Infectious Diseases Service, Department of Medicine, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland

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Edited by:
Liwu Li,
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***Correspondence:**
Thierry Roger
thierry.roger@chuv.ch

†ORCID:
Thierry Roger
orcid.org/0000-0002-9358-0109

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Sirtuin 2 (SIRT2) and SIRT3 are cytoplasmic and mitochondrial NAD-dependent deacetylases. SIRT2 and SIRT3 target proteins involved in metabolic, proliferation and inflammation pathways and have been implicated in the pathogenesis of neurodegenerative, metabolic and oncologic disorders. Both pro- and anti-inflammatory effects have been attributed to SIRT2 and SIRT3, and single deficiency in SIRT2 or SIRT3 had minor or no impact on antimicrobial innate immune responses. Here, we generated a SIRT2/3 double deficient mouse line to study the interactions between SIRT2 and SIRT3. SIRT2/3$^{-/-}$ mice developed normally and showed subtle alterations of immune cell populations in the bone marrow, thymus, spleen, blood and peritoneal cavity that contained notably more anti-inflammatory B-1a cells and less NK cells. *In vitro*, SIRT2/3$^{-/-}$ macrophages favored fatty acid oxidation (FAO) over glycolysis and produced increased levels of both proinflammatory and anti-inflammatory cytokines. In line with metabolic adaptation and increased numbers of peritoneal B-1a cells, SIRT2/3$^{-/-}$ mice were robustly protected from endotoxemia. Yet, SIRT2/3 double deficiency did not modify endotoxin tolerance. Overall, these data suggest that sirtuins can act in concert or compensate each other for certain immune functions, a parameter to be considered for drug development. Moreover, inhibitors targeting multiple sirtuins developed for clinical purposes may be useful to treat inflammatory diseases.

Keywords: sirtuins, innate immunity, macrophage, sepsis, cytokine, inflammation, metabolism, endotoxemia

INTRODUCTION

Sentinel immune cells like monocytes/macrophages, dendritic cells (DCs) and polymorphonuclear neutrophils (PMNs) sense microbial- and danger-associated signals through pattern recognition receptors (PRRs) expressed at cell surface, in endosomes and in cytoplasm. The main families of PRRs are Toll-like receptors (TLRs), C-type lectin receptors, NOD-like receptors, RIG-I-like receptors and cytosolic DNA sensors (1, 2). Triggering PRRs through microbial and endogenous

agonists activates mitogen-activated protein kinase (MAPK), NF-κB and interferon-related factor signal transduction pathways. Signaling leads to the production of effector molecules, such as cytokines, critical to activate innate and adaptive immunity. Tight regulation of inflammation and innate immune responses is vital for controlling microbial invasion while ensuring prompt tissue repair and return to homeostasis. Hence, innovative immunomodulatory therapies have been proposed to fight severe infections and sepsis (3–8).

Mammals express seven sirtuins (SIRT1-7). Sirtuins are NAD^+-dependent enzymes, originally described as histone deacetylases (HDACs). Each sirtuin potentially targets thousands of non-histone proteins (9). SIRT1, SIRT6, and SIRT7 localize mainly in the nucleus, SIRT2 in the cytoplasm, and SIRT3-5 in the mitochondria. Shuttling between organelles have been observed for several sirtuins, for example SIRT2 and SIRT3 into the nucleus and SIRT5 into the cytoplasm (10). Besides a deacetylase activity described for all but SIRT4, sirtuins function as decrotonylase (SIRT1-3), demyristylase (SIRT2), ADP-ribosyltransferase (SIRT4, SIRT6), lipoamidase (SIRT4), demalonylase, deglutarylase, and desuccinylase (SIRT5), and deacylase (SIRT6) (11).

SIRT2 is the most expressed sirtuin in the brain and myeloid cells (12, 13). SIRT2 regulates cellular stability and division by targeting tubulin in the cytoplasm (14) and acting as a mitotic checkpoint in the nucleus during the G2/M phase transition (15). SIRT2 is involved in myelogenesis and other brain functions and is a promising target for treating neurodegenerative conditions among which Parkinson's disease and Huntington's disease (16). SIRT2 is a tumor suppressor gene but has also been associated with tumorigenesis (17). SIRT2 is likely linked to disease progression through cell metabolism regulation. SIRT2 inhibits glycolysis and adipogenesis and promotes lipolysis, gluconeogenesis and the pentose phosphate pathway (PPP), possibly in a cell and disease dependent manner (13, 18–22). Accordingly, SIRT2 plays a role in obesity, type 2 diabetes and other metabolic disorders (18). The picture is not clear regarding inflammatory processes. SIRT2 deficiency has been reported to stimulate, inhibit and have no effect on the activation NF-κB p65 and MAPKs, the expression of cytokines and the development of inflammatory and autoimmune diseases (13, 23–32).

SIRT3 is expressed ubiquitously and its expression increases upon caloric restriction and other stress conditions. SIRT3 is the main mitochondrial deacetylase and a major regulator of cell metabolism. SIRT3 promotes tricarboxylic acid cycle and electron transport chain, ketogenesis, fatty acid oxidation, brown adipose tissue thermogenesis and urea cycle (33–37). SIRT3 inhibits oxidative stress by activating isocitrate dehydrogenase 2 (IDH2) and superoxide dismutase 2 (SOD2) (38–40). Through the regulation of metabolism and redox homeostasis, SIRT3 protects from age-associated metabolic, cardiovascular and neurodegenerative diseases. SIRT3 also counteracts the development of inflammation-related disorders, although some studies suggest that SIRT3 does not impact on inflammatory responses (41–46). Finally, SIRT3 was shown to drive both pro-tumorigenic and tumor-suppressive effects (47).

HDACs may act in concert or compensate each other as suggested by the role played by SIRT1, HDAC5, HDAC6, and HDAC9 in dampening regulatory T cells (Tregs) and by SIRT1, SIRT3, and SIRT6 in regulating metabolic adaptation to inflammation (48–51). As a consequence, deficiency in several sirtuins might amplify or reveal phenotypes undetectable in single knockouts. Indeed, dual deletion of SIRT3 and SIRT5 showed some impact on antimicrobial host defense mechanisms not seen in - SIRT3 and SIRT5 single deficient mice (43, 52, 53). SIRT2 and SIRT3 do not seem to share targets, but they both impact on ROS detoxification at different levels. SIRT2 regulates through FOXO1 the transcription of genes encoding for ROS detoxifying enzymes while SIRT3 regulates the activity of the enzymes (38–40). Thus, the absence of SIRT2 or SIRT3 could be compensated by an increased activity of the other sirtuin. To challenge our assumption, we generated a SIRT2/3 double deficient mouse line. We show that the double deletion of SIRT2 and SIRT3 impacts on some metabolic and immune parameters not observed in single knockouts. Importantly from a translational perspective, SIRT2/3 deficient mice were protected from endotoxemia. This information is valuable considering that inhibitors targeting multiple sirtuins are developed for clinical purposes.

MATERIALS AND METHODS

Ethics Statement

Animal experiments were approved by the Service des Affaires Vétérinaires, Direction Générale de l'Agriculture, de la Viticulture et des Affaires Vétérinaires (DGAV), état de Vaud (Epalinges, Switzerland) under authorizations 876.9 and 877.9 and performed according to Swiss and ARRIVE guidelines.

Mice

$SIRT2^{-/-}$ and $SIRT3^{-/-}$ C57BL/6J mice have been described (54, 55). $SIRT3^{-/-}$ females were crossed with $SIRT2^{-/-}$ males to obtain 53 female and 47 male $SIRT2/3^{+/-}$ mice. $SIRT2/3^{+/-}$ mice were crossed to obtain 312 F2 mice. We identified 7 female and 3 male double knockout mice that were used to establish the $SIRT2/3^{-/-}$ mouse line. For genotyping purposes, DNA was extracted and analyzed by PCR using the Mouse Direct PCR Kit (Bimake, Houston, TX), primers pairs (**Supplementary Information**) and a QuantStudio™ 12K Flex system (Life Technologies, Carlsbad, CA). Mice used in this study were 7–14-week old, housed under specific pathogen-free conditions and exempt of mouse hepatitis virus and murine norovirus.

Cells and Reagents

Bone marrow (BM) cells were cultured for 7 days in RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with 1% penicillin-streptomycin (Invitrogen, Carlsbad CA), 10% heat inactivated fetal bovine serum (FBS; Biochrom GmbH, Berlin, Germany) and 50 U/ml macrophage colony-stimulating factor (M-CSF; ImmunoTools, Friesoythe, Germany) to obtain BM derived macrophages (BMDMs) (56). BMDMs were seeded in 96-well or 24-well plates (2×10^5 or 1×10^6 cells/well) without

M-CSF for stimulation and phagocytosis/killing experiments, respectively. Peritoneal cells were obtained through a peritoneal lavage performed using 5 ml ice-cold RPMI (57). Cells were enumerated and seeded in 96-well plates (2×10^5 cells/well) in RPMI containing 1% penicillin-streptomycin and 10% FBS. BMDMs and peritoneal cells were stimulated with ultrapure LPS from *Salmonella minnesota* (List Biological Laboratories, Campbell, CA), Pam_3CSK_4 (EMC microcollections, Tübingen, Germany), and CpG A 1585 (Microsynth, Balgach, Switzerland).

RNA Analyses

RNA was extracted with the RNeasy kit (Qiagen, Hilden, Germany) and reverse transcribed with the QuantiTect reverse transcription kit (Qiagen). Real-time PCR was performed using primers (**Supplementary Information**) and KAPA SYBR Green Fast (Kapa Biosystems, Wilmington, MA) as described (58). Reactions were run on a QuantStudio™ 12K Flex system (Life Technologies). Gene expression was normalized to actin expression.

Western Blot Analyses

Proteins were extracted, submitted to PAGE and transferred to nitrocellulose membranes as described (59). Membranes were incubated with primary antibodies against β-actin (4967, Cell Signaling, Danvers, MA), NF-κB p65 (8242, Cell Signaling) p44/42 MAPK (ERK1/2; 9102, Cell Signaling), phospho-p44/42 (ERK1/2; 9101, Cell Signaling), p38 MAPK (9102, Cell Signaling), phosho-p38 MAPK (9211, Cell Signaling), SIRT2 (ab67299; Abcam, Cambridge, United Kingdom), SIRT3 (5490; Cell Signaling), α-tubulin (T5168; Sigma-Aldrich, Darmstadt, Germany), and HRP-coupled secondary antibodies (31430 and 31460; Invitrogen). Blots were revealed with the enhanced chemiluminescence Western blotting system (Advansta, San Jose, CA). Images were recorded with the Fusion Fx system (Viber Lourmat, Collégien, France). Full length blots are presented in **Supplementary Figure 1**.

Flow Cytometry

Single cell suspensions were incubated with Fc blocker, stained with antibodies listed in **Supplementary Information** and fixed with 2% paraformaldehyde. Data were acquired with an Attune Nxt flow cytometer (ThermoFisher, Waltham, MA) and analyzed using FlowJo 10.2 (FlowJo LLC, Ashland, OR). Gating strategies are presented in **Supplementary Figure 2** and in Heinonen et al. (52).

Metabolic Activity

Four $\times 10^4$ BMDMs per well were plated in Seahorse XFe96 plates. Glycolytic activity, mitochondrial respiration and mitochondrial flexibility were analyzed using Seahorse Glycolysis Stress and Mito Fuel Flex Test kits (Agilent, Santa Clara, CA) as recommended by the manufacturer. Two $\times 10^4$ BMDMs were plated in 96-well plates and grown in RPMI (Sigma-Aldrich) with 5 mM glucose. Glucose and lactate were measured with the Glucose-Glo and Lactate-Glo kits (J6021 and J5021, Promega, Madison, WI) and luminescence was recorded with a Synergy plate reader (BioTek, Winooski, VT).

Cytokine Measurements

Cytokines and chemokines were measured in cell supernatant and plasma by ELISA (IL-6 and TNF: R&D systems, Minneapolis, MN; IL-10: Mabtech, Nacka Strand, Sweden) or by Luminex (Mouse Custom ProcartaPlex 17-plex: ENA-78/CXCL5, G-CSF, IFNγ, IL-1α, IL-1β, IL-3, IL-6, IL-10, IL-12p40, IL-17A, IL-18, IP-10/CXCL10, KC/CXCL1, MCP-1/CCL2, MIP-1α/CCL3, MIP-2/CXCL2, TNF) (Invitrogen) and recorded with a bioplex 200 (Bio-Rad, Hercules, CA) (60).

Phagocytosis and Killing Assay

E. coli O18 (*E. coli*) was grown in brain heart infusion broth (Oxoid Limited, Hampshire, United Kingdom) and washed in 0.9% NaCl (61). BMDMs were incubated with Fluoresbrite® Yellow Green Microspheres (Polysciences Inc., Warrington, PA, USA) or live *E. coli* at a bead or bacteria-to-cell ratio of 10:1 for 1 h to quantify phagocytosis and for 6 h to quantify killing as described (62).

In vivo Models

SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ male mice (n = 8–10 per group) were challenged intraperitoneally with 10 mg/kg LPS from *E. coli* O111:B4. Blood was collected 0, 1, 3, and 7 days post-challenge to quantify cytokines by Luminex, analyze cell populations by flow cytometry, and perform whole blood assays as described (63). Body weight loss, severity score and survival were monitored at least twice daily. The severity score was graded from 0 to 6 based on animal motility and aspect. Two to three operators performed animal follow-up (64).

Statistical Analyses

Graphics represent data obtained from individual mice (dots), or box with min to max whiskers. Data from different groups were analyzed for normal distribution and homogeneity of variances and compared with the appropriate parametric (two-tailed unpaired Student's t-test) or non-parametric (two-tailed Mann-Whitney test) statistical test. For gravity score, area under the curve was used for analysis. Survival was analyzed using the Kaplan-Meier method. P-values were two-sided, and $P < 0.05$ was considered to indicate statistical significance. $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.005$. Analyses were performed using PRISM 8.0.1 (GraphPad Software, San Diego, CA).

RESULTS

SIRT2/3$^{-/-}$ Mice Develop Without Abnormalities

SIRT2$^{-/-}$ and SIRT3$^{-/-}$ mice were crossed to generate a F2 population (see **Materials and Methods**). Among 312 F2 mice, we identified 10 double knockouts that were used to establish the SIRT2/3$^{-/-}$ mouse line. The truncation of the *Sirt2* and *Sirt3* genes and the absence of SIRT2 and SIRT3 protein expression were confirmed by PCR (**Figure 1A**) and Western blotting (**Figure 1B**). The size of litters (**Figure 1C**) and the female/male sex ratio of litters (**Figure 1D**) were similar in the SIRT2/3$^{+/+}$, SIRT2$^{-/-}$, SIRT3$^{-/-}$, and SIR2/3$^{-/-}$ mouse lines. The weight of adult female and male mice were comparable among mouse

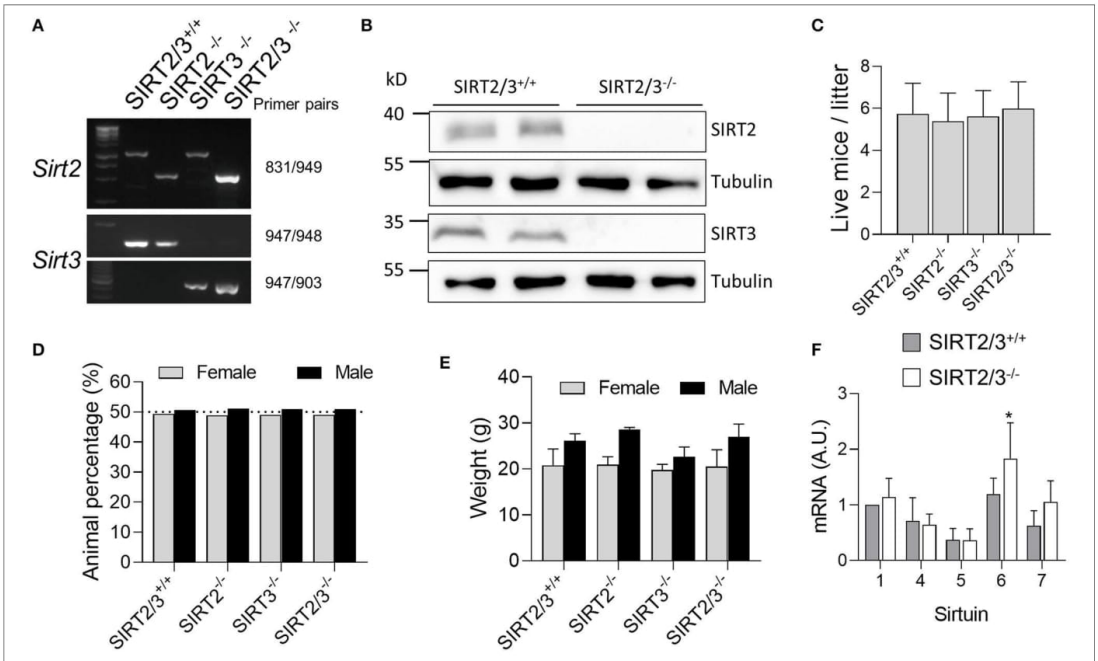

FIGURE 1 | Establishment of a SIRT2/3$^{-/-}$ mouse line. **(A)** Genotyping of SIRT2/3$^{+/+}$, SIRT2$^{-/-}$, SIRT3$^{-/-}$, and SIRT2/3$^{-/-}$ mice. Genomic DNA was amplified by PCR using the primer pairs specified on the right. Reaction mixtures were electrophoresed through agarose gels before imaging. **(B)** Western blot analysis of SIRT2, SIRT3 and tubulin expression in SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ BMDMs (Full blots are presented in **Supplementary Figure 1**). **(C,D)** Number of individuals **(C)** and female/male distribution **(D)** of SIRT2/3$^{+/+}$, SIRT2$^{-/-}$, SIRT3$^{-/-}$ and SIRT2/3$^{-/-}$ litters. **(E)** Weight of SIRT2/3$^{+/+}$, SIRT2$^{-/-}$, SIRT3$^{-/-}$, and SIRT2/3$^{-/-}$ adult female and male mice. **(F)** mRNA expression of SIRT1 and SIRT4-7 in SIRT2/3$^{-/-}$ BMDMs were quantified by RT-qPCR and normalized to actin mRNA. Data are mean ± SD of eight mice analyzed in triplicate. *$P < 0.05$.

lines (**Figure 1E**). Mouse development was normal, and no macroscopic abnormalities were detected upon autopsy. The expression levels of Sirt1, 4, 5, and 7 mRNA were not affected while the expression of Sirt6 mRNA was increased 1.5-fold in SIRT2/3$^{-/-}$ BMDMs (**Figure 1F**).

SIRT2/3$^{-/-}$ Mice Have Minor Alterations of Leukocyte Subpopulations

SIRT2 and SIRT3 are expressed by all major immune cell subpopulations (13, 43) but their role in the development of myeloid cells is unknown. We quantified leukocyte populations in primary and secondary lymphoid organs, blood and peritoneum of SIRT2/3$^{+/+}$, SIRT2$^{-/-}$, SIRT3$^{-/-}$, and SIRT2/3$^{-/-}$ mice. In the bone marrow, the number of CD45^{+} cells was similar in all mouse lines (**Table 1**). The frequency of T cells, conventional DCs (cDCs), plasmacytoid DCs (pDCs) and granulocytes was not affected in knockout mouse lines. The proportion of Ly6C^{low} (alternative) monocytes was slightly reduced (<10%) in SIRT2/3$^{-/-}$ mice, as it was in SIRT2$^{-/-}$ and SIRT3$^{-/-}$ mice. In contrast, the frequency of B cells was 30% higher in SIRT2/3$^{-/-}$ mice. In the thymus, SIRT2/3$^{-/-}$ mice expressed normal proportions and absolute numbers of CD4/CD8 double negative (DN) thymocytes, DN1-DN4 thymocytes, and CD4 and CD8 single positive thymocytes and 4% less CD4^{+} CD8^{+} double positive thymocytes (**Table 2**). The spleen of SIRT2/3$^{+/+}$, SIRT2$^{-/-}$, SIRT3$^{-/-}$, and SIRT2/3$^{-/-}$ mice contained comparable populations of CD4^{+} and CD8^{+} T cells and monocytes (**Table 3**). However, the spleen of SIRT2/3$^{-/-}$ mice contained 19% more naïve CD4^{+} T cells and 11% more B cells, as seen in the bone marrow, but 17% less CD11c^{+} DCs and 39% less Ly6G^{+} granulocytes (**Table 3**). Taken all together, SIRT2/3 deficiency had no dramatic impact on leukocyte development.

Blood leukocytes play a key role in sensing MAMPs/DAMPs and are quickly recruited to inflamed tissues. The number of leukocytes and the frequencies of B cells, T cells, PMNs, monocytes and NK cells were similar in SIRT2/3$^{+/+}$, SIRT2$^{-/-}$, SIRT3$^{-/-}$, and SIRT2/3$^{-/-}$ mice (**Figures 2A,B**), although we could observe an increased frequency of B cells and decreased frequency of PMNs in the blood of SIRT2/3$^{-/-}$ mice. The egress of PMNs from the bone marrow follows a circadian rhythm, a process that is modulated by sirtuins (65). Aged neutrophils express increased levels of CD11b (66). CD11b mean fluorescence intensity (MFI) of SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$

TABLE 1 | Bone marrow cell subsets.

	SIRT2/3$^{+/+}$ ($n = 4$)	SIRT2$^{-/-}$ ($n = 4$)	SIRT3$^{-/-}$ ($n = 4$)	SIRT2/3$^{-/-}$ ($n = 4$)
B220^{+} B cells	13.2 ± 1.9	15.5 ± 3.8	17.2 ± 0.7	17.4 ± 1.3
CD3^{+} T cells	4.6 ± 0.9	3.5 ± 1.1	5.2 ± 1.1	3.6 ± 1.1
CD11c^{+} DCs	3.8 ± 0.3	3.3 ± 0.2	3.8 ± 0.3	3.6 ± 0.2
pCDs	63.6 ± 2.2	64.3 ± 2.6	62.2 ± 0.9	67.4 ± 4.0
cDCs	33.4 ± 2.0	32.8 ± 2.7	34.2 ± 0.9	29.9 ± 3.7
cDC1 (CD11b^{-})	51.0 ± 2.9	49.7 ± 8.2	48.0 ± 4.6	52.7 ± 3.6
cDC2 (CD11b^{+})	45.8 ± 2.9	47.4 ± 8.5	48.0 ± 4.6	44.3 ± 3.4
Ly6G^{+} Ly6C^{-} granulocytes	48.1 ± 3.3	48.2 ± 4.2	44.3 ± 0.9	44.9 ± 1.0
Ly6G^{-} Ly6C^{+} monocytes	15.4 ± 0.8	15.3 ± 2.4	15.0 ± 0.7	15.3 ± 0.7
Ly6C^{low} alternative monocytes	44.9 ± 0.9	40.4 ± 2.0	38.9 ± 2.2	40.7 ± 2.8
Ly6C^{high} classical monocytes	48.0 ± 0.5	51.8 ± 1.7	52.2 ± 1.8	50.1 ± 2.9

Data are mean ± SD of four mice per group and are expressed as the percentage of CD45^{+} cells (B220^{+}, CD3^{+}, CD11c^{+}, Ly6G^{+}Ly6C^{-}, Ly6G^{-}Ly6C^{+}) or the percentage of parental cells. The number of CD45^{+} cells per leg was 14.4 ± 2.5, 14.9 ± 2.3, 12.0 ± 3.1, and 12.6 ± 4.2 × 10^{6} cells for SIRT2/3$^{+/+}$, SIRT2$^{-/-}$, SIRT3$^{-/-}$, and SIRT2/3$^{-/-}$ mice. Gray background: P < 0.05 vs. SIRT2/3$^{+/+}$ mice.

TABLE 2 | Thymic cell subsets.

	SIRT2/3$^{+/+}$ ($n = 4$)	SIRT2$^{-/-}$ ($n = 4$)	SIRT3$^{-/-}$ ($n = 4$)	SIRT2/3$^{-/-}$ ($n = 4$)
CD4^{-} CD8^{-}	3.3 ± 0.2	3.3 ± 0.3	3.9 ± 0.6	4.1 ± 0.4
DN1 (CD25^{-} CD44^{+})	20.2 ± 3.2	21.1 ± 0.6	21.9 ± 6.8	21.5 ± 1.7
DN2 (CD25^{+} CD44^{+})	21.0 ± 2.4	19.8 ± 2.3	20.9 ± 1.7	21.2 ± 2.1
DN3 (CD25^{+} CD44^{-})	35.7 ± 2.5	35.3 ± 1.2	32.6 ± 2.0	32.0 ± 1.1
DN4 (CD25^{-} CD44^{-})	23.1 ± 1.5	23.8 ± 2.7	24.6 ± 3.6	25.3 ± 1.9
CD4^{+} CD8^{+}	84.6 ± 1.2	82.4 ± 0.7	80.9 ± 3.1	81.1 ± 0.2
CD4^{+}	6.8 ± 0.7	8.1 ± 0.4	8.7 ± 1.9	8.2 ± 0.5
CD8^{+}	1.6 ± 0.3	2.3 ± 0.2	2.1 ± 0.7	2.0 ± 0.4

Data are mean ± SD of four mice per group expressed as percentage of total thymocytes (CD4^{-} CD8^{-}, CD4^{+} CD8^{+}, CD4^{+}, CD8^{+}) or the percentage of CD4^{-} CD8^{-} cells (CD25^{-} CD44^{+}, CD25^{+} CD44^{+}, CD25^{+} CD44^{-}, CD25^{-} CD44^{-}). Thymus weights were 71.3 ± 8.6, 107.0 ± 20.9, 90.5 ± 15.6, and 89.5 ± 5.3 mg for SIRT2/3$^{+/+}$, SIRT2$^{-/-}$, SIRT3$^{-/-}$, and SIRT2/3$^{-/-}$ mice. Gray background: P < 0.05 vs. SIRT2/3$^{+/+}$ mice.

TABLE 3 | Splenic cell subsets.

	SIRT2/3$^{+/+}$ ($n = 4$)	SIRT2$^{-/-}$ ($n = 4$)	SIRT3$^{-/-}$ ($n = 4$)	SIRT2/3$^{-/-}$ ($n = 4$)
B220^{+} B cells	47.1 ± 0.8	48.9 ± 1.3	47.1 ± 2.5	52.2 ± 2.2
Mature (CD23^{+} IgD^{+})	70.0 ± 1.8	67.2 ± 1.6	66.8 ± 2.4	67.3 ± 2.4
Immature (non-CD23^{+}IgD^{+})	30.0 ± 1.8	32.8 ± 1.6	33.2 ± 2.4	32.7 ± 2.4
CD3^{+} T cells (%)	35.6 ± 1.6	36.3 ± 1.9	37.9 ± 2.7	34.0 ± 1.6
CD4^{+}	56.0 ± 2.0	58.4 ± 1.6	58.1 ± 0.8	59.8 ± 3.0
Naive	50.0 ± 2.0	61.4 ± 4.1	56.6 ± 5.3	59.3 ± 4.5
Memory	18.4 ± 2.2	13.6 ± 1.3	13.7 ± 1.8	15.2 ± 1.4
CD8^{+}	38.6 ± 1.7	36.6 ± 2.0	37.0 ± 0.7	35.0 ± 3.3
Naive	53.0 ± 3.7	58.7 ± 4.7	55.3 ± 4.4	61.2 ± 5.5
Memory	9.5 ± 1.2	7.9 ± 1.5	9.0 ± 1.6	8.4 ± 2.3
CD4^{-} CD8^{-}	5.0 ± 0.4	4.7 ± 0.5	4.7 ± 0.2	5.0 ± 0.8
CD11c^{+} DCs	4.2 ± 0.3	3.9 ± 0.2	4.2 ± 0.7	3.5 ± 0.3
pDCs	22.1 ± 2.3	23.4 ± 2.5	19.8 ± 1.6	24.3 ± 3.1
cDC	77.6 ± 2.3	76.2 ± 2.5	79.8 ± 1.6	75.2 ± 3.0
cDC1 (CD11b^{-})	41.1 ± 8.9	34.4 ± 1.3	32.3 ± 0.9	35.5 ± 3.0
cDC2 (CD11b^{+})	40.5 ± 5.4	39.9 ± 2.9	45.8 ± 1.3	38.2 ± 2.6
Ly6G^{+} Ly6C^{-} granulocytes	2.8 ± 0.7	2.5 ± 0.9	1.3 ± 0.1	1.7 ± 0.3
Ly6G^{-} Ly6C^{+} monocytes	2.4 ± 0.6	2.8 ± 0.1	3.3 ± 0.2	2.7 ± 0.4
Ly6C^{low} alternative monocytes	41.7 ± 7.1	30.9 ± 9.3	48.0 ± 2.1	35.3 ± 6.5
Ly6C^{high} classical monocytes	21.8 ± 7.9	31.2 ± 4.3	13.8 ± 2.6	21.5 ± 3.9

Data are mean ± SD of four mice per group and expressed as percentage of total cells (B220^{+}, CD3^{+}, CD11c^{+}, Ly6G^{+} Ly6C^{-}, Ly6G^{-} Ly6C^{+}) or percentage of parental cells. Spleen weights were 103.8 ± 10.3, 119.3 ± 20.0, 94.5 ± 7.5, and 108.8 ± 10.0 mg for SIRT2/3$^{+/+}$, SIRT2$^{-/-}$, SIRT3$^{-/-}$, and SIRT2/3$^{-/-}$ mice. Gray background: P < 0.05 vs. SIRT2/3$^{+/+}$ mice.

PMNs was alike (**Figure 2C**), suggesting that SIRT2 and SIRT3 do no impact on PMNs release from the bone marrow. The frequency of Ly6C^{low}, Ly6C^{int}, and Ly6C^{high} monocyte subpopulations were similar in all mouse lines (**Figure 2D**). Hence, the reduced number of Ly6C^{low} monocytes in the bone marrow of knockout mice (**Table 1**) had no perceptible impact on blood monocytes. CD62L, a homing receptor for secondary lymphoid organs, is expressed by NK cells at steady-state (67). CD62L MFI was 1.7- to 2-fold higher in NK cells from SIRT2/3$^{-/-}$ mice (**Figure 2E**).

The peritoneal cavity of mice contains mainly B cells, T cells, macrophages and NK cells (68). Peritoneal macrophages are divided into large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs). LPMs are self-renewing macrophages with homeostatic functions and represent the main macrophage population (90% of all macrophages) expressed at baseline in the peritoneum. SPMs derived from blood inflammatory monocytes quickly exceed LPMs upon infection or inflammation (69). The peritoneal cavity of SIRT2/3$^{-/-}$ mice contained 1.7-fold less NK cells, while the other leukocyte subpopulations were not statistically significantly different (**Figure 2F**). Beside a reduced number of NK cells, the MFI of the activation marker CD43 expressed by NK cells was 1.3-fold lower in SIRT2/3$^{-/-}$ mice (**Figure 2G**). Similar to what was observed in the bone marrow and spleen, there was 1.8-fold more B cells in the peritoneum of SIRT2/3$^{-/-}$ mice (**Figure 2F**). B cells are divided into B1 (B-1a and B-1b) cells producing natural antibodies and B2 cells (70). Interestingly, the peritoneal cavity of SIRT2/3$^{-/-}$ mice contained proportionally more B-1a cells and less B-1b cells than the one of SIRT2/3$^{+/+}$ mice (**Figure 2H**). This resulted in 3.3-fold more B-1a cells, 1.4-fold more B-1b cells and subnormal number of B2 cells in SIRT2/3$^{-/-}$ mice (SIRT2/3$^{+/+}$ vs. SIRT2/3$^{-/-}$: 0.9 ± 0.2 vs. 3.0 ± 0.5 × 10^{5} B-1a cells, 2.1 ± 0.8 vs. 3.0 ± 0.6 × 10^{5} B-1b cells, 0.8 ± 0.4 vs. 1.9 ± 1.2 × 10^{5} B-2 cells, $P = 0.007$, 0.05, and 0.08). To substantiate the relevance of this observation, we quantified IL-10, TNF and IL-6 production by peritoneal cells stimulated *ex vivo* for 24 h with 10 ng/ml LPS (**Figure 2I**). SIRT2/3$^{-/-}$

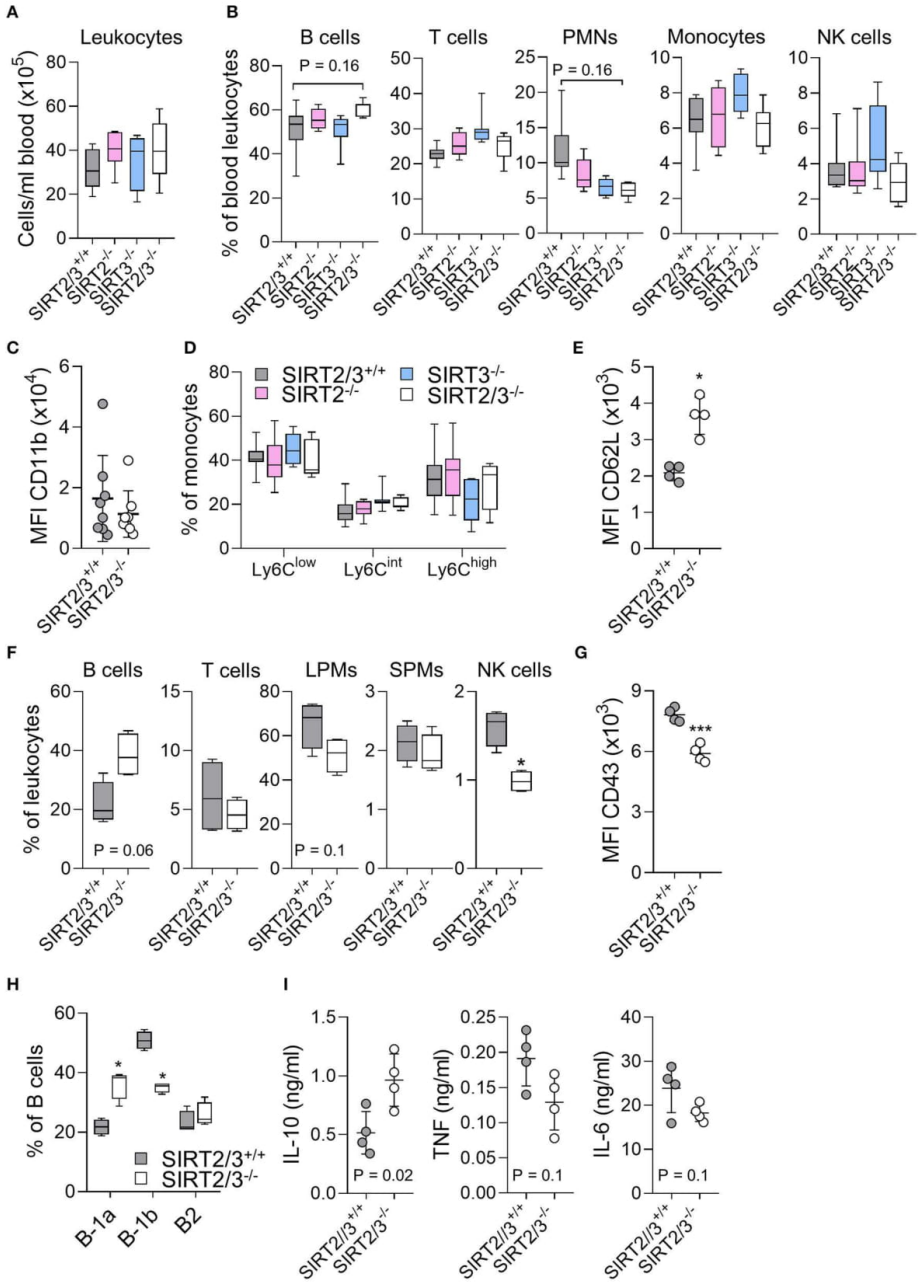

FIGURE 2 | SIRT2/3$^{-/-}$ mice have minor alterations of blood and peritoneal leukocyte subpopulations. **(A–E)** Absolute number of CD45^{+} leukocytes **(A)** and percentages of CD19^{+} B cells, CD3^{+} T cells, Ly6G^{+} PMNs, CD11b^{+}Ly6G^{-} monocytes and NK1.1^{+} NK cells **(B)** in peripheral blood of SIRT2/3$^{+/+}$, SIRT2$^{-/-}$, SIRT3$^{-/-}$, and SIRT2/3$^{-/-}$ mice. **(C)** Mean fluorescence intensity (MFI) of CD11b expressed by SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ PMNs. **(D)** Percentage of Ly6C low,

(Continued)

FIGURE 2 | intermediate and high expressing monocytes. **(E)** MFI of CD62L expressed by blood SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ NK cells. **(F)** Percentage of B cells, T cells, large peritoneal macrophages (LPMs), small peritoneal macrophages (SPMs) and NK cells in the peritoneal cavity of SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ mice. **(G)** MFI of CD43 expressed by peritoneal SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ NK cells. **(H)** Percentage of B-1a (CD23^{-} CD5^{+}), B-1b (CD23^{-} CD5^{-}) and B-2 (CD23^{+}) cells among B cells. **(I)** Peritoneal cells were exposed for 24 h to 10 ng/ml LPS. The concentrations of TNF, IL-6 and IL-10 in cell culture supernatants were measured by ELISA. Data were obtained from eight **(A–D)** or four **(E–I)** mice per group. Each dot represents one mouse. *$P < 0.05$; ***$P < 0.005$. Gating strategies are presented in **Supplementary Figure 3** and in Heinonen et al. (52).

peritoneal cells produced more IL-10 and less TNF and IL-6 than SIRT2/3$^{+/+}$ peritoneal cells ($P < 0.02$ for IL-10). These results were in line with the fact that B-1 cells, and particularly B-1a cells, are considered to produce high levels of IL-10 at baseline and upon microbial stimulation (70–72). The proportion of LPMs was slightly reduced in the peritoneal cavity of SIRT2/3$^{-/-}$ mice (**Figure 2F**). Overall, SIRT2/3 deficiency resulted in minor organ-specific alterations of the main leukocyte subpopulations, apart from an increased, functionally relevant, number of B-1a cells in the peritoneum.

SIRT2/3 Deficiency Enhances Cytokine Secretion, Phagocytosis, and Killing by Macrophages

Previous studies demonstrated that the deletion of SIRT2 and SIRT3 had no consequence on the expression of PRRs and microbial product-induced cytokine production by immune cells including BMDMs (13, 43). To test if SIRT2/3 deletion influenced cytokine production, BMDMs were exposed to LPS, Pam_3CSK_4, and CpG (i.e., agonists of TLR4, TLR1/2, and TLR9, respectively) for 24 h before measuring the concentrations of TNF, IL-6 and IL-10 in cell culture supernatants (**Figure 3A**). SIRT2/3$^{-/-}$ BMDMs produced significantly more TNF in response to LPS and CpG, more IL-6 in response to LPS, Pam_3CSK_4 and CpG, and more IL-10 in response to CpG. To test whether an increased intracellular signaling was associated with increased cytokine production, we quantified by Western blotting the phosphorylation of ERK1/2 and p38 MAPKs and the nuclear translocation of NF-κB p65 in BMDMs exposed to LPS for 0, 10, 30, and 60 min (**Figure 3B**). SIRT2/3 double deletion resulted in higher NF-κB p65 nuclear content at baseline and increased phosphorylation of ERK1/2 after 30 min of stimulation. p38 was not differentially phosphorylated in SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ BMDMs. In line with the increased intracellular signaling and cytokine production, the expression level of TLR1, TLR2, TLR4, and TLR9 mRNA was increased in SIRT2/3$^{-/-}$ BMDMs when compared to SIRT2/3$^{+/+}$ BMDMs (**Figure 3C**).

Since SIRT2 deficiency promoted phagocytosis (13), we compared the phagocytic activity over a period of 1 h of SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ BMDMs. The analysis by flow cytometry of BMDMs incubated with fluorescent beads showed a 1.4-fold higher proportion of SIRT2/3$^{-/-}$ BMDMs having phagocytosed beads ($P = 0.03$; **Figure 3D**, left panel). To better reflect infectious conditions, BMDMs were exposed to live *E. coli*, and intracellular bacteria were quantified by plating cell lysates followed by the enumeration of colonies. SIRT2/3$^{-/-}$ BMDMs ingested 2.6-fold more *E. coli* (SIRT2/3$^{+/+}$ vs. SIRT2/3$^{-/-}$ BMDMs: 4.4 ± 2.5 vs. 11.3 ± 4.8 × 10^4 *E. coli*, $P = 0.03$; **Figure 3D**, right panel). The increased phagocytic activity of SIRT2/3$^{-/-}$ BMDMs was independent from an increased expression of the phagocytic receptors integrin αM/Itgam (CD11b), Itgβ1/Itgb1 (CD29), Itgβ2/Itgb2 (CD18), Itgα5/Itga5 (CD49e), and ItgαX/Itgax (CD11c) (**Supplementary Figure 2A**). Since a main function of phagocytes is to kill invading microorganisms, we tested the killing of *E. coli* by BMDMs following 6 h of incubation. SIRT2/3$^{-/-}$ BMDMs showed around 2-fold more efficient killing capacity when compared to SIRT2/3$^{+/+}$ BMDMs as demonstrated by a reduced percentage recovery of ingested bacteria by SIRT2/3$^{-/-}$ BMDMs (SIRT2/3$^{+/+}$ vs. SIRT2/3$^{-/-}$ BMDMs: 25 ± 6 vs. 11 ± 2% recovery of ingested *E. coli*, $P = 0.03$; **Figure 3E**, left panel), and similar total numbers of bacteria in SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ BMDMs (9.3 vs. 1.2 × 10^4 total *E. coli*, $P = 0.5$; **Figure 3E**, right panel).

SIRT2/3 Deficiency Alters Macrophage Metabolism

Macrophages acquire energy mainly through oxidative metabolism under steady-state and switch to glycolysis upon activation (73, 74). Since SIRT2/3 deficiency increased the cytokine response of BMDMs, though both proinflammatory and anti-inflammatory cytokines, we assumed that their glycolytic parameters were affected. The metabolic parameters of SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ BMDMs were measured using the Seahorse technology. Unexpectedly, SIRT2/3$^{-/-}$ BMDMs displayed reduced glycolysis, glycolytic capacity and glycolytic reserve (**Figure 4A**). In line with this observation, SIRT2/3$^{-/-}$ BMDMs expressed slightly reduced mRNA levels of Solute carrier family 2, member 1 also known as Glucose transporter 1 (Glut1) while the mRNA levels of Hypoxia inducible factor 1α (Hif1a) were slightly increased (1.6-fold, **Figure 4B**). To assess whether the lower glycolysis of SIRT2/3$^{-/-}$ BMDMs was compensated by an increased metabolism of other sources of energy, we assessed the dependency (i.e., the necessity for a fuel to meet metabolic demand) and the flexibility (i.e., the ability to increase the usage of fuel when access to other energy sources is inhibited) of BMDMs toward glucose, fatty acids (FA) and glutamine. SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ BMDMs were equally dependent and flexible toward glucose (**Figure 4C**). However, SIRT2/3$^{-/-}$ BMDMs were less dependent than SIRT2/3$^{+/+}$ BMDMs toward FA and glutamine, but were more flexible than SIRT2/3$^{+/+}$ BMDMs toward FA (**Figure 4C**). FA are metabolized in the mitochondria through FA oxidation (FAO). FAO is controlled by the rate-limiting enzyme Carnitine palmoyltransferase I (Cpt1) that facilitates FA transport to the mitochondria (74). SIRT2/3$^{-/-}$ BMDMs expressed higher levels of Cpt1 mRNA when compared to SIRT2/3$^{+/+}$ BMDMs, but

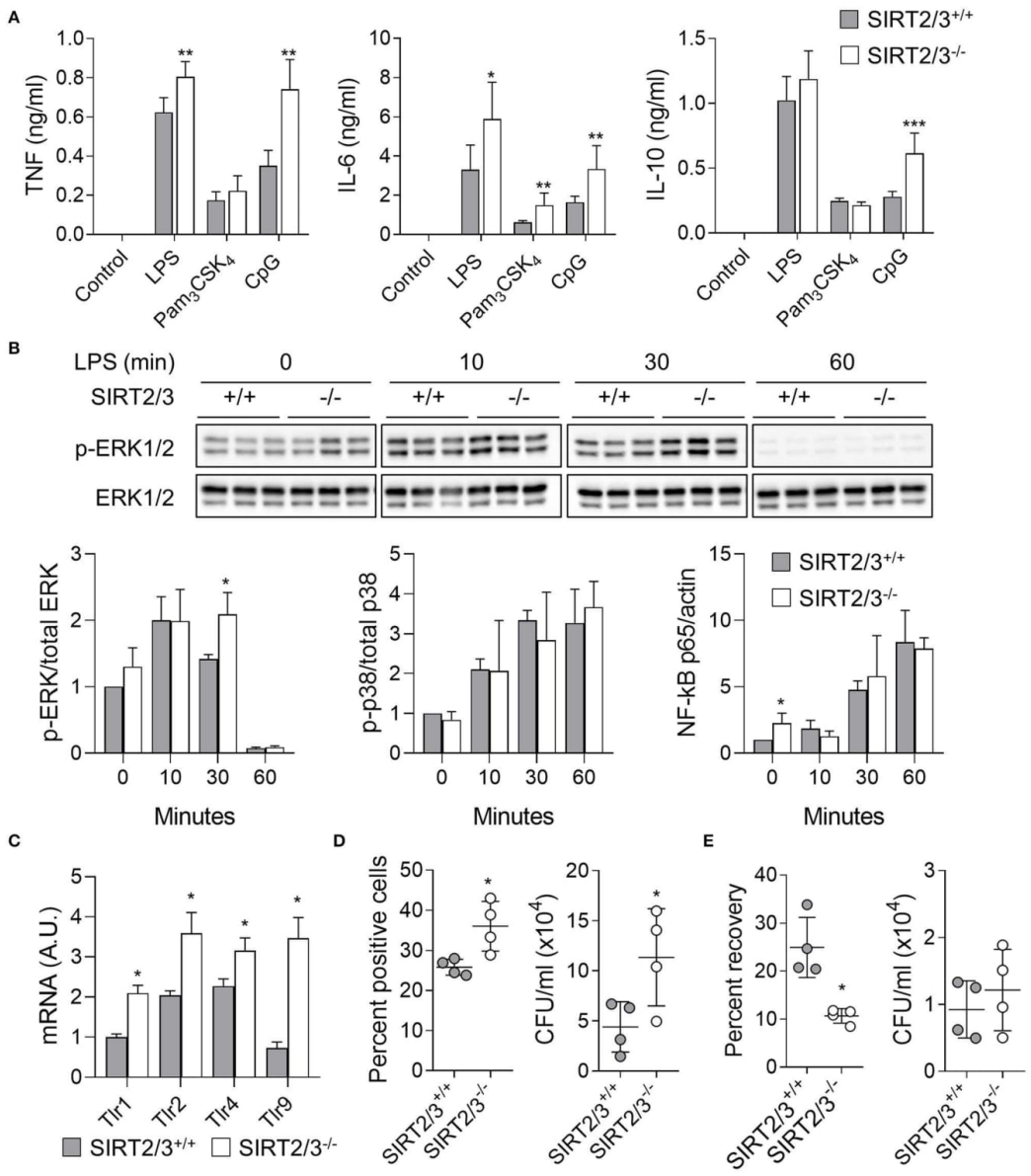

FIGURE 3 | SIRT2/3 deficiency increases cytokine secretion, phagocytosis and killing by macrophages. **(A)** BMDMs were exposed to LPS (10 ng/ml), Pam_3CSK_4 (100 ng/ml), and CpG (1 μg/ml) for 24 h. The concentrations of TNF, IL-6, and IL-10 in cell culture supernatants were measured by ELISA. **(B)** BMDMs were exposed for 0, 10, 30, and 60 min to 10 ng/ml LPS. Phosphorylation of ERK1/2 and p38, and nuclear translocation of NF-κB p65 were measured by Western blotting. Representative blots are presented for ERK1/2 phosphorylation (Full blots are available in **Supplementary Figure 1**). Quantitative data are means ± SD of three mice. **(C)** Tlr1, Tlr2, Tlr4, and Tlr9 mRNA levels in BMDMs were quantified by RT-qPCR and normalized to actin mRNA levels. Data are mean ± SD of four mice analyzed in triplicate. **(D)** Phagocytosis by BMDMs exposed for 1 h to fluorescent beads (left) and live *E. coli* (right) at a bead or bacteria-to-cell ratio of 10:1. Data were analyzed by flow cytometry and colony enumeration, respectively. **(E)** Killing of *E. coli* by BMDMs. The percentage of recovery (left) was calculated by dividing the number of bacteria recovered after 6 h by the number of bacteria recovered after 1 h. Absolute counts are presented on the right. Data are mean ± SD of four mice analyzed in triplicate. Each dot represents a mouse. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

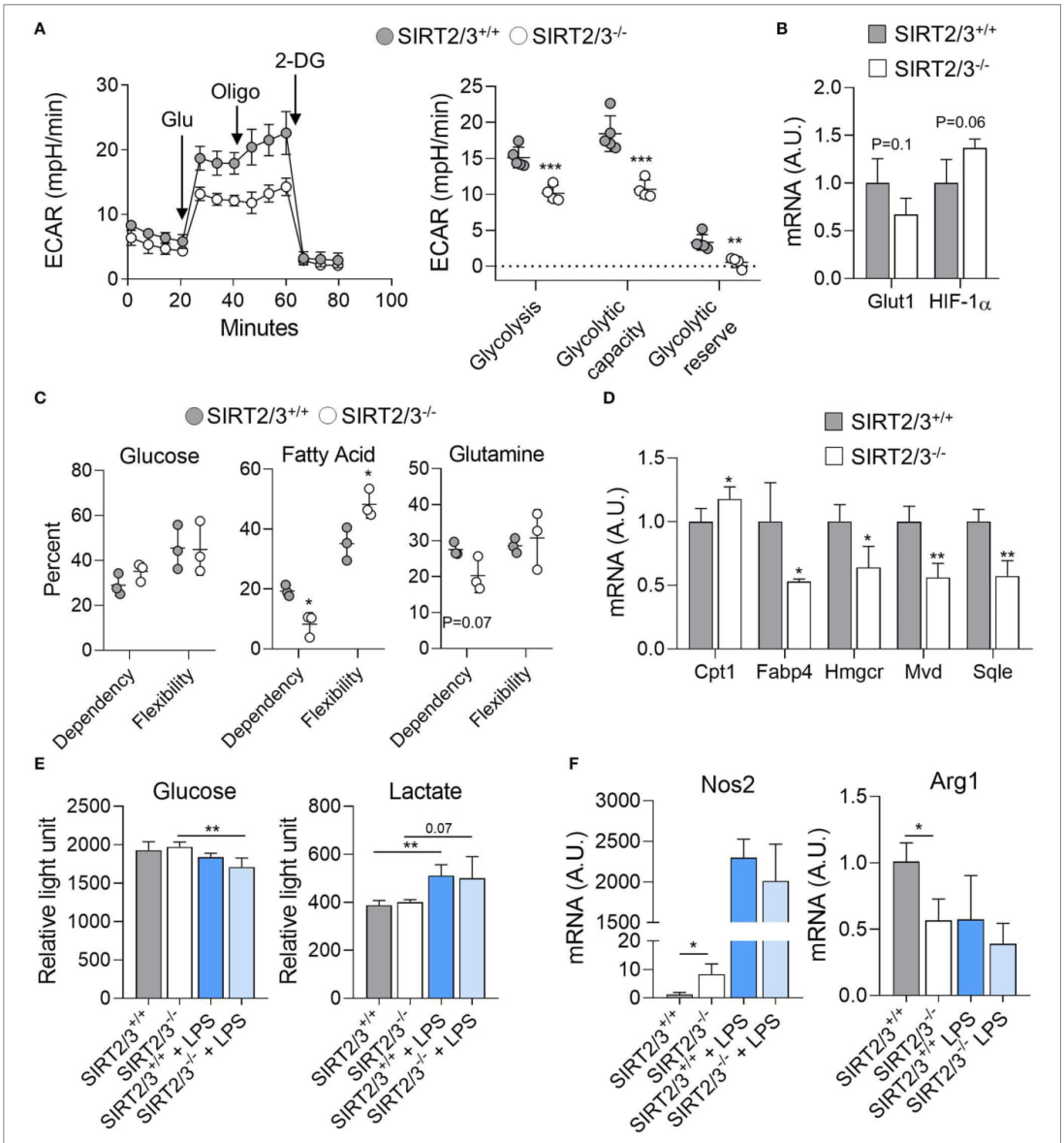

FIGURE 4 | SIRT2/3 deficiency alters macrophage metabolism. **(A)** The extracellular acidification rate (ECAR) of SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ BMDMs was measured using the Seahorse technology (left) and used to calculate the glycolysis, glycolytic capacity and glycolytic reserve (right). Glu, glucose; Oligo, oligomycin; 2-DG, 2-deoxyglucose. **(B)** Glut1 and Hif1a mRNA levels in BMDMs were quantified by RT-qPCR and normalized to actin mRNA levels. **(C)** Dependency and flexibility of SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ BMDMs toward glucose, fatty acids (FA) and glutamine. **(D)** Cpt1, Fabp4, Hmgcr, Mvd and Sqle mRNA levels in BMDMs were quantified by RT-qPCR and normalized to actin mRNA levels. **(E)** Glucose consumption and lactate production by SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ BMDMs left unstimulated or stimulated for 8 h (glucose) or 24 h (lactate) with 10 ng/ml LPS. Glucose and lactate were detected using a luminescence-based assay. **(F)** Nos2 and Arg1 were quantified by RT-qPCR and normalized to actin mRNA levels. Data are means ± SD of 3–5 mice analyzed in triplicate. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

lower levels of mRNA encoding for carrier protein and enzymes involved in cholesterol synthesis FA binding protein 4 (Fabp4), 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (Hmgcr), Mevalonate decarboxylase (Mvd), and Squalene epoxidase (Sqle) (**Figure 4D**). Therefore, SIRT2/3 deficiency possibly favored FAO over cholesterol synthesis.

LPS stimulation induces macrophage polarization toward a classical, M1, phenotype which is linked to a metabolic shift from oxidative phosphorylation to glycolysis (73, 74). Thus, we measured the consumption of glucose and the production of lactate upon LPS stimulation. Glucose concentration in medium decreased when BMDMs were exposed for 8 h to LPS, with a slightly superior effect observed with SIRT2/3$^{-/-}$ BMDMs (Control vs. LPS: $P = 0.2$ and 0.008 for SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ BMDMs; **Figure 4E**). In line with a metabolic shift, the levels of lactate increased in the medium of LPS-simulated BMDMs (**Figure 4E**). Overall, glucose consumption and lactate production were not different between SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ BMDMs. Although these results were unanticipated considering ECAR measurements, they could also reflect the small increase in the production of both proinflammatory and anti-inflammatory cytokines by double knockout BMDMs exposed to LPS. Finally, we questioned whether SIRT2/3$^{-/-}$ altered macrophage polarization by measuring the mRNA expression levels of Nitric oxide synthase 2 (Nos2) and Arginase 1 (Arg1) as markers of M1 and M2 phenotypes, respectively. SIRT2/3$^{-/-}$ macrophages expressed higher levels of Nos2 and lower levels of Arg1 at baseline, but similar levels of both mRNAs after stimulation with LPS (**Figure 4F**).

SIRT2/3$^{-/-}$ Mice Are Protected From Endotoxic Shock

Acute inflammation induced by LPS is characterized by an early anabolic glycolytic phase followed by a catabolic adaptation phase involving FAO (50). SIRT2$^{-/-}$ and SIRT3$^{-/-}$ mice were shown to behave like wild-type mice in models of endotoxemia (13, 43). Yet, considering that SIRT2/3$^{-/-}$ BMDMs stimulated with microbial products produced more proinflammatory and anti-inflammatory cytokines, and that the peritoneum of SIRT2/3$^{-/-}$ mice contained more anti-inflammatory B-1a cells and less activated NK cells, we hypothesized that SIRT2/3 deficiency may protect mice from acute inflammation. A model of endotoxic shock was developed by challenging mice intraperitoneally with 10 mg/kg LPS. While 93% of SIRT2/3$^{-/-}$ mice survived endotoxemia, only 58% of SIRT2/3$^{+/+}$ mice did ($P = 0.004$; **Figure 5A**). In agreement, SIRT2/3$^{-/-}$ mice showed lower severity scores than SIRT2/3$^{+/+}$ mice (**Figure 5B**), but there was no noticeable differential effect on mouse weight (**Figure 5C**). Depletion of macrophages by clodronate liposomes injected i.p. (90% depletion, $n = 4$; $P < 0.001$) did not modify the survival profiles ($P = 0.6$), suggesting a minor impact of macrophages in our model. To assess the immune status of endotoxemic mice, blood was collected 1 day post-challenge to quantify cytokines (**Figure 5D**) and leukocyte subpopulations (**Figure 5E**). Nine out of the fourteen detectable cytokines measured by Luminex (IFNγ, IL-6, IL-10, IL-12p40, IL-17A, IL-18, KC/CXCL1, MIP-2/CXCL2, IP-10/CXCL10) were present at significantly lower concentrations in the blood of SIRT2/3$^{-/-}$ mice. Endotoxemia was associated with a strong reduction of all blood leukocyte subpopulations (**Figure 5E**). Finally, we questioned whether SIRT2/3 deletion would affect endotoxin tolerance as reported for other sirtuins (24, 75–77). To address that question, blood was collected from mice challenged 0, 1, 3, and 7 days earlier with LPS. Blood was used to quantify leukocytes and to measure TNF and IL-10 response upon *ex vivo* exposure for 6 h to 10 ng/ml LPS or 10 μM CpG. At baseline, the blood of SIRT2/3$^{-/-}$ mice contained slightly more leukocytes (**Figure 5F**) and produced more TNF and IL-10 in response to LPS and CpG stimulation (**Figure 5G**). Confirming the induction of endotoxin tolerance, TNF production induced by LPS and CpG was decreased while IL-10 production induced by LPS was enhanced when comparing the reactivity of blood from SIRT2/3$^{+/+}$ and SIRT2/3$^{-/-}$ mice challenged 1 day earlier with LPS with that of untouched mice (**Figure 5G**). The production of TNF and IL-10 returned roughly to those measured under baseline conditions using blood collected from mice challenged 1 week earlier with LPS. Overall, SIRT2/3 double deletion did not seem to affect the induction of endotoxin tolerance.

DISCUSSION

Sirtuins share structural and functional features and shuttle between cellular compartments. Hence they may compensate the absence of one sirtuin in single knockout mice. Here, we described a germinal SIRT2/3 double deficient mouse line. We elected to delete SIRT2 and SIRT3 because SIRT2 is the overall most expressed sirtuin and SIRT3 the predominant mitochondrial sirtuin in myeloid cells (13, 43). The new SIRT2/3$^{-/-}$ mouse line developed normally without apparent defects. Yet, SIRT2/3 deficiency altered to some extent the frequency of immune cells in several immune compartments and the metabolism and functions of macrophages. Importantly, SIRT2/3$^{-/-}$ mice were protected from endotoxemia, contrary to SIRT2$^{-/-}$ and SIRT3$^{-/-}$ mice that behaved like wild-type mice (13, 43). Altogether, these data suggest a subtle, concerted role for SIRT2 and SIRT3 to be considered when developing drugs targeting multiple sirtuins.

Additive or synergistic effects between SIRT2 and SIRT3 could be anticipated since, for example, both SIRT2 and SIRT3 impact on ROS detoxification albeit through different transcriptional and post-transcriptional mechanisms, and both have been reported to dampen inflammatory responses (13, 23–31, 38, 39, 41–44). However, most of the effects observed here were rather slight. The most consistent effect of SIRT2/3 deficiency on immune cell distribution was on B cells that were increased slightly in the bone marrow, the spleen, the blood and more strongly in the peritoneum. Whether these variations impact on the development of plasma cells and humoral responses will be addressed in future studies. An impact on the levels of natural antibodies might be expected since B-1a cells are main producers of natural polyreactive IgM antibodies biased toward bacterial

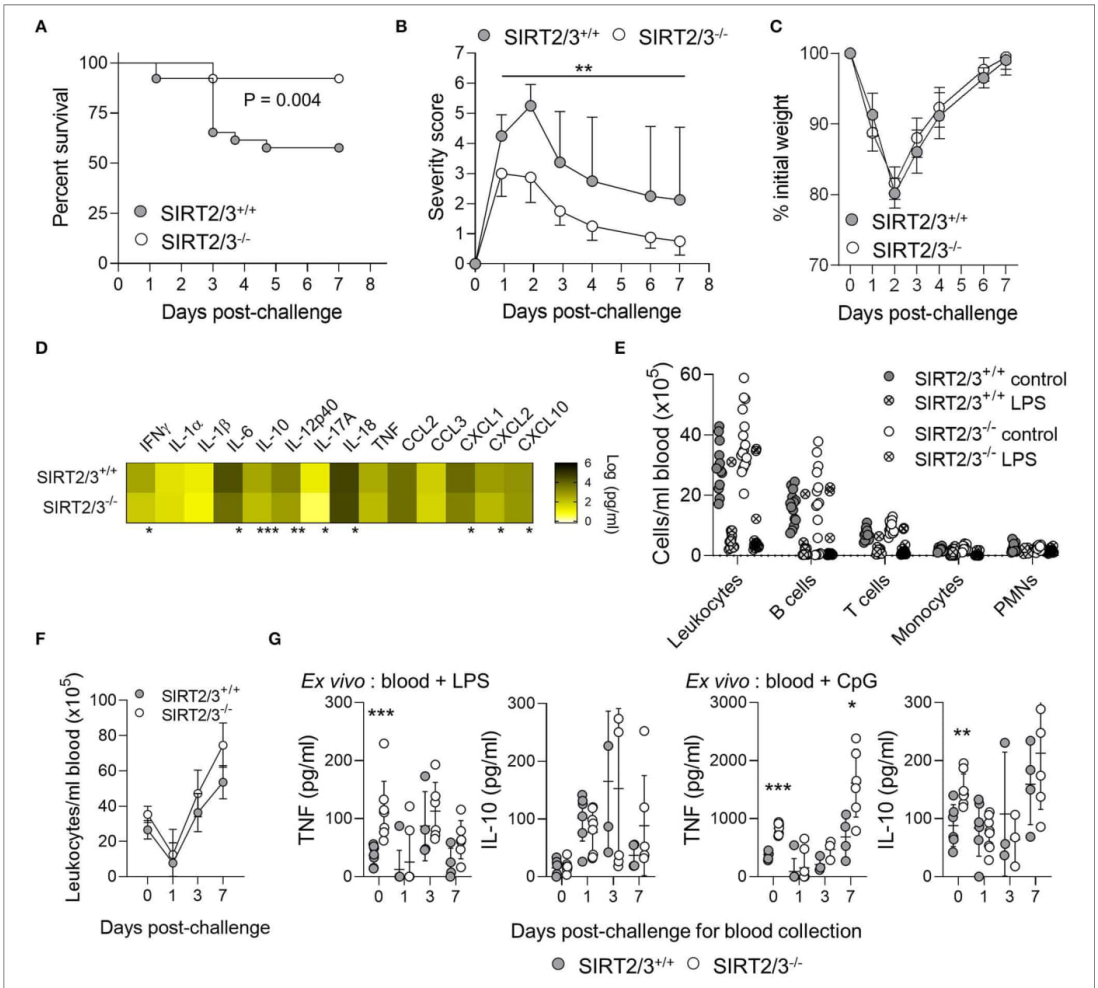

FIGURE 5 | SIRT2/3$^{-/-}$ mice are protected from endotoxic shock. Mice were challenged with 10 mg/kg LPS i.p. Survival **(A)**, severity score **(B)**, and weight loss **(C)** were recorded daily. Data are mean ± SD **(B,C)**. **(D–G)** Blood was collected 0, 1, 3, and 7 days post-challenge to quantify the concentrations of cytokines by Luminex (**D**, at day 1 post-challenge) and leukocytes by flow cytometry (**E,F**, at day 1 post-challenge in **E**). Blood collected 0, 1, 3, and 7 days post-LPS challenge was stimulated *ex vivo* for 6 h with 10 ng/ml LPS and 10 μM CpG. TNF and IL-10 concentrations were measured by ELISA **(G)**. TNF and IL-10 concentrations in blood cultured for 6 h without stimulation were below the detection limit (not depicted on the graphs). Data were obtained from 26 **(A)**, 7–8 **(B,C,F,G)**, 4 **(D)**, and 14–16 **(E)** mice per group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

and self-antigens (70). Considering that B-1 B cells cooperate to protect from pneumococcal diseases through the generation of anti-streptococcal natural antibodies by B-1a cells and the generation of acquired anticapsule response by B-1b cells (78), it will be interesting to test whether SIRT2/3$^{-/-}$ mice are resistant to *Streptococcus pneumoniae* pneumonia (79).

The bone marrow of SIRT3$^{-/-}$ mice, but not SIRT2$^{-/-}$ mice, contained more B cells. Up to now, the role of sirtuins in B cell biology has been mainly studied using B cell lymphoma giving rise to contrasting observations. Overexpression of SIRT1 and SIRT2 promoted the survival of chronic lymphocytic leukemia (CLL) B cells and correlated with poor outcome in patients with CLL (80, 81). On the contrary, SIRT3 acts as a tumor suppressor since reduced SIRT3 expression was associated with B cell proliferation and worsening of patients with B cell malignancies (82). However, sirtuins may have a differential impact on malignant cells and primary cells.

Additional leukocyte subsets were affected in SIRT2/3$^{-/-}$ mice, however with small organ specific effects, for example a reduced proportion of alternative monocytes in the bone marrow and spleen, of PMNs in the blood and spleen and of DCs in the spleen. In general, these effects were observed in one of the two parental mouse lines (SIRT2$^{-/-}$ or SIRT3$^{-/-}$ mice), indicative of dominant effects of the knockouts. A more robust effect was observed on NK cells. The expression of sirtuins in NK cells has not been reported, but the fact that caloric restriction modulated NK cell phenotype suggested that sirtuins may impact on NK cell development or functions (83). NK cells from the blood of SIRT2$^{-/-}$, SIRT3$^{-/-}$, and SIRT2/3$^{-/-}$ mice showed increased expression of the homing receptor CD62L, while the peritoneum of SIRT2/3$^{-/-}$ mice contained less NK cells with reduced expression of CD43. CD43 is a maturation marker of NK cells, and reduced CD43 expression reflects decreased IFNγ production by NK cells (84). Thus, in the peritoneum, SIRT2/3$^{-/-}$ NK cells potentially display a reduced inflammatory profile. It will be interesting to assess the functional consequences of SIRT2/3 deficiency on NK cell-mediated immunity, for instance in models of viral infections induced by murine cytomegalovirus (MCMV), lymphocytic choriomeningitis virus (LCMV) or influenza virus during which NK cells play a protective role. Whether SIRT2/3 deficiency affects NK cell antitumor cell cytotoxicity and has a role in graft-vs-host-disease would be additional interesting areas of investigations.

We previously reported that SIRT2$^{-/-}$ and SIRT3$^{-/-}$ BMDMs produced normal levels of a large panel of cytokines (13, 43). Conversely, SIRT2/3$^{-/-}$ BMDMs produced increased levels of both proinflammatory (TNF, IL-6) and anti-inflammatory (IL-10) cytokines when compared to control BMDMs. A plausible mechanism underlying augmented cytokine response was the increased activation of ERK1/2 and NF-κB p65 intracellular signaling in SIRT2/3$^{-/-}$ BMDMs. Like SIRT2$^{-/-}$ BMDMs (13), SIRT2/3$^{-/-}$ BMDMs phagocytosed better inert beads and live bacteria than control BMDMs. Moreover, they killed ingested *E. coli* to a higher rate. Of note, divergent observations have been reported in the literature, with SIRT2 and SIRT3 associated with both anti-inflammatory and proinflammatory activities also in macrophages (13, 23–31, 41–44). As an example of a possible antagonism between SIRT2 and SIRT3, silencing SIRT2 reduced NF-κB activation and induced macrophage alternative (anti-inflammatory) activation (85) while SIRT3 upregulation reduced macrophage inflammatory responses (86). This dichotomy might explain the mixed phenotype of SIRT2/3$^{-/-}$ BMDMs which displayed characteristics of classically and alternatively activated macrophages.

Sirtuins are intrinsically linked to cell metabolism. The deletion of SIRT2 and SIRT3 stimulated HIF-1α expression and activity, thereby promoting Glut1 expression, glucose uptake and tumor growth (87, 88). In line, Hif1a mRNA levels were increased 1.6-fold in SIRT2/3$^{-/-}$ BMDMs. However, this increase did not translate into an increase of glucose metabolism since Glut1 expression and glycolytic parameters, measured using the Seahorse technology, were all reduced in SIRT2/3$^{-/-}$ BMDMs. This was surprising since inflammatory cytokine response is normally supported by glycolysis. Yet one has to take into account that SIRT2/3$^{-/-}$ BMDMs were not purely "proinflammatory" since they increased IL-10 production, which in alternatively activated macrophages is fueled by oxidative phosphorylation (73). Nonetheless, glucose consumption and lactate production measured by luminescent assays were not affected in SIRT2/3$^{-/-}$ BMDMs. Differences in the time point analyzed, plastic support and medium used for cell culture in relation with the technology used may have affected metabolic measurements. Overall, dual deletion of SIRT2 and SIRT3 may have disrupted metabolic control in BMDMs. A possible explanation for our observations is that post-translational mechanisms destabilized HIF-1α and impaired its transcriptional activity in SIRT2/3$^{-/-}$ BMDMs. Attractive mediators could be SIRT6 and SIRT7 which expression was enhanced to some extent in SIRT2/3$^{-/-}$ BMDMs. Indeed, SIRT6 functions as a co-repressor of HIF-1α, and SIRT7 inhibits the activity of HIF-1α through a mechanism independent of proteasomal or lysosomal degradation of HIF-1α (89, 90). SIRT2/3$^{-/-}$ BMDMs were less dependent on FA and glutamine but more flexible toward FA, indicating that both glycolysis and FA metabolism were altered in SIRT2/3$^{-/-}$ BMDMs. The expression of genes essential for the cholesterol synthesis pathway was decreased in SIRT2/3$^{-/-}$ BMDMs, signifying that SIRT2/3 deficiency influenced lipid metabolism of macrophages by favoring FAO over cholesterol synthesis. Although counterintuitive at first glance, a similar antagonism between FAO and cholesterol synthesis has been observed in macrophages deficient in carnitine palmitoyltransferase 1 and 2 (CPT1, CPT2) (91).

Metabolic adaptation shapes immune cell functions and influences the acute phase and the resolution phase of inflammation that are fueled predominantly by glycolysis and oxidative phosphorylation, respectively (92, 93). Indeed, mice treated with 2-deoxyglucose to block glycolysis or with the sirtuin inhibitor cambinol were protected from endotoxemia (94, 95). Going well along with a reduced glycolytic activity of macrophages *in vitro*, SIRT2/3$^{-/-}$ mice had a strong survival advantage during endotoxemia, which was associated with reduced blood levels of cytokines. Even so, SIRT2/3 double deletion did not seem to affect endotoxin tolerance as reported for SIRT1, SIRT4, and SIRT5 (24, 75–77). Interestingly, SIRT6, together with SIRT1, coordinated the switch from glucose to FAO during the acute inflammatory response (50), and transcriptional activation of SIRT6 via FOXO3a inhibited the Warburg effect in glioblastoma cells (96). Consequently, the increased expression of SIRT6 in SIRT2/3$^{-/-}$ macrophages may favor FAO and contribute to dampen the cytokine storm involved in the pathological process of endotoxemia. An additional possibility is about a role played by B-1a cells, which were around 3-times more numerous in the peritoneum of SIRT2/3$^{-/-}$ mice. Indeed, B-1a cells are an important source of IL-10 and may circumvent local cytokine burst in response to LPS challenge. Further, B-1a cells were shown to protect mice from experimental sepsis (97, 98). Finally, peritoneal NK cells in SIRT2/3$^{-/-}$ mice were not

only less, but also likely less activated as pinpointed by a reduced expression of CD43. As a consequence, the NK compartment potentially produced reduced levels of IFNγ which was shown to strongly potentiate TNF production and mortality during experimental endotoxemia (84, 99, 100). Altogether, several factors likely contributed to reduce systemic inflammatory parameters, morbidity and mortality of SIRT2/3$^{-/-}$ endotoxemic mice.

Overall, we report that SIRT2/3 dual deletion revealed a phenotype not observed in single deficient mice, indicating that sirtuins act in concert or compensate each other for certain immune functions. Considering the link between SIRT2 and SIRT3, metabolism and age-associated dysfunctions, it will be of great interest to investigate the impact of SIRT2/3 deficiency in the pathogenesis of metabolic, oncologic, neurodegenerative and chronic inflammatory disorders. Importantly from a translational perspective, SIRT2/3$^{-/-}$ mice were protected from endotoxemia. Thus, inhibitors targeting multiple sirtuins developed for clinical purposes may be useful to treat inflammatory diseases.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Service des Affaires Vétérinaires, Direction Générale de l'Agriculture, de la Viticulture et des Affaires Vétérinaires (DGAV), état de Vaud (Epalinges, Switzerland) under authorizations 876.9 and 877.9.

AUTHOR CONTRIBUTIONS

TH, EC, ER, JR, and DL performed the *in vitro* experiments. TH, EC, and DL performed the *in vivo* experiments. TH, EC, and TR conceived the project and designed the experiments. TH and TR wrote the paper. All the authors revised the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02713/full#supplementary-material

Supplementary Figure 1 | Full blots used to create **Figure 1**.

Supplementary Figure 2 | mRNA expression levels of phagocytic receptors by resting BMDMs. ItgaM, Itgb1, Itgb2, Itga5, and ItgaX mRNA levels were quantified by RT-qPCR and normalized to actin mRNA levels. Data are mean ± SD of four mice analyzed in triplicate.

Supplementary Figure 3 | Gating strategies for flow cytometry analyses.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.6 Impact of the dual deletion of the mitochondrial sirtuins SIRT3 and SIRT5 on anti-microbial host defenses

Tytti Heinonen, Eleonora Ciarlo, Didier Le Roy, Thierry Roger

Infectious Diseases Service, Department of Medicine, Lausanne University Hospital, Epalinges, Switzerland

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Summary

SIRT3 and SIRT5 are the main mitochondrial deacetylase and desuccinylase, respectively. SIRT3 and SIRT5 are linked to age-associated and oncologic diseases. However, the single deficiency in SIRT3 or SIRT5 impacted neither the responses of macrophages nor the survival of mice in preclinical models of infections. Therefore, we decided to test whether SIRT3 and SIRT5 compensated each other.

We crossed SIRT3 and SIRT5 deficient mice to generate SIRT3/5 double deficient mice. Macrophages and neutrophils derived from these mice produced higher levels of ROS and inflammatory cytokines. The blood of SIRT3/5 deficient mice showed increased killing of *Listeria monocytogenes* and produced higher levels of cytokines. In line, SIRT3/5 double deficient mice were to some extent protected from listeriosis.

Altogether, our data reveal a subtle effect of SIRT3/5 double deficiency on innate immune responses. These results suggest that therapies targeting SIRT3 and/or SIRT5 should not impact host defenses.

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Impact of the Dual Deletion of the Mitochondrial Sirtuins SIRT3 and SIRT5 on Anti-microbial Host Defenses

*Tytti Heinonen, Eleonora Ciarlo, Didier Le Roy and Thierry Roger**†*

Infectious Diseases Service, Department of Medicine, Lausanne University Hospital and University of Lausanne, Epalinges, Switzerland

The sirtuins SIRT3 and SIRT5 are the main mitochondrial lysine deacetylase and desuccinylase, respectively. SIRT3 and SIRT5 regulate metabolism and redox homeostasis and have been involved in age-associated metabolic, neurologic and oncologic diseases. We have previously shown that single deficiency in either SIRT3 or SIRT5 had no impact on host defenses in a large panel of preclinical models of sepsis. However, SIRT3 and SIRT5 may compensate each other considering that they share subcellular location and targets. Here, we generated a SIRT3/5 double knockout mouse line. SIRT3/5 deficient mice multiplied and developed without abnormalities. Hematopoiesis and immune cell development were largely unaffected in SIRT3/5 deficient mice. Whole blood, macrophages and neutrophils from SIRT3/5 deficient mice displayed enhanced inflammatory and bactericidal responses. In agreement, SIRT3/5 deficient mice showed somewhat improved resistance to *Listeria monocytogenes* infection. Overall, the double deficiency in SIRT3 and SIRT5 has rather subtle impacts on immune cell development and anti-microbial host defenses unseen in single deficient mice, indicating a certain degree of overlap between SIRT3 and SIRT5. These data support the assumption that therapies directed against mitochondrial sirtuins, at least SIRT3 and SIRT5, should not impair antibacterial host defenses.

Keywords: sirtuin, innate immunity, cytokine, infection, sepsis, metabolism, macrophage, neutrophil

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Liwu Li,
Virginia Tech, United States

Reviewed by:
Xiaohu Huang,
University of Maryland, Baltimore, United States
Chaofeng Han,
Second Military Medical University, China

****Correspondence:***
Thierry Roger
thierry.roger@chuv.ch

†ORCID:
Thierry Roger
orcid.org/0000-0002-9358-0109

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INTRODUCTION

The innate immune system plays a central role in host defenses. Innate immune cells among which monocytes/macrophages, granulocytes and dendritic cells (DCs) sense microbial and danger associated molecular patterns (MAMPs/DAMPs) through pattern recognition receptors (PRRs) expressed at the cell surface, in the cytoplasm and in endosomes. The best characterized PRRs belong to the families of Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and cytosolic DNA sensors (1, 2). The binding of MAMPs/DAMPs to PRRs activates intracellular signaling cascades that induce the production of effector molecules involved in inflammation and host defense mechanisms, as well as the resolution of inflammation and tissue repair (3, 4). Immune cells are plastic and adapt their metabolism and responsiveness to their environment to execute their biological functions (5, 6).

Sirtuins belong to the family of so-called histone deacetylases (HDACs) that target lysine posttranscriptional modifications. Classical HDACs (HDAC1-11) are Zn^{2+}-dependent, while sirtuins are NAD^{+}-dependent lysine deacetylases. Sirtuins are homologs to yeast Sir2 that gained tremendous attention when it was shown to be activated upon caloric restriction and to increase lifespan (7). The mammalian genome encodes for seven sirtuins that target proteins by removing acetyl functional groups, but also acyl, glutaryl, malonyl, and succinyl groups as demonstrated lately (8). The list of targets of sirtuins has increased dramatically over the years, and high throughput proteomics analyses pinpointed to thousands of substrates for sirtuins. Accordingly, sirtuins are involved in the regulation of many biological and pathological processes and in the development of metabolic, neurodegenerative, cardiovascular, and oncologic diseases (9, 10).

SIRT3 and SIRT5 are mainly localized in the mitochondrial matrix, where SIRT3 is the main deacetylase (11) and SIRT5 is the main desuccinylase (12, 13). Of note, SIRT5 also catalyzes lysine demalonylation and deglutarylation (13, 14). SIRT3 promotes glucose and fatty acid metabolism, urea cycle and the activity of the electron transport chain. During caloric restriction, SIRT3 regulates mitochondrial acetylome and multiple metabolic pathways in the liver (15, 16). SIRT3 protects from oxidative stress by activating the reactive oxygen species (ROS) detoxifying enzyme superoxide dismutase 2 (SOD2) and the redox controlling enzyme isocitrate dehydrogenase 2 (IDH2) (17, 18). Similar to SIRT3, SIRT5 activates enzymes involved in ROS detoxification (i.e., SOD1, IDH1, and IDH2), promotes mitochondrial functions and integrity and regulates the urea cycle and other metabolic pathways (14, 19–25). The genetic ablation of SIRT3 or SIRT5 in mice has been associated with increased susceptibility to age-associated diseases including insulin resistance, obesity, neurodegeneration, cardiac dysfunction and fibrosis, while contrasting context-dependent effects have been reported for tumorigenesis (10, 26–30). Deficiencies in SIRT3 or SIRT5 have also been reported to promote colitis, acute lung injury and ischemia reperfusion injury (10, 31–36). Overall, targeting the activity of sirtuins and particularly mitochondrial sirtuins is viewed as an attractive therapeutic strategy to tackle the development of age-related disorders (10, 28–30). Considering that inflammation is an essential component of innate immune defenses, we analyzed the impact of SIRT3 and SIRT5 deficiencies on the response of mice subjected to a broad panel of preclinical models of bacterial and fungal sepsis (37, 38). Neither SIRT3 nor SIRT5 was critical to fight against infections. Additionally, $SIRT3^{-/-}$ mice were not particularly susceptible to cecal ligation and puncture (CLP), a stringent model of sepsis (39, 40). Hence, SIRT3 and SIRT5 appear to have a more prominent influence on chronic metabolic and inflammation-related disorders than on infectious diseases characterized by acute inflammatory reactions.

SIRT3 and SIRT5 share subcellular location and targets, so they might compensate each other in single knockout mice. To bypass this hurdle, we generated a SIRT3/5 deficient mouse line. $SIRT3/5^{-/-}$ mice were fertile and developed without apparent abnormalities. *In vitro* and *in vivo* investigations revealed somewhat enhanced inflammatory and bactericidal responses of whole blood, macrophages, and neutrophils and a moderate improved resistance to *Listeria monocytogenes* in the double knockouts. Altogether SIRT3 and SIRT5 have subtle, redundant roles during antimicrobial host defenses. Overall, therapies directed against mitochondrial sirtuins should not dramatically impact on antimicrobial host defenses.

MATERIALS AND METHODS

Key Resources

See **Supplementary Information**.

Ethics Statement

Animal experiments were approved by the Service des Affaires Vétérinaires, Direction Générale de l'Agriculture, de la Viticulture et des Affaires Vétérinaires (DGAV), état de Vaud (Epalinges, Switzerland; authorizations 876.9 and 877.9) and performed according to Swiss and ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines).

Mice

C57BL/6J mice were from Charles River Laboratories (Saint-Germain-sur-l'Arbresle, France). $SIRT3^{-/-}$ and $SIRT5^{-/-}$ C57BL/6J mice were described (41, 42) and obtained from Prof. Johan Auwerx, Laboratory for Integrative and Systems Physiology, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland. $SIRT3^{-/-}$ males were crossed with $SIRT5^{-/-}$ females. Thirty-two $SIRT3/5^{+/-}$ females were crossed with 16 $SIRT3/5^{+/-}$ males. Among the 205 F2 mice, 4 males and 8 females were double knockout mice and used to establish the $SIRT3/5^{-/-}$ mouse line. All mice used in this study were 7–14-week old, housed under specific pathogen-free conditions and exempt of mouse hepatitis virus and murine norovirus. For genotyping purposes, DNA was extracted and analyzed by PCR using the Mouse Direct PCR Kit (Bimake, Houston, TX) and primers pairs described in **Supplementary Information**.

Cells and Reagents

Bone marrow (BM) cells were cultured 7 days in IMDM or RPMI 1640 supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen, San Diego, CA), 10% heat inactivated fetal bovine serum (Biochrom GmbH, Berlin, DE) and 50 U/ml macrophage colony-stimulating factor (ImmunoTools, Friesoythe, Germany) or 30% L929 cell supernatant to generate BM-derived macrophages (BMDMs) (43, 44). Cells were seeded in half-area 96-well plates (2.5×10^4 cells/well), 96-well plates (2×10^5 cells/well) and 6-well plates (3×10^6 cells/well) without growth factors. Neutrophils were isolated from the bone marrow using the Neutrophil isolation kit (Miltenyi, Bergisch Gladbach, Germany) and plated in 96-well plates (10^5 cells/well). *Salmonella minnesota* ultra pure lipopolysaccharide (LPS) was from List Biologicals Laboratories (Campbell, CA), Pam_3CSK_4 from EMC microcollections GmBH (Tübingen, Germany), and CpG ODN 1826 (CpG) and poly(I:C) from Invivogen (San Diego, CA). Monosodium urate (MSU) crystals were prepared as described (45). *Listeria monocytogenes* 10403 s was grown

in brain heart infusion broth (BD Biosciences, Erembodegem, Belgium). Bacteria were washed with 0.9% NaCl and adjusted at 10^{10} cfu/ml. When required, bacteria were heat-inactivated for 2 h at 70°C.

RNA Analyses

RNA was extracted (RNeasy kit) and reverse transcribed (QuantiTect reverse transcription kit) (Qiagen, Hilden, Germany). PCRs were performed in triplicate with 1.25 µl cDNA, 1.25 µl H_2O, 0.62 µl primers [**Supplementary Information** and (46)] and 3.12 µl KAPA SYBR Green Fast (Kapa Biosystems, Wilmington, MA) using a QuantStudio™ 12K Flex system (Life Technologies, Carlsbad, CA). Gene expression was normalized to actin expression.

Western Blot Analyses

Total and nuclear proteins were extracted, submitted to PAGE and transferred onto membranes as described (47, 48). Membranes were incubated with primary and secondary HRP-coupled antibodies and revealed by chemiluminescence [**Supplementary Information** and (49)]. Images were recorded with a Fusion Fx system (Vilber Lourmat, Collégien, France). Full length blots are presented in **Supplementary Figure S1**.

Flow Cytometry

Single cell suspensions from thymus, spleen and BM were incubated with 2.4 G2 to block Fc receptors and stained with antibodies described in **Supplementary Information** (50). For hematopoietic stem cells (HSC) and progenitor cells, lineage cocktail contained antibodies directed against CD45R (B220), CD3e, CD11b, CD19, Ly6C/G, Ter119/Ly-76. Data were acquired using an Attune Nxt flow cytometer (ThermoFisher, Waltham, MA) and analyzed using FlowJo version 10.2 (FlowJo LLC, Ashland, OR). Gating strategies are presented in **Supplementary Figure S2**.

ROS Measurement

BMDMs were plated in half-area black 96-well plates in RPMI without phenol red (Invitrogen). Cells were incubated for 10 min at 37°C with 5 µM MitoSOX (Thermofisher). Stimuli were added and fluorescence (Ex_{510}, Em_{580}) recorded using a Synergy plate reader (BioTek, Winooski, VT). Neutrophils in HBSS without calcium and magnesium (ThermoFisher) were incubated for 1 h with 100 nM PMA (Enzo Life Sciences, Farmingdale, NY) and 5 µM MitoSOX during the last 10 min of incubation. ROS were measured by flow cytometry.

Cytokine Measurement

Cytokines were quantified by ELISA (**Supplementary Information**) or Luminex using a custom ProCarta kit (ENA-78/CXCL5, G-CSF, IFNγ, IL-1α, IL-1β, IL-3, IL-6, IL-10, IL-12p40, IL-17A, IL-18, IP-10/CXCL10, KC/CXCL1, MCP-1/CCL2, MIP-1α/CCL3, MIP-2/CXCL2, TNF) (Invitrogen, Carlsbad, CA) and a bioplex 200 system (Bio-Rad, Hercules, CA) (51).

Metabolic Activity

The metabolic activity of BMDMs was measured using the XF Cell Mito Stress, Glycolysis Stress and Mito Fuel Flex Test Kits on a 96-well format Seahorse XFe® system (Agilent Technologies, Santa Clara, CA) (46).

Neutrophil Killing and NETosis Assays

Neutrophils were incubated with live *L. monocytogenes* for 1 h in RPMI medium. Serial dilutions of reaction mixtures were plated on blood agar plates (BD Biosciences). Twenty-four hours later, colonies were enumerated. To measure NETosis, neutrophils were incubated for 3 h with 100 nM PMA and 5 µM of the cell impermeable dye Sytox green. Fluorescence (Em_{504}, Ex_{523}) was recorded using the Synergy plate reader.

In vivo Models

Listeriosis was induced by challenging intravenously (i.v.) age and sex-matched mice with a low (7.3×10^3 cfu) or a high ($0.9–1 \times 10^5$ cfu) inoculum of *L. monocytogenes*. Blood and organs were collected 1–3 days post-infection to quantify bacteria and cytokines and analyze cell populations. A model of endotoxemia was developed by challenging mice intraperitoneally (i.p.) with 10 mg/kg LPS. Body weight loss, severity score and survival were registered at least twice daily (52, 53) by 2–3 operators. The severity score was graded from 0 to 4 based on the mobility, the posture, the appearance and the weight loss of mice (detailed criteria were approved by the Service des Affaires Vétérinaires, DGAV, and are available upon request). Mice were sacrificed when they met a severity score of 4. A mice found dead was assigned a score of 5.

Statistical Analyses

Groups were compared by variance analysis followed by two-tailed unpaired Student's *t*-test or a Mann-Whitney test when appropriate. Survival was analyzed using the Kaplan-Meier method. $P < 0.05$ was used to indicate statistical significance. Analyses were performed using PRISM 8.0.1 (GraphPad Software, San Diego, CA).

RESULTS

SIRT3/5 Deficiency Has No Dramatic Impact on Mouse Development and Macrophage Metabolism

We generated a SIRT3/5 double knockout mouse line ($SIRT3/5^{-/-}$, see Materials and Methods) to study the interaction between SIRT3 and SIRT5. Genomic-DNA based PCR genotyping (**Figure 1A**) and Western blotting analyses (**Figure 1B**) confirmed the truncation of the *Sirt3* and *Sirt5* genes and the absence of SIRT3 and SIRT5 protein expression in $SIRT3/5^{-/-}$ mice. Fecundity and development were normal. The size (**Figure 1C**) and the female/male sex ratio (**Figure 1D**) of the litters as well as the weight of adult female and male mice (**Figure 1E**) were like those of $SIRT3/5^{+/+}$, $SIRT3^{-/-}$, and $SIRT5^{-/-}$ mouse lines. Autopsy did not reveal gross abnormalities in $SIRT3/5^{-/-}$ mice.

The dual deletion of SIRT3 and SIRT5 was not compensated by an increased expression of mRNA encoding for SIRT1, SIRT2, SIRT4, SIRT6, and SIRT7 in bone marrow derived macrophages (BMDMs) (**Figure 1F**). There was around 20% increase of total protein acetylation in $SIRT3/5^{-/-}$

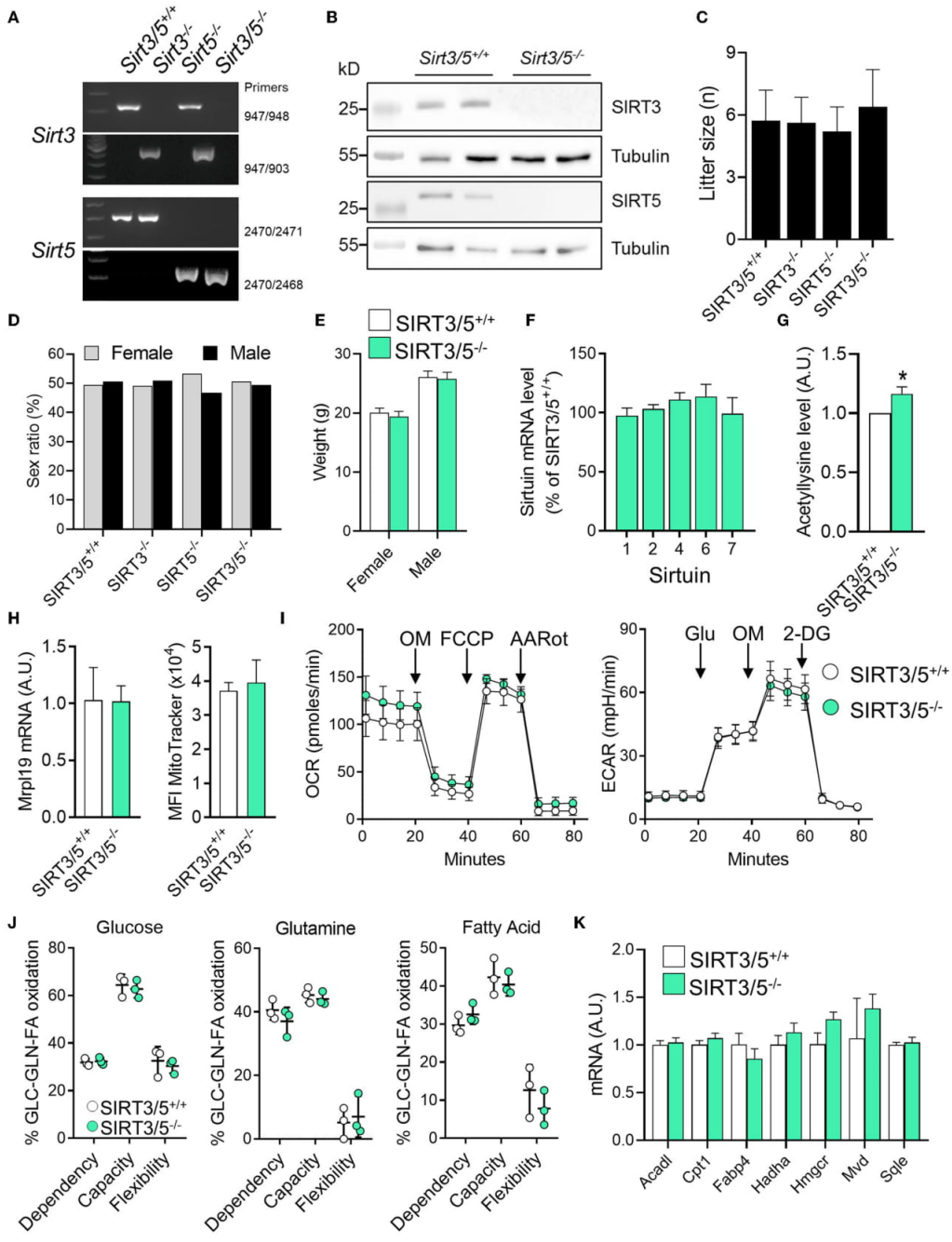

FIGURE 1 | SIRT3/5 deficiency has no evident impact on mouse development and macrophage metabolism. **(A)** Genotyping of SIRT3/5$^{+/+}$, SIRT3$^{-/-}$, SIRT5$^{-/-}$, and SIRT3/5$^{-/-}$ mice by PCR. Genomic DNA was amplified by PCR using the primer pairs indicated on the right and reaction mixtures were electrophoresed through

(Continued)

FIGURE 1 | agarose gels. **(B)** SIRT3, SIRT5, and tubulin expression in the liver of SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ mice measured by Western blotting (Full blots are presented in **Supplementary Figure S1**). **(C,D)** Size **(C)** and sex ratio (in percentage, **D**) of litters from SIRT3/5$^{+/+}$, SIRT3$^{-/-}$, SIRT5$^{-/-}$, and SIRT3/5$^{-/-}$ mouse lines. **(E)** Weight of adult SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ female and male mice. **(F)** Sirtuin mRNA expression levels in SIRT3/5$^{-/-}$ BMDMs, expressed relative to the mRNA levels in SIRT3/5$^{+/+}$ BMDMs set at 100%. Data are means ± SD from one experiment performed with three mice analyzed in triplicate. **(G)** Acetyllysine levels in total protein extracts from SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ BMDMs were measured by Western blotting and imaging. Values were normalized to those obtained using SIRT3/5$^{+/+}$ BMDMs set at 1. Data are means ± SD from one experiment performed with three mice. $P = 0.009$. **(H)** Mrpl19 mRNA expression levels assessed by RT-PCR and median fluorescence intensity (MFI) of MitoTracker measured by flow cytometry in SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ BMDMs. Data are means ± SD from four mice aged 10–12 weeks. **(I)** Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Seahorse technology. OM, oligomycin; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; AARot, rotenone+Antimycin A; Glu, glucose; 2-DG, 2-deoxyglucose. Data are means ± SD from four mice aged 10–12 weeks analyzed in quadruplicate. **(J)** Mitochondrial fuel usage by SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ BMDMs measured using the Seahorse technology. **(K)** Acadl, Cpt1, Fabp4, Hadha, Hmgcr, Mvd, and Sqle mRNA expression levels were quantified by RT-PCR. Gene expression levels were normalized to actin levels. A.U., arbitrary unit. Data are means ± SD from three mice aged 10–12 weeks analyzed in triplicate.

BMDMs (**Figure 1G**). SIRT3/5 deficiency did not alter the mitochondrial mass, evaluated by measuring mitochondrial ribosomal protein L19 (Mrpl19) mRNA levels and the fluorescence intensity of the mitochondrial dye MitoTracker (**Figure 1H**). The oxygen consumption rate (OCR), which reflects mitochondrial respiration, was weakly increased in SIRT3/5$^{-/-}$ BMDMs (**Figure 1I**). The extracellular acidification rate (ECAR), a readout of the glycolytic activity, was not affected in resting and LPS stimulated SIRT3/5$^{-/-}$ BMDMs (**Figure 1I** and **Supplementary Figure S3A**). OCR and ECAR were similarly affected in resting SIRT3$^{-/-}$ BMDMs (**Supplementary Figure S3B**) and SIRT5$^{-/-}$ BMDMs (38). The dependency, capacity, and flexibility of BMDMs to oxidize the mitochondrial fuels glucose, glutamine and fatty acids were identical for SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ BMDMs (**Figure 1J**). SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ BMDMs expressed similar levels of a number of genes encoding for molecules involved in the fatty acid metabolism, i.e., Acadl (acyl-CoA dehydrogenase long chain), Cpt1 (carnitine palmitoyltransferase 1), Fabp4 (fatty acid binding protein 4), Hadha (hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha), Hmgcr (3-hydroxy-3-methylglutaryl-CoA reductase), Mvd (mevalonate diphosphate decarboxylase) and Sqle (squalene epoxidase) (**Figure 1K**). Finally, the OCR of SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ BMDMs was not different before and after addition of etimoxir, an inhibitor of Cpt1 (88.0 vs. 85.5% inhibition in SIRT3/5$^{+/+}$ vs. SIRT3/5$^{-/-}$ BMDMs; $n = 6$; $P = 0.3$). Hence, SIRT3/5 deficiency had no strong impact on basic metabolic parameters of BMDMs.

SIRT3/5 Deficient Mice Have Minor Abnormalities of Leukocyte Development

The bone marrow is the main source of hematopoietic stem cells (HSC) and progenitors of immune cells during adulthood (54, 55). The number of CD45^{+} hematopoietic cells per leg (femur + tibia) was identical in SIRT3/5$^{+/+}$, SIRT3$^{-/-}$, SIRT5$^{-/-}$, and SIRT3/5$^{-/-}$ mice (**Figure 2A**), as well as the composition of the HSC pool which is made of lineage negative, Sca1 positive, c-kit positive (LSK) cells, long-term (LT)-HSC, short-term (ST)-HSC, and multipotent progenitors (MPP) (**Figure 2B**). Accordingly, the percentage and the absolute number of T cells, B cells, neutrophilic granulocytes and monocytes were similar in SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ mice, while some minor changes were observed in SIRT3$^{-/-}$ and SIRT5$^{-/-}$ mice (decreased CD3^{+} T cells and CD19^{+} B cells and increased monocytes) (**Figure 2C** and **Table 1**).

SIRT3/5$^{-/-}$ mice, but not single knockout mice, showed a slight reduction of thymus cellularity when compared to SIRT3/5$^{+/+}$ mice (SIRT3/5$^{+/+}$ vs. SIRT3/5$^{-/-}$ mice: 12.7 ± 1.8 vs. 8.3 ± 1.2 million cells; $P = 0.03$). However, the proportion of CD4/CD8 single positive, double positive and double negative (DN1-DN4) thymocytes was comparable in all mouse lines (**Table 2**). The size of the spleen (SIRT3/5$^{+/+}$, SIRT3$^{-/-}$, SIRT5$^{-/-}$, and SIRT3/5$^{-/-}$ mice: 6.1 ± 1.5, 5.3 ± 1.1, 7.3 ± 1.6, and 7.0 ± 1.8 × 10^7 cells; $P > 0.05$ for all) and the proportion of total T cells, B cells, dendritic cells (DCs), neutrophilic granulocytes and monocytes were not affected in SIRT3/5$^{-/-}$ mice (**Table 3**). Small, statistically significant, differences were noticed between SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ mice, i.e., reduced percentages of effector memory CD4^{+} T cells (23.1 ± 3.3 vs. 17.2 ± 2.3% of CD4^{+} T cells), CD4^{-}CD8^{-} T cells (4.2 ± 0.9 vs. 3.0 ± 0.2% of CD3^{+} T cells) and conventional DC1 (cDC1, 35.4 ± 6.3 vs. 27.2 ± 3.0% of CD11c^{+} DCs) (**Table 3**). Like SIRT3/5$^{-/-}$ mice, SIRT3$^{-/-}$ mice showed a reduced percentage of cDC1, while SIRT5$^{-/-}$ mice showed an increased percentage of Ly6C^{low} (alternative) monocytes at the expense of Ly6C^{high} (inflammatory) monocytes. Collectively, these results suggested that the dual deletion of SIRT3 and SIRT5 had a subtle impact on the development of immune cells.

SIRT3/5 Deficiency Increases the Inflammatory Profile of Macrophages and the Killing Activity of Neutrophils

Macrophages and neutrophils are proficient at sensing microbial products and play key defense roles during infections. SIRT3 and SIRT5 single deficiency did not influence antimicrobial host defenses (37, 38). Therefore, we asked whether dual deficiency of SIRT3 and SIRT5 would reveal a phenotype unseen in single knockouts. SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ BMDMs were exposed to LPS, CpG, and poly(I:C), which are sensed through TLR4, TLR9, and TLR3, respectively. SIRT3/5$^{-/-}$ BMDMs produced higher levels of TNF, IL-6, and IL-12p40 (as a trend for CpG-induced IL-12p40) and lower levels of IL-10 than SIRT3/5$^{+/+}$ BMDMs in response to LPS and CpG, while SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ BMDMs produced similar levels of TNF and IL-6 in response to poly(I:C) (**Figure 3A**).

To address whether the increased inflammation driven by SIRT3/5-deficiency was linked to increased intracellular signaling, we quantified by Western blotting the nuclear

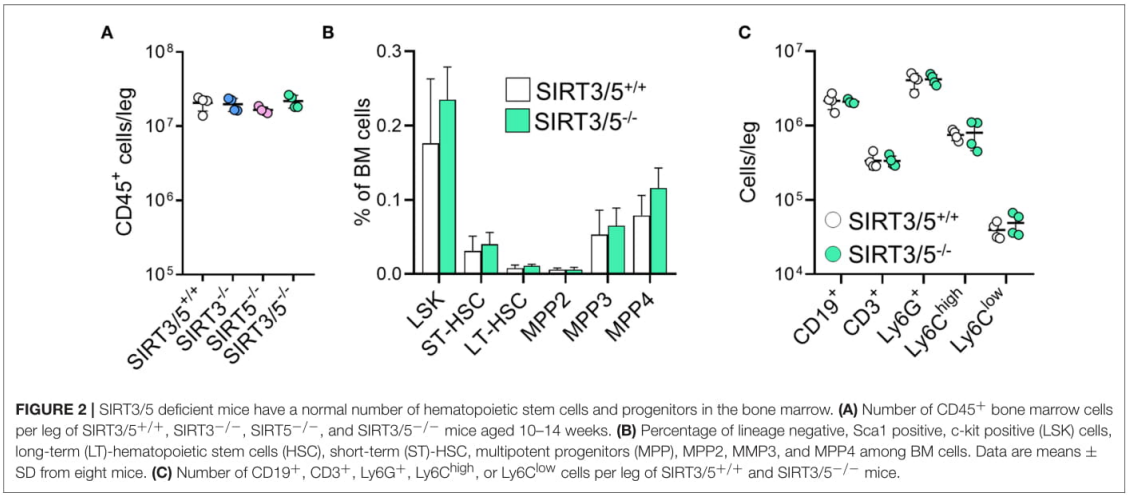

FIGURE 2 | SIRT3/5 deficient mice have a normal number of hematopoietic stem cells and progenitors in the bone marrow. **(A)** Number of $CD45^+$ bone marrow cells per leg of $SIRT3/5^{+/+}$, $SIRT3^{-/-}$, $SIRT5^{-/-}$, and $SIRT3/5^{-/-}$ mice aged 10–14 weeks. **(B)** Percentage of lineage negative, Sca1 positive, c-kit positive (LSK) cells, long-term (LT)-hematopoietic stem cells (HSC), short-term (ST)-HSC, multipotent progenitors (MPP), MPP2, MMP3, and MPP4 among BM cells. Data are means ± SD from eight mice. **(C)** Number of $CD19^+$, $CD3^+$, $Ly6G^+$, $Ly6C^{high}$, or $Ly6C^{low}$ cells per leg of $SIRT3/5^{+/+}$ and $SIRT3/5^{-/-}$ mice.

TABLE 1 | Bone marrow leukocyte subsets.

	$SIRT3/5^{+/+}$ ($n = 4$)	$SIRT3^{-/-}$ ($n = 4$)	$SIRT5^{-/-}$ ($n = 4$)	$SIRT3/5^{-/-}$ ($n = 4$)
$CD3^+$ T cells	2.8 ± 0.4	1.9 ± 0.5	1.6 ± 0.4	2.8 ± 0.5
$CD19^+$ B cells	17.6 ± 1.0	14.8 ± 1.1	16.9 ± 2.8	17.3 ± 1.2
$Ly6G^+$ $Ly6C^-$ granulocytes	33.5 ± 4.2	39.4 ± 1.5	35.9 ± 4.3	34.6 ± 4.0
$Ly6C^+$ $Ly6G^-$ monocytes	6.8 ± 0.4	9.7 ± 1.7	7.0 ± 1.4	8.0 ± 1.9
$Ly6C^{high}$ inflammatory/classical monocytes	93.8 ± 0.5	95.1 ± 0.3	93.2 ± 1.4	93.2 ± 0.8
$Ly6C^{low}$ alternative/patrolling monocytes	6.2 ± 0.5	4.9 ± 0.3	6.9 ± 1.4	6.8 ± 0.8

Data are means ± SD of four mice (aged 10–14 weeks) per group and expressed as the percentage of total cells ($CD3^+$, $CD19^+$, $Ly6G^+$, $Ly6C^+$) or the percentage of parental cells. Gray background: $P < 0.05$ vs. $SIRT3/5^{+/+}$.

TABLE 2 | Thymic cell subsets.

	$SIRT3/5^{+/+}$ ($n = 4$)	$SIRT3^{-/-}$ ($n = 4$)	$SIRT5^{-/-}$ ($n = 4$)	$SIRT3/5^{-/-}$ ($n = 4$)
$CD4^+$	13.0 ± 1.7	15.7 ± 0.7	16.1 ± 2.4	13.3 ± 1.6
$CD8^+$	2.4 ± 0.4	3.4 ± 0.1	3.3 ± 0.7	2.9 ± 0.5
$CD4^+$ $CD8^+$	73.0 ± 1.6	67.4 ± 1.8	67.6 ± 3.2	71.9 ± 2.5
$CD4^-$ $CD8^-$	7.9 ± 0.9	8.9 ± 2.2	8.7 ± 1.3	8.3 ± 2.3
DN1: $CD25^-$ $CD44^+$	10.6 ± 1.8	12.2 ± 5.4	9.1 ± 1.9	10.7 ± 2.7
DN2: $CD25^+$ $CD44^+$	7.7 ± 1.1	8.9 ± 2.2	6.2 ± 1.2	6.4 ± 1.9
DN3: $CD25^+$ $CD44^-$	15.3 ± 2.1	16.8 ± 3.3	14.6 ± 2.2	16.0 ± 2.4
DN4: $CD25^-$ $CD44^-$	66.4 ± 4.8	62.1 ± 10.9	70.1 ± 4.5	66.8 ± 6.7

Data are means ± SD of four mice (aged 7–8 weeks) per group and expressed as the percentage of total thymocytes ($CD4^+$, $CD8^+$, $CD4^+$ $CD8^+$, $CD4^-$ $CD8^-$) or the percentage of $CD4^-$ $CD8^-$ cells. No statistically significant differences in subsets' percentages were detected.

translocation of NF-κB p65 and the phosphorylation of ERK1/2 and p38 MAPKs in BMDMs exposed for 0, 10, 30, and 60 min to LPS (**Figure 3B**). In $SIRT3/5^{-/-}$ BMDMs, there was an increased NF-κB p65 nuclear content at baseline and, albeit not statistically significant, after 30 and 60 min of stimulation. The level of phospho-ERK1/2 was also increased after 30 min of exposure to LPS. Overall, SIRT3/5 deficiency increased inflammatory intracellular signaling pathways and inflammatory cytokine production by BMDMs.

ROS activate the NOD-like receptor pyrin domain-containing-3 (NLRP3) inflammasome that cleaves pro-IL-1β into mature IL-1β that is secreted. Considering that SIRT3 and SIRT5 activate enzymes playing a role in the detoxification process of ROS (i.e., IDH1, IDH2, SOD1, and SOD2) (17–21),

TABLE 3 | Splenic cell subsets.

	SIRT3/5$^{+/+}$ (n = 4)	SIRT3$^{-/-}$ (n = 4)	SIRT5$^{-/-}$ (n = 4)	SIRT3/5$^{-/-}$ (n = 4)
CD3^{+} T cells	21.5 ± 2.6	23.3 ± 3.9	25.0 ± 4.2	24.4 ± 1.5
CD4^{+}	58.7 ± 4.8	56.0 ± 1.9	61.2 ± 2.0	60.9 ± 1.2
CD44^{low} CD62L^{high} naïve	43.0 ± 12.6	48.4 ± 10.3	53.7 ± 8.1	49.5 ± 7.4
CD44^{high} CD62L^{low} memory	23.1 ± 3.3	19.1 ± 2.7	20.2 ± 3.0	17.2 ± 2.3
CD8^{+}	29.6 ± 5.6	34.0 ± 1.8	31.7 ± 0.7	32.2 ± 0.7
CD44^{low} CD62L^{high} naïve	70.4 ± 6.3	73.4 ± 4.7	71.4 ± 3.7	71.3 ± 5.1
CD44^{high} CD62L^{low} memory	3.6 ± 0.9	3.5 ± 0.7	4.8 ± 1.2	3.9 ± 1.0
CD4^{-} CD8^{-}	4.2 ± 0.9	3.8 ± 0.5	3.3 ± 0.6	3.0 ± 0.2
CD4^{+} CD8^{+}	7.6 ± 4.3	6.2 ± 2.1	3.8 ± 1.3	4.0 ± 0.8
B220^{+} B cells	56.3 ± 3.5	52.4 ± 3.9	58.6 ± 1.1	53.5 ± 2.1
IgD^{-} CD23^{+} mature	17.0 ± 5.3	18.0 ± 3.4	24.7 ± 2.9	16.7 ± 3.1
Non-IgD^{+}/CD23^{+} immature	83.0 ± 5.3	82.0 ± 3.4	75.3 ± 2.9	83.3 ± 3.1
CD11c^{+} DCs	2.9 ± 0.4	3.0 ± 0.4	3.3 ± 0.1	2.8 ± 0.2
B220^{+} pDCs	19.1 ± 2.6	19.6 ± 4.8	21.0 ± 2.1	16.8 ± 1.6
B220^{-} cDCs	79.7 ± 2.9	78.9 ± 5.1	77.8 ± 2.2	82.0 ± 1.8
cDC1	35.4 ± 6.3	27.7 ± 5.4	33.6 ± 2.8	27.2 ± 3.0
cDC2	55.1 ± 6.4	63.0 ± 6.2	55.9 ± 3.4	63.4 ± 4.1
Ly6G^{+} Ly6C^{-} granulocytes	6.2 ± 5.5	5.5 ± 3.7	1.2 ± 0.3	5.9 ± 2.2
Ly6C^{+} Ly6G^{-} monocytes	3.7 ± 1.1	3.9 ± 0.7	3.1 ± 0.5	4.7 ± 0.8
Ly6C^{high}	34.1 ± 14.7	25.5 ± 6.4	19.1 ± 3.5	35.9 ± 1.8
Ly6C^{int}	26.0 ± 6.2	30.2 ± 4.0	33.2 ± 2.9	27.7 ± 1.7
Ly6C^{low}	33.5 ± 9.5	37.1 ± 3.5	42.3 ± 2.6	31.1 ± 0.9

Data are means ± SD of four mice (aged 10–14 weeks) per group and expressed as the percentage of total splenocytes (CD3^{+} T cells, B220^{+} B cells, CD11c^{+} DCs, Ly6G^{+} granulocytes, monocytes) or the percentage of parental cells. Gray background: $P < 0.05$ vs. SIRT3/5$^{+/+}$.

we questioned whether SIRT3/5$^{-/-}$ BMDMs produce increased levels of mitochondrial ROS (mtROS) and IL-1β. SIRT3/5$^{+/+}$, SIRT3$^{-/-}$, SIRT5$^{-/-}$, and SIRT3/5$^{-/-}$ BMDMs were exposed to monosodium urate (MSU) crystals, a commonly used activator of the NLRP3 inflammasome before measuring mtROS (**Figure 3C**). MSU crystals induced mtROS equally in SIRT3/5$^{+/+}$ and SIRT5$^{-/-}$ BMDMs, 1.5-fold more in SIRT3$^{-/-}$ BMDMs and 2-fold more in SIRT3/5$^{-/-}$ BMDMs. mRNA levels of Idh1, Idh2, Sod1, and Sod2 were similar in SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ BMDMs (**Figure 3D**), in agreement with the fact that sirtuins target the activity rather than the expression of IDH1, IDH2, SOD1, and SOD2. As expected from the above, SIRT3/5$^{-/-}$ BMDMs secreted higher levels of IL-1β than SIRT3/5$^{+/+}$ BMDMs (**Figure 3E**).

The role of sirtuins in the development and functions of granulocytes is scarce and has not been reported for SIRT5 (56, 57). The mRNA expression levels of SIRT3 and SIRT5 decreased gradually from common myeloid progenitors (CMP) to granulocyte-monocyte progenitor (GMP; 1.6-fold) and from GMP to neutrophilic granulocytes (5.3–5.7-fold) (**Figure 4A**). Compared to SIRT3/5$^{+/+}$ mice, SIRT3/5$^{-/-}$ mice expressed in the bone marrow statistically significantly more CMP (P = 0.03) but not GMP (P = 0.06) (**Figure 4B**). Accordingly, SIRT3/5$^{-/-}$ mice expressed normal numbers of neutrophilic granulocytes in the bone marrow and spleen (**Figure 2C** and **Table 3**). We then addressed whether SIRT3/5 deficiency affected neutrophil functions. We setup a killing assay in which neutrophils were incubated for 1 h with *Listeria monocytogenes* before quantifying bacteria. As shown in **Figure 4C**, 115, 96, 77, and 66% of the starting inoculum were recovered from assays using SIRT3/5$^{+/+}$, SIRT3$^{-/-}$, SIRT5$^{-/-}$, and SIRT3/5$^{-/-}$ neutrophils, respectively. Hence, the dual deletion of SIRT3 and SIRT5 promoted the killing of *L. monocytogenes* by neutrophils. This prompted us to analyze two main mechanisms through which neutrophils kill bacteria, i.e., the production of ROS and the release of neutrophil extracellular traps (NETs). SIRT3/5$^{-/-}$ neutrophils, and to a lesser extent SIRT3$^{-/-}$ and SIRT5$^{-/-}$ neutrophils, produced increased levels of ROS when compared to SIRT3/5$^{+/+}$ neutrophils (**Figure 4D**). In contrast, SIRT3/5$^{+/+}$, SIRT3$^{-/-}$, SIRT5$^{-/-}$, and SIRT3/5$^{-/-}$ neutrophils produced similar amounts of NETs (**Figure 4E**). Thus, the proficient killing of *L. monocytogenes* by SIRT3/5$^{-/-}$ neutrophils was more likely related to an increased generation of ROS than NETs. Lastly, we measured by Luminex the cytokines released by whole blood exposed to heat-killed *L. monocytogenes*. Fifteen of 17 mediators were produced at measurable levels: CCL2, CCL3, CXCL1, CXCL5, CXCL10, G-CSF, IFNγ, IL-1α, IL-1β, IL-6, IL-10, IL-12p40, IL-17A, IL-18, and TNF. Going well along with an increased inflammatory response of BMDMs (**Figure 3**), SIRT3/5$^{-/-}$ whole blood produced more G-CSF and showed a trend toward producing more IL-1α, IL-6, and IFNγ than SIRT3/5$^{+/+}$ whole blood (**Figure 4F**). The production of other cytokines was not affected.

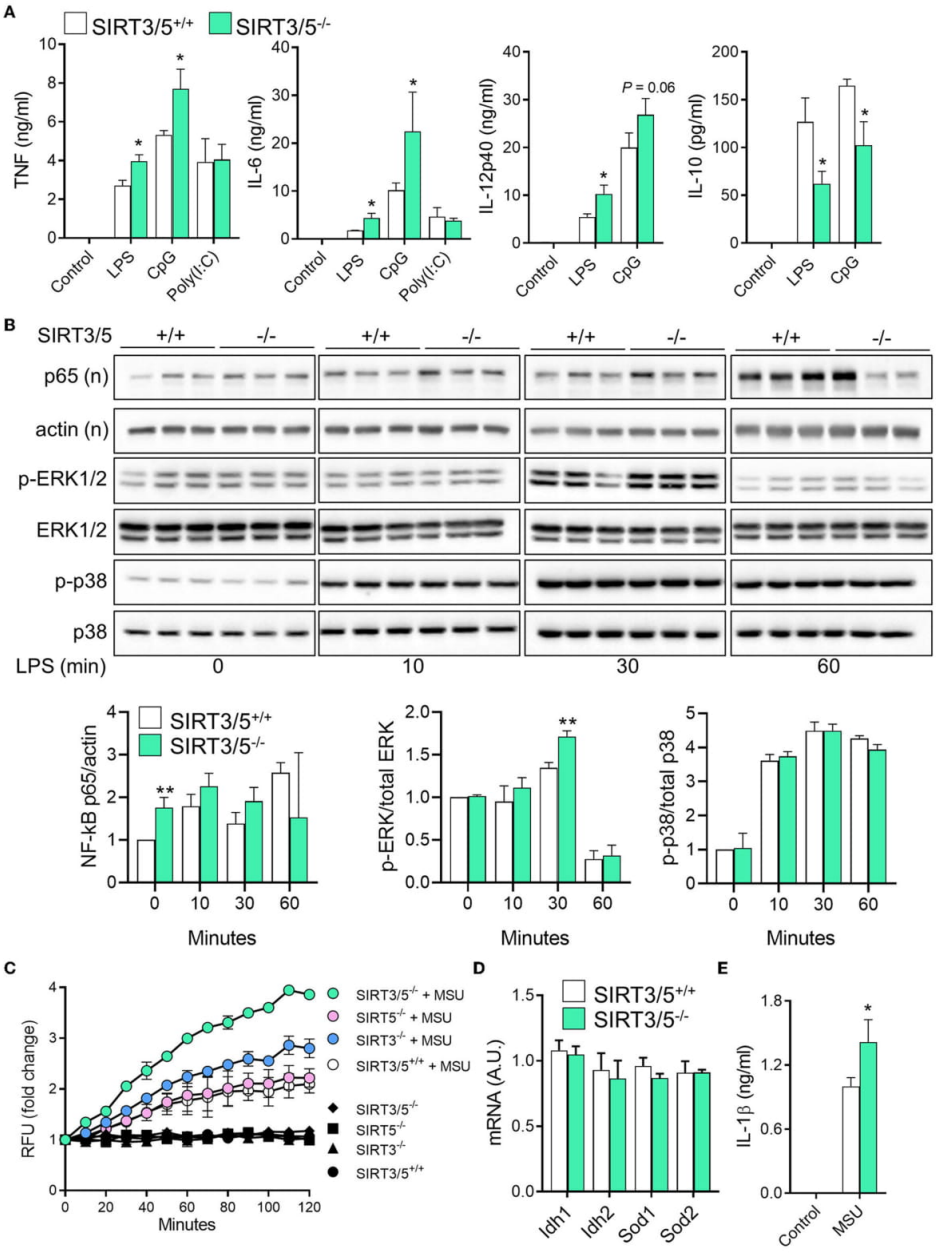

FIGURE 3 | SIRT3/5 deficient macrophages display an enhanced proinflammatory profile. **(A)** SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ BMDMs were exposed for 24 h to LPS (10 ng/ml), CpG (1 μg/ml), and poly(I:C) (10 μg/ml). TNF, IL-6, IL-12p40, and IL-10 concentrations in cell culture supernatants were quantified by ELISA. Data are means ± SD from one experiment performed with four mice. **(B)** SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ BMDMs were exposed for 0, 10, 30, and 60 min to LPS (10 ng/ml). Nuclear (n) and total protein extracts were used to analyze NF-κB p65, and phosphorylated (p) and total ERK1/2 and p38. Signals were quantified by

(Continued)

FIGURE 3 | imaging and results (ratio p65/actin, p-ERK1/2/ERK1/2, and p-p38/p38) expressed relative to the results obtained in resting SIRT3/5$^{+/+}$ BMDMs set at 1. Data are means ± SD from one experiment performed with three mice. Full blots are presented in **Supplementary Figure S1**. **(C–E)** BMDMs were primed with Pam_3CSK_4 (10 ng/ml) for 18 h and exposed (colored and white symbols) or not (black symbols) to MSU crystals for the indicated time **(C)** or 6 h **(E)**. mtROS were quantified using MitoSOX **(C)**, Idh1, Idh2, Sod1, and Sod2 mRNA levels by RT-PCR **(D)** and IL-1β by ELISA **(E)**. Gene expression levels were normalized to actin levels. A.U, arbitrary unit. Data are mean ± SD of four mice aged 10–12 weeks analyzed in triplicate **(C–E)**. *$P < 0.05$; **$P < 0.01$.

SIRT3/5 Deficiency Provides a Modest Protection to Listeriosis

Myeloid cells play a crucial role in protecting from *L. monocytogenes* infection (58, 59). Considering that SIRT3/5 deficiency increased *L. monocytogenes* killing by neutrophils (**Figure 4C**) and cytokine production by macrophages and to some extent by whole blood (**Figures 3A**, **4F**), we tested the relevance of these observations *in vivo* using a model of listeriosis. Mice challenged intravenously with a high inoculum of *L. monocytogenes* (0.9–1.5 × 10^5 cfu) exhibited signs of disease 36–40 h post-infection and were severely sick after 65 h as shown by elevated severity scores in most animals. SIRT3/5$^{-/-}$ mice were not as ill as SIRT3/5$^{+/+}$ mice ($P = 0.01$; **Figure 5A**) and had 2.3-fold less *L. monocytogenes* in blood collected 48 h after infection (SIRT3/5$^{+/+}$ vs. SIRT3/5$^{-/-}$: 3.5 ± 0.9 × 10^3 cfu/ml vs. 1.5 ± 0.3 × 10^3 cfu/ml; median ± SEM; $P = 0.005$) (**Figure 5B**). In line with these observations, SIRT3/5$^{-/-}$ mice had a modest, statistically significant, delayed mortality rate compared to SIRT3/5$^{+/+}$ mice (median survival of SIRT3/5$^{+/+}$ vs. SIRT3/5$^{-/-}$: 3.0 vs. 3.12 days; $P = 0.01$) (**Figure 5C**). Going well along with an improved response to infection, SIRT3/5$^{-/-}$ mice had significantly higher blood concentrations of TNF at day 1 ($P = 0.001$), TNF and IL-1β at day 2 ($P = 0.004$ and 0.03) and KC/CXCL1 at day 3 ($P = 0.05$). Albeit not statistically significant, the levels of G-CSF, IL-1α, MCP-1/CCL2, MIP-2/CXCL2 at day 1, G-CSF, KC, MCP-1, and MIP-2 at day 2, and IL-1α, IL-1β, IL-6, IL-10, MCP-1, MIP-2, and TNF at day 3 post-infection were 1.5–4.4-fold higher in SIRT3/5$^{-/-}$ than in SIRT3/5$^{+/+}$ mice (**Figure 5D**). As expected, 2 days after the onset of infection, listeriosis induced a dramatic drop of circulating leukocytes including B cells, T cells, neutrophils and Ly6C^{high} inflammatory monocytes that rebounded at day 3 (**Figure 5E**). No differences were observed between SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ mice, suggesting that the reactivity rather than the number of blood leukocytes conferred some protection to SIRT3/5$^{-/-}$ mice during listeriosis. Interestingly, there was no statistically significant difference of mortality when SIRT3/5$^{-/-}$ and SIRT3/5$^{+/+}$ mice were challenged with a low inoculum (7.3 × 10^3 cfu) of *L. monocytogenes* responsible for <25% death (**Figure 5C**). Finally, we tested the mouse lines in a model of endotoxemia induced by an intraperitoneal challenge with 10 mg/kg LPS. Surprisingly, there was no statistically significant difference between the SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ groups ($n = 21$–22 mice per group; $P = 0.1$).

DISCUSSION

This is the first report about the impact of the dual deficiency of SIRT3 and SIRT5 on immune cell development and antimicrobial host defenses. Double knockout mice developed normally and showed subtle, minor alterations of immune cell subpopulations and host responses to infection. Together with the fact that SIRT3$^{-/-}$ and SIRT5$^{-/-}$ mice are susceptible to bacterial sepsis like wild-type mice (37–40), these observations strengthen the development of pharmacological modulators of the activity of mitochondrial sirtuins for clinical purposes.

Notwithstanding that SIRT3 and SIRT5 orchestrate metabolism and oxidative stress responses, SIRT3 and SIRT5 whole body knockout mice have no macroscopic abnormalities (41, 42). The SIRT3/5$^{-/-}$ mouse line we generated here developed normally. No problem of fertility, sex distribution and growth were noticed. Surprisingly, the metabolism of SIRT3/5$^{-/-}$ BMDMs was similar to that of SIRT3/5$^{+/+}$ BMDMs. Yet, the impact of SIRT3 and SIRT5 on metabolism was mainly demonstrated in cells or tissues such as the liver and the heart that are rich in mitochondrial sirtuins when compared to macrophages (38, 41, 60). Another SIRT3/5$^{-/-}$ mouse line has been recently generated. In line with our observations, no developmental defects were reported. Moreover, these SIRT3/5$^{-/-}$ mice were susceptible to streptozotocin-induced hyperglycemia like controls, while showing only a modest inner retinal dysfunction (61, 62).

Studies on the role of sirtuins in hematopoiesis and immune cell development are scarce. SIRT3/5$^{-/-}$ mice had a normal pool of HSCs and MPPs and a slightly increased number of CMP (and GMP as a trend) in their bone marrow. The primary and secondary immune organs of SIRT3/5$^{-/-}$ mice were largely unaffected, according to absolute numbers and proportions of immune cell subpopulations. There was only a slight reduction of thymus size, which did not impact the proportion of thymocyte subpopulations. This reminds the phenotype of SDHD-ESR mice with deletion of the *succinate dehydrogenase, subunit D* gene encoding for one of the subunits of the mitochondrial complex II (63). SDHD mice have a thymic atrophy without perturbation of thymocyte development. Overall, deficiencies in SIRT3, SIRT5, and SIRT3/5 do not seem to have a dramatic impact on immune cell development and/or functions [(37, 38) and present study]. Nonetheless, a role for these enzymes might come to light under stress or stimulatory conditions, or in aged mice. For example, SIRT1 shaped the T helper (Th) and T regulatory (Treg) responses of naïve T cells (64–68). Furthermore, in SIRT1$^{-/-}$ mice, the percentages of CD4$^+$, CD8$^+$, and CD4$^+$CD8$^+$ T cell subpopulations were normal, but thymocytes were at increased sensitivity to ionizing radiation induced DNA damaging (69). Finally, SIRT3$^{-/-}$ mice of 18–24 months had a lower frequency of bone marrow hematopoietic progenitors than mice of 12 weeks (70).

SIRT3/5$^{-/-}$ macrophages exposed to TLR agonists produced more inflammatory cytokines and less IL-10 than SIRT3/5$^{+/+}$

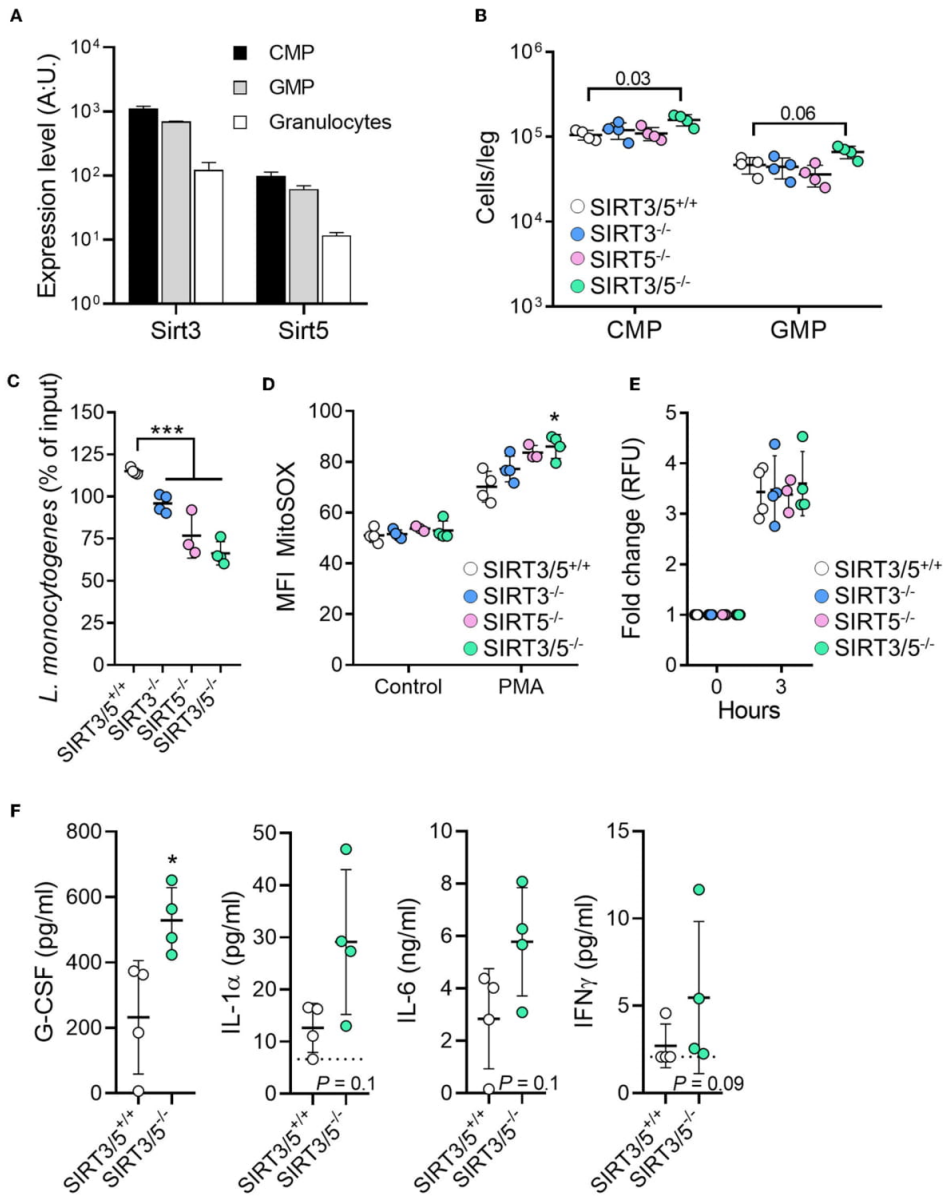

FIGURE 4 | Increased killing of *Listeria monocytogenes* by SIRT3/5 deficient neutrophils. **(A)** Sirt3 and Sirt5 mRNA expression levels in common myeloid progenitors (CMP), granulocyte-monocyte progenitor (GMP), and granulocytes. Data were extracted from BioGPS (http://biogps.org). **(B)** Number of CMP (Lin^- c-kit^+ $CD34^+$ cells) and GMP (Lin^- c-kit^+ $CD135^+$ $CD34^+$ cells) per leg of $SIRT3/5^{+/+}$, $SIRT3^{-/-}$, $SIRT5^{-/-}$, and $SIRT3/5^{-/-}$ mice aged 8–9 weeks. The gating strategies are presented in **Supplementary Figure S2. (C–E)** Killing of bacteria **(C)** and production of mtROS **(D)** and NETs **(E)** by neutrophils. Neutrophils were incubated for 1 h with *L. monocytogenes* (0.1 cfu/cell) **(C)**, for 50 min with PMA (100 nM) and MitoSOX **(D)** or for 3 h with PMA (100 nM) and SYTOX **(E)**. Bacteria were enumerated and results expressed relative to the initial inoculum set at 100% **(C)**. $^*P < 0.05$; $^{***}P < 0.005$. **(F)** G-CSF, IL-1α, IL-6, and IFNγ concentrations, determined by Luminex, in blood from $SIRT3/5^{+/+}$ and $SIRT3/5^{-/-}$ mice incubated with heat-killed *L. monocytogenes* for 24 h. The concentrations of CCL2, CCL3, CXCL1, CXCL5, CXCL10, IL-1β, IL-10, IL-12p40, IL-17A, IL-18 and TNF were similar for $SIRT3/5^{+/+}$ and $SIRT3/5^{-/-}$ blood.

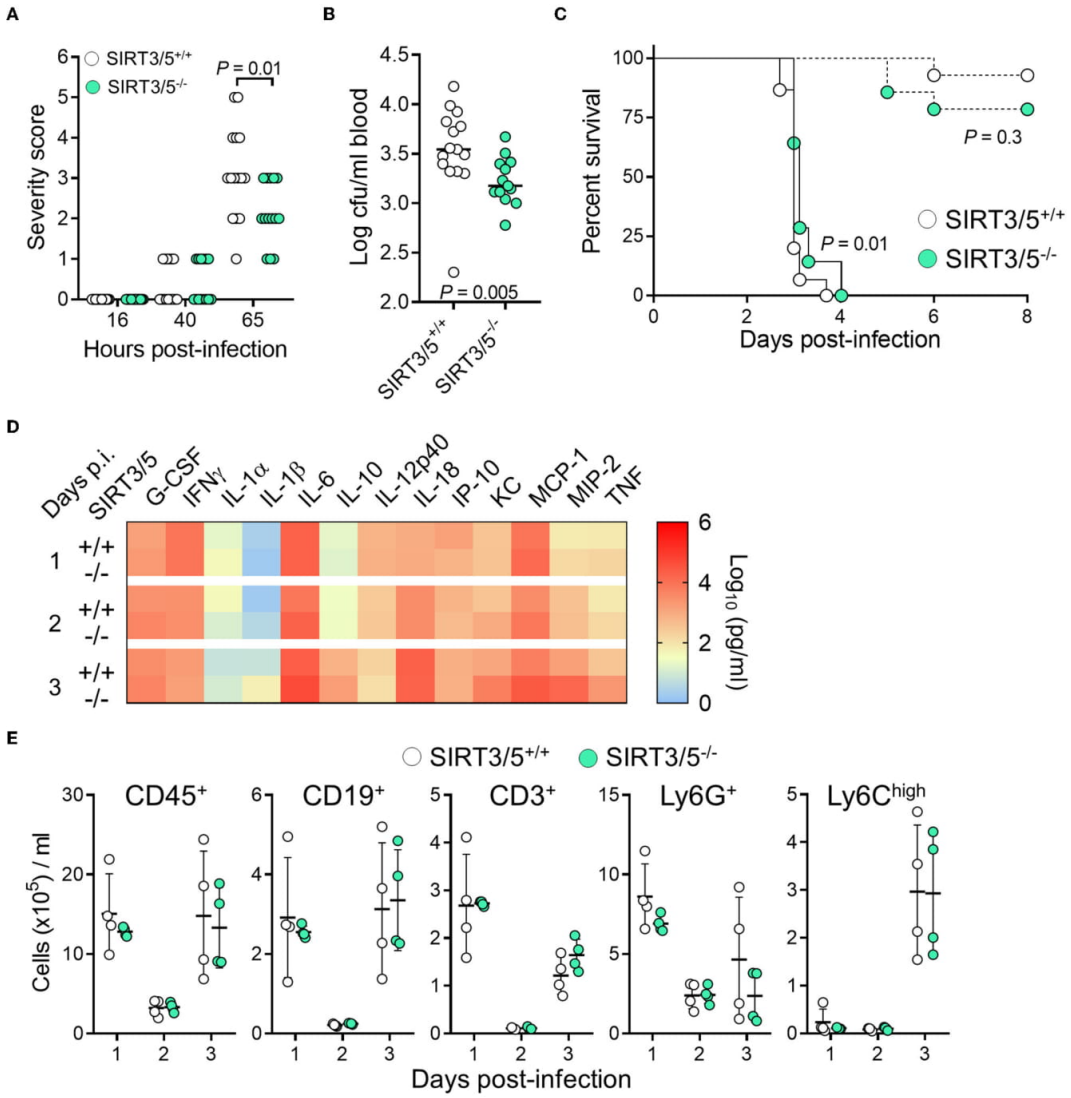

FIGURE 5 | SIRT3/5 deficiency confers some protection to listeriosis. **(A–E)** SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ mice (n = 14–15 per group, aged 8–12 weeks) were injected intravenously with 7.3 × 10^3 cfu (**C**, dashed lines) or 0.9–1.5 × 10^5 cfu *L. monocytogenes* (**A–E**, plain lines in **C**). **(A)** Severity scores were recorded. **(B)** *L. monocytogenes* in blood collected 2 days post-infection. **(C)** Survival of mice. **(D)** Cytokines, quantified by Luminex, in blood collected 1, 2, and 3 days post-infection (p.i.). The horizontal bar represents the median. Data are mean ± SD of four mice per group. P = 0.001 for TNF at day 1 p.i. 0.004 and 0.03 for TNF and IL-1β at day 2 p.i. and 0.05 for KC at day 3 p.i. ENA-78, IL-3, IL-17A, and MIP-1α were not detected. **(E)** CD45$^+$, CD19$^+$, CD3$^+$, Ly6G$^+$ and Ly6C^{high} leukocytes in blood collected 1, 2, and 3 days post-infection. Each dot represents a mouse, and line and bars mean ± SD.

macrophages, contrary to SIRT3$^{-/-}$ and SIRT5$^{-/-}$ macrophages that behaved like wild-type cells (37, 38). Accordingly, NF-κB and MAPK signaling pathways were increased in resting and/or LPS-stimulated SIRT3/5$^{-/-}$ macrophages. These data somehow support the possibility that SIRT3 and SIRT5 compensate each other in single knockout animals. Sirtuins are generally considered to drive anti-inflammatory responses. However, as nicely reviewed recently for SIRT1 (68), sirtuins may promote proinflammatory and anti-inflammatory effects depending on the context and whether myeloid or lymphoid cells are

considered. For example, SIRT5 deficiency was associated with both increased and decreased innate inflammatory response *in vivo* (33, 71). More generally, contrasting observations have been reported for most sirtuins (SIRT1-3, SIRT5-6). Methodological differences may explain these differences when studying monocytes/macrophages (37, 38, 46): the origin/fate of the cells (BMDMs vs. peritoneum macrophages vs. established macrophage cell lines, growth factors used for differentiation and maturation state of macrophages), strategies to delete or overexpress sirtuins or to modulate sirtuin activity (siRNA, shRNA, expression vectors, full or cell-specific knockouts, pharmacological activators, and inhibitors), readouts, and subtle variations in NAD^+ concentrations and circadian rhythm known to affect sirtuin activity or expression.

The expression levels of SIRT3 and SIRT5 decreased gradually from CMP to GMP and from GMP to granulocytes, which mirror the decline of mitochondrial mass and mitochondrial DNA during hematopoietic differentiation (72). Granulopoiesis relies on the expression of CCAAT/enhancer binding protein (C/EBP). The expression of SIRT1, which deacetylates C/EBPε and represses neutrophil terminal differentiation (73), declines during granulopoiesis (727 ± 13, 643 ± 20, and 307 ± 61 mRNA arbitrary units in CMP, GMP, and granulocytes, respectively). Thus, the downregulation of sirtuins seems to be a general feature associated with neutrophil development. The fact that neutrophil counts were normal and not increased in SIRT3/5$^{-/-}$ mice suggests either the implementation of compensatory mechanisms, possibly through SIRT1, or that SIRT3 and SIRT5 have a modest influence on granulopoiesis.

Neutrophils produce cytotoxic compounds that target pathogenic bacteria and fungi but are harmful for host tissues (74). Contrary to SIRT3/5$^{+/+}$ neutrophils, and better than SIRT3$^{-/-}$ and SIRT5$^{-/-}$ neutrophils, SIRT3/5$^{-/-}$ neutrophils killed *L. monocytogenes*, which was associated with an augmented production of ROS but not of NETs. In comparison, neutrophils deficient in SIRT3 had a mild increase of intracellular ROS but performed either normal or increased NETosis (56, 57). SIRT1 deficiency did not impact on neutrophil functions, and the role of the remaining sirtuins has not been reported. Whereas, SIRT3$^{-/-}$ mice had increased neutrophil infiltration in lungs during sterile injury (36, 75) and mycobacterial infection impairing the survival of mice (76), SIRT5$^{-/-}$ had reduced inflammation and ischemia/reperfusion brain injury (77). Finally, SIRT3$^{-/-}$ and SIRT5$^{-/-}$ mice behaved like wild-type mice in models of sepsis requiring neutrophils to fight the infectious agents (37–40). Interestingly, SIRT3/5$^{-/-}$ mice resisted better than SIRT3/5$^{+/+}$ mice to acute listeriosis, showing decreased signs of morbidity, reduced blood bacterial loads and significant albeit modest delayed mortality. SIRT3/5$^{-/-}$ mice expressed higher concentrations of cytokines but normal counts of leukocytes in blood, suggesting that the reactivity rather than the number of leukocytes protected SIRT3/5$^{-/-}$ mice from listeriosis. Interestingly, SIRT3/5$^{-/-}$ mice were not more resistant to mild listeriosis than their wild type counterparts, and behaved like wild type mice in a model of endotoxemia. These observations support the assumption that drugs targeting SIRT3 and SIRT5 should not have a deletary impact on host defenses, which would contrast with drugs targeting classical HDACs that strongly impaired innate immune defenses against infections in preclinical models and clinical settings (78–84).

Overall, the double deficiency in SIRT3 and SIRT5 had rather modest and subtle impacts on immune cell development and anti-microbial host defenses. It might be that SIRT4, the remaining mitochondrial sirtuin in SIRT3/5$^{-/-}$ mice, compensated for SIRT3 and SIRT5 absence. Unfortunately, whether SIRT4 affects immune responses has not been reported. Considering the link between sirtuins, metabolism and age-associated pathologies, it is possible that phenotypes will emerge in aged mice or in mice submitted to metabolic stress. SIRT3/5$^{-/-}$ mice should be tested in other preclinical models of sepsis. Nonetheless, putting together the data from the present study together with the fact that single deficiencies in SIRT3 and SIRT5 had no impact in a large panel of experimental sepsis (37–40), one may foresee that therapies directed against mitochondrial sirtuins or concomitant targeting of SIRT3 and SIRT5 activity should have no deep impact on antibacterial host defenses.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

The animal study was reviewed and approved by Service des Affaires Vétérinaires, Direction Générale de l'Agriculture, de la Viticulture et des Affaires Vétérinaires (DGAV), état de Vaud (Epalinges, Switzerland; authorizations 876.9 and 877.9).

AUTHOR CONTRIBUTIONS

TH and EC performed *in vitro* experiments. TH, EC, and DLR performed *in vivo* experiments. TR and TH conceived the project, designed the experiments and wrote the paper. All the authors revised the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02341/full#supplementary-material

Supplementary Figure S1 | Full blots used to extract the panels shown in **Figures 1B**, **3B**.

Supplementary Figure S2 | Gating strategies for flow cytometry analysis. CD117 encodes for c-kit.

Supplementary Figure S3 | (A) Extracellular acidification rate (ECAR) by SIRT3/5$^{+/+}$ and SIRT3/5$^{-/-}$ BMDMs exposed to LPS (10 ng/ml) measured using the Seahorse technology. **(B)** Oxygen consumption rate (OCR) and ECAR by SIRT3/5$^{+/+}$ and SIRT3$^{-/-}$ BMDMs measured using the Seahorse technology. Data are means ± SD from three mice aged 10–12 weeks analyzed in quadruplicate.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.7 Trained immunity confers broad-spectrum long-term protection against infections

Eleonora Ciarlo[1], Tytti Heinonen[1], Charlotte Théroude[1], Fatemeh Asgari[1], Jacobus Herderschee[1], Didier Le Roy[1], Mihai G. Netea[3,4], Thierry Roger[1]

[1]Infectious Diseases Service, Department of Medicine, Lausanne University Hospital and University of Lausanne, Epalinges, Switzerland

[2]Department of Biomedical Sciences, Humanitas University, Milan, Italy

[3]Radboud Center for Infectious Diseases (RCI), and Department of Internal Medicine, Radboud University Medical Center, Nijmegen, The Netherlands

[4]Department for Genomics & Immunoregulation, Life and Medical Sciences Institute (LIMES), University of Bonn, Bonn, Germany

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Summary

Innate immune cells can recall a first insult to mount a stronger response to a subsequent challenge, a process called “trained immunity”. Trained immunity involves metabolic, epigenetic and functional reprogramming of immune cells and myeloid stem cells. However, the breadth of the protection afforded by trained immunity remains unknown.

Based on the literature, we developed a model of training by two intraperitoneal injections of zymosan, a yeast extract rich in β-glucan. Trained mice had increased numbers of inflammatory monocytes and neutrophils in their blood and bone marrow, which was associated with an enhanced bone marrow myelopoisesis. The blood of trained mice controlled better the growth of *Listeria monocytogenes* and produced higher levels of cytokines. In line, trained mice were powerfully protected from systemic infections, peritonitis, enteritis and pneumonia induced by *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Citrobacter rodentium* and *Pseudomonas aeruginosa*. The protection against listeriosis was dependent on monocytes/macrophages but not neutrophils, and required intact IL-1 signaling. Finally, trained immunity protected mice from listeriosis and *E. coli* peritonitis for at least 5 weeks.

Our data demonstrate that trained immunity protects mice from a broad range of infections. Therefore, trained immunity based therapies might be used to improve host antimicrobial defenses.

My contribution to this work: I analysed cell populations in the blood, peritoneum and bone marrow by flow cytometry. I developed the *in vitro* training and blood stimulation/killing models. I performed ELISA and Luminex analyses and participated to the follow-up of mice.

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MAJOR ARTICLE

Trained Immunity Confers Broad-Spectrum Protection Against Bacterial Infections

AQ6 Eleonora Ciarlo,[1] Tytti Heinonen,[1] Charlotte Théroude,[1] Fatemeh Asgari,[2] Didier Le Roy,[1] Mihai G. Netea,[3,4] and Thierry Roger[1]

[1]Infectious Diseases Service, Department of Medicine, Lausanne University Hospital and University of Lausanne, Epalinges, Switzerland, [2]Department of Biomedical Sciences, Humanitas University, Milan, Italy, [3]Radboud Center for Infectious Diseases, and Department of Internal Medicine, Radboud University Medical Center, Nijmegen, The Netherlands, and [4]Department for Genomics & Immunoregulation, Life and Medical Sciences Institute, University of Bonn, Bonn, Germany

Background. The innate immune system recalls a challenge to adapt to a secondary challenge, a phenomenon called *trained immunity*. Training involves cellular metabolic, epigenetic and functional reprogramming, but how broadly trained immunity protects from infections is unknown. For the first time, we addressed whether trained immunity provides protection in a large panel of preclinical models of infections.

Methods. Mice were trained and subjected to systemic infections, peritonitis, enteritis, and pneumonia induced by *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Citrobacter rodentium*, and *Pseudomonas aeruginosa*. Bacteria, cytokines, leukocytes, and hematopoietic precursors were quantified in blood, bone marrow, and organs. The role of monocytes/macrophages, granulocytes, and interleukin 1 signaling was investigated using depletion or blocking approaches.

Results. Induction of trained immunity protected mice in all preclinical models, including when training and infection were initiated in distant organs. Trained immunity increased bone marrow hematopoietic progenitors, blood Ly6C^{high} inflammatory monocytes and granulocytes, and sustained blood antimicrobial responses. Monocytes/macrophages and interleukin 1 signaling were required to protect trained mice from listeriosis. Trained mice were efficiently protected from peritonitis and listeriosis for up to 5 weeks.

Conclusions. Trained immunity confers broad-spectrum protection against lethal bacterial infections. These observations support the development of trained immunity-based strategies to improve host defenses.

Keywords. innate immunity; infection; sepsis; trained immunity; peritonitis; *Listeria*; pneumonia; monocyte/macrophage; neutrophil; stem cell.

We used preclinical models to demonstrate that trained immunity confers broad-spectrum protection against bacterial infections. Trained immunity increased myeloid progenitors and circulating inflammatory monocytes and neutrophils, and depletion or neutralization of monocytes/macrophages and interleukin 1 signaling impaired trained immunity-mediated protection.

Innate immune cells express pattern recognition receptors specific for microbial-associated and danger-associated molecular patterns that are released by stressed or injured cells. The interaction of pattern recognition receptors with microbial- or danger-associated molecular patterns activates intracellular signaling pathways that coordinate metabolic adaptation, epigenetic changes, and gene expression. The cellular and soluble mediators mobilized on infection regulate the development of the inflammatory response, the establishment of antimicrobial cellular and humoral responses, and the restoration of homeostasis once the pathogen has been contained or eradicated. Dysfunctions in these processes may have dramatic consequences for the infected host, as observed in patients with sepsis [1–5].

It has long been thought that immunological memory was restricted to antigen-specific memory and a privilege of the adaptive immune system carried by lymphocytes. However, the description of systemic acquired resistance in plants, specific memory in invertebrates, antigen-specific memory by natural killer cells, and heterologous protection conferred by BCG, smallpox, and measles vaccines suggested the existence of a form of innate immune memory [6–13]. The term *trained immunity* was proposed to reflect the fact that the innate immune system recalls or adapts to a first challenge to mount a robust response to a secondary challenge by a similar or dissimilar microbial stimulus [14, 15]. The concept of innate immune training was poised by showing that a nonlethal challenge by

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Correspondence: Thierry Roger, Infectious Diseases Service, Lausanne University Hospital and University of Lausanne, CLED.04.407, Chemin des Boveresses 155, CH-1066 Epalinges, Switzerland (Thierry.Roger@chuv.ch).

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Candida albicans improved innate immune response of mice [16].

The molecular mechanisms underlying trained immunity include metabolic, epigenetic, and functional reprogramming of bone marrow myeloid precursors and innate immune cells. β-Glucan, a fungal cell wall compound commonly used to study trained immunity, is detected by monocytes/macrophages through the dectin 1 receptor. Dectin 1 triggering activates a PI3K/AKT/mTOR/HIF-1α pathway that induces a metabolic shift toward aerobic glycolysis, increases glutaminolysis that replenishes the tricarboxylic acid cycle, activates the cholesterol synthesis pathway, and blocks the itaconate pathway [16–21]. As a consequence, metabolites such as fumarate, succinate, and mevalonate accumulate and act as cofactors of epigenetic modifiers and as amplifiers of trained immunity [19, 22]. β-Glucan, *C. albicans,* and BCG vaccine induce genome-wide epigenetic changes, including monomethylation and trimethylation of histone (H) 3 lysine (K) 4 and acetylation of H3K27 at promoters and enhancers of genes associated with metabolic, immune, and host defense pathways [16, 23, 24]. Hence, trained monocytes/macrophages produce increased levels of cytokines (tumor necrosis factor [TNF], interleukin 1β (IL-1β), and interleukin 6 [IL-6]) when challenged with microbial compounds [16, 19, 24, 25].

Whether the induction of trained immunity confers a wide-ranging advantage during infections is unknown. Therefore, we questioned to which extent trained immunity protects from heterologous infections and at anatomic sites distant from the priming training site. Our results showed that a unique training scheme potently protected mice from clinically relevant pathogens inoculated through diverse routes to induce peritonitis, systemic infections, enteritis and pneumonia. Trained immunity was particularly efficient at protecting mice from lethal listeriosis, which was dependent on monocytes/macrophages and interleukin 1 (IL-1) signaling.

MATERIALS AND METHODS

Products used in this study are described in Supplementary Table 1.

Ethics Statement

Animal experimentation was approved by the Service des Affaires Vétérinaires, Direction Générale de l'Agriculture, de la Viticulture et des Affaires Vétérinaires, état de Vaud (Epalinges, Switzerland) under authorizations 876.9 and 877.9 and performed according to Swiss and ARRIVE guidelines.

Mice, Cells, and Bacteria

C57BL/6J (wild-type, MyD88$^{-/-}$, and Toll-like receptor [TLR] 2$^{-/-}$), and BALB/cByJ female mice (Charles River Laboratories) were 8–10 weeks old. Mice were housed under specific pathogen-free conditions (license VD-H04). Mice were free of mouse hepatitis and norovirus. Bone marrow cells were cultured in Roswell Park Memorial Institute medium [26], supplemented with 50 IU/mL macrophage colony-stimulating factor (M-CSF) (ImmunoTools) to generate bone marrow–derived macrophages (BMDMs). BMDMs were trained as described elsewhere [18, 23]. Bone marrow cells were cultured for 24 hours with 10 μg/mL zymosan and M-CSF, washed, cultured 6 days in fresh medium containing M-CSF, detached, enumerated, and seeded (2×10^6 cells/mL) in 96-well plates.

Peritoneal cells obtained by a peritoneal lavage were plated at 10^5 cells/well in 96-well plate in 100 μL of Roswell Park Memorial Institute, washed after 4 hours, and stimulated for 24 hours. *Listeria monocytogenes* 10403S, methicillin-resistant *Staphylococcus aureus* AW7, and *Escherichia coli* O18 were grown in brain-heart infusion broth, *Citrobacter rodentium* DBS100 in LB Broth Miller, *Pseodomonas aeruginosa* PAO1 in LB Broth Lennox, and *C. albicans* 5102 in yeast extract–peptone-dextrose [27–30]. Heat inactivation was performed for 2 hours at 70°C.

Flow Cytometry

Cells were collected and stained as described in the **Supplementary Methods**, using antibodies described in Supplementary Table 1 [31, 32]. Data were acquired using an Attune NxT Flow Cytometer (Thermo Fisher Scientific) and analyzed using FlowJo_V10_CL software (FlowJo). Gating strategies are presented in Supplementary Figure 1.

Whole-Blood Bactericidal Assay and Cytokine Production

The whole-blood assay is described in the **Supplementary Methods.** Cytokines were quantified by means of enzyme-linked immunosorbent assay, a ProCarta kit (Invitrogen), and a Bio-Plex 200 system (Bio-Rad) [33].

Isolation of Bone Marrow Monocytes and Chromatin Immunoprecipitation

The isolation of bone marrow monocytes and chromatin immunoprecipitation were performed as described in the **Supplementary Methods** [34].

In Vivo Models

Age-matched female mice were randomly divided into groups. Mice were injected intraperitoneally with 1 mg of zymosan or heat-killed *C. albicans* or intravenously with 0.1 mg of zymosan before bacterial challenge. Staphylococcal sepsis was also tested in BALB/cByJ mice. Enteritis was induced in TLR2$^{-/-}$ mice deprived of food for 8 hours before bacterial challenge. The role of monocytes/macrophages was assessed using mice injected intraperitoneally with 200 μL of clodronate or phosphate-buffered saline liposomes (LIPOSOMA research) 7 and 4 days before infection. The role of polymorphonuclear neutrophils (PMNs) was assessed using mice injected intraperitoneally with 100 μg of 1A8 anti-Ly6G monoclonal antibody (mAb) or 2A3 immunoglobulin G2a isotype control mAb (Bio X Cell) 6 days, 3 days, and 1 day before infection.

Cell depletion (≥95% and ≥60% for monocytes/macrophages and PMNs, respectively) was evaluated on the day of infection by means of flow cytometry. Depletion in trained mice reduced PMNs to levels similar to those measured in isotype control mAb-treated untrained mice (mean [standard deviation], 3.7 [1.2] vs 2.7 [0.7] × 10^6 PMNs/mL; n = 8; $P > .05$). To assess the role of IL-1 signaling, mice were injected intraperitoneally with 500 μg of anakinra (Kineret; Sobi) 6, 5, 4, and 3 days before infection. Body weight, severity score, and survival were registered at least once daily [35].

Statistical Analyses

Graphics represent data obtained from individual mice, or boxes with minimum-to-maximum whiskers. Data were analyzed for normal distribution and homogeneity of variances and compared with the appropriate parametric (2-tailed unpaired Student *t*) or nonparametric (2-tailed Mann-Whitney) statistical test. The false discovery rate was controlled, when necessary. The Kaplan-Meier method was used for building survival curves, and differences were analyzed using the log-rank sum test. Analyses were performed using PRISM software (GraphPad Software). *P* values were 2 sided.

RESULTS

Trained Immunity Protects From *E. coli* Peritonitis

C57BL/6J mice were trained with zymosan, a cell wall preparation rich in β-glucans, given intraperitoneally 7 and 3 days before infection [18] unless specified otherwise (Figure 1A). In a first model, control and trained mice were challenged intraperitoneally with *E. coli*. Trained mice coped much better with peritonitis than control mice, showing lower severity scores, absence of *E. coli* dissemination in the blood, and improved survival rates (92% vs 23% survival; $P < .001$) (Figure 1B). Cytokines were increased in the blood of trained mice (Figure 1B), likely reflecting diffusion from the peritoneal cavity in which trained cells responded massively to *E. coli*. Supporting this assumption, peritoneal cells from trained mice produced high levels of TNF and IL-6 in response to lipopolysaccharide (LPS) stimulation (Figure 1C). Moreover, the peritoneal cavity of trained mice contained more leukocytes, among which were more phagocytes (Figure 1D). PMNs were increased 8.2-fold, and there was a shift in the macrophage population. In control mice, the peritoneal cavity contained mainly homeostatic large peritoneal macrophages (LPMs) that virtually disappeared in trained mice at the expense of inflammatory and bactericidal small peritoneal macrophages (SPMs).

Trained Immunity Protects From Systemic Staphylococcal and *Listeria* Infections

To explore whether trained immunity protected from systemic infections, C57BL/6J mice were injected intravenously with methicillin-resistant *S. aureus*. Trained mice survived better than control mice (31% vs 0% survival; $P = .006$) and had 10-fold less bacteria in blood 2 days after infection (Figure 2A). Very similar results were obtained using BALB/cByJ mice (Supplementary Figure 2A), and subsequent experiments were all performed using C57BL/6J mice.

Mice were challenged intravenously with a lethal dose of *L. monocytogenes*. Most strikingly, all trained mice survived infection, whereas all control mice died within 5 days (Figure 2B). Bacteria were not detected in blood collected from trained mice 2 and 3 days after infection, whereas up to 10^5 colony-forming units/mL were measured in the circulation of control mice (Figure 2B and Supplementary Figure 2B). Trained mice had 2–3-log lower counts of *L. monocytogenes* in spleen, liver, and kidney (Figure 2B), and *L. monocytogenes* was undetectable in organs collected from mice surviving infection for 1–2 months.

Confirming an efficient control of bacterial burden in trained mice, cytokines and chemokines were detected at much lower concentrations in blood collected 3 days after infection (Figure 2B). Training mice with zymosan given intravenously as a single dose 7 days before infection also efficiently reduced bacterial burden, suggesting that training can be induced through diverse routes (Figure 2C). Because training was demonstrated by challenging mice with heat-killed *C. albicans* [16], we questioned whether a similar approach would protect from listeriosis. Mice trained with heat-killed *C. albicans* were powerfully protected from lethal listeriosis ($P = .002$) and had greatly reduced bacteremia (Figure 2D). Thus, trained immunity efficiently protected mice from systemic bacterial infections.

Trained Immunity Protects From Enteritis and Pneumonia

To extend the panel of microorganisms and routes of inoculation tested, we developed models of enteritis and pneumonia. Enteritis induced by *C. rodentium* was established in TLR2$^{-/-}$ mice, because the bacteria are cleared quickly in immunocompetent animals . All mice lost weight and had some diarrhea, but none died. Trained and control mice recovered their initial weights 7 and 18 days after infection, respectively. Trained mice showed improved weight from day 7 to day 30 ($P < .01$) (Figure 3A). Pneumonia was induced by an intranasal challenge with *P. aeruginosa*. The survival of trained mice was largely improved ($P = .02$) (Figure 3B). Overall, trained immunity protected mice in all the preclinical models tested, suggesting the enhancement of broad mechanisms of defense.

Trained Immunity Increases Blood Antimicrobial Activity and Stimulates Myelopoiesis

Trained immunity protected mice remarkably well from systemic listeriosis (92 of 96 trained mice survived vs 0 of 74 control mice; n = 5 experiments). We reasoned that blood should provide an efficient barrier against *L. monocytogenes* burden. Ex vivo, the blood of trained mice limited the growth of *L. monocytogenes* better than that of control mice (Figure 4A),

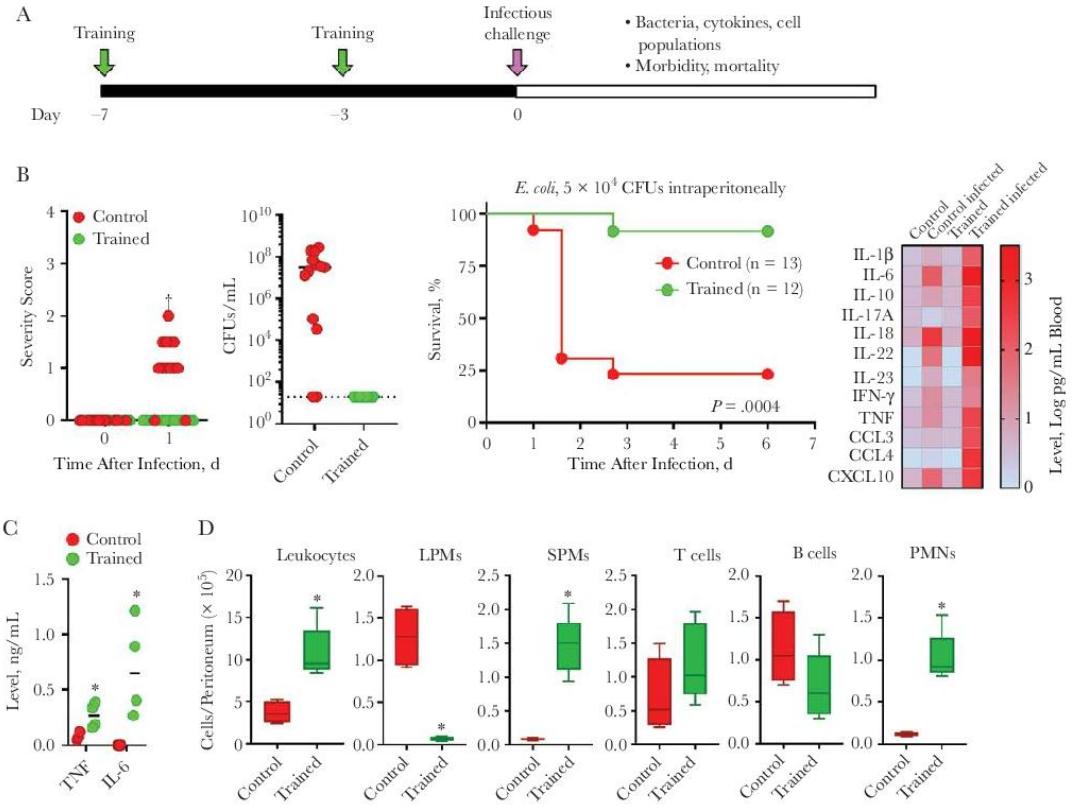

Figure 1. Trained immunity protects from *Escherichia coli* peritonitis. *A*, Experimental model to study protection from infection mediated by trained immunity. Unless specified otherwise, training was induced by 2 intraperitoneal injections of 1 mg zymosan, performed 4 days apart (at –7 and –3 days) using C57BL/6J mice. *B*, Control and trained mice were challenged intraperitoneally with 10^4 colony-forming units (CFUs) of *E. coli*. Severity score and survival were recorded. Blood was collected 18 hours after infection to quantify bacteria and cytokines using Luminex (n = 6 mice per group). *C*, Tumor necrosis factor (TNF) and interleukin 6 (IL-6) production by peritoneal cells from control and trained mice exposed ex vivo for 6 hours (TNF) or 24 hours (IL-6) to 10 ng/mL lipopolysaccharide. *D*, Leukocytes in the peritoneal cavity of control and trained mice before infection (n = 4–5 mice). *$P \leq .05$; †$P \leq .001$. Abbreviations: IFN, interferon; IL-1β (etc), interleukin 1β (etc); LPMs, large peritoneal macrophages; PMNs, polymorphonuclear neutrophils; SPMs, small peritoneal macrophages.

and it was more reactive to microbial products, as shown by increased production of TNF and IL-6 in response to LPS, CpG, Pam_3CSK_4, *L. monocytogenes*. and *C. albicans* (Figure 4B).

We then quantified leukocytes in control and trained mice, using blood collected before and 2 days after infection with *L. monocytogenes* (Figure 4C). Training increased leukocyte counts 1.6 fold, reflecting more $Ly6C^{high}$, $Ly6C^{int}$, and $Ly6C^{low}$ monocytes (inflammatory, intermediate, and nonclassic monocytes) and PMNs (7.6-, 3.9-, 1.9-, and 4.7-fold increase, respectively, vs control; $P < .05$). In trained mice, $Ly6C^{high}$ and $Ly6C^{int}$ monocytes expressed more CD11b/Itgam (2.5- and 1.8-fold higher mean fluorescence intensity), indicative of a primed/activated phenotype (Supplementary Figure 3). Conversely, PMNs were expressed lower CD11b (2.5 lower mean fluorescence intensity), which is associated with an immature status [36]. The absolute numbers of T and B lymphocytes were not affected.

L. monocytogenes induced a massive depletion of leukocytes 2 days after infection, which was less pronounced in trained mice (2.9- vs 11.5-fold decrease in trained vs control mice) (Figure 4C). $Ly6C^{high}$ and $Ly6C^{low}$ monocytes were more preserved in trained mice. PMNs were rather stable in trained mice, but they decreased 9.9-fold in control mice. T cells and B cells decreased 4–7-fold in trained mice, but 40-fold in control mice. The relative preservation of blood leukocytes suggested an enhanced hematopoiesis in trained mice. Indeed, the bone marrow of trained mice contained more long-term hematopoietic stem cells and more multipotent progenitors (MPPs), including more myeloid-biased MPP3 and lymphoid-biased MPP4 (Figure 4D).

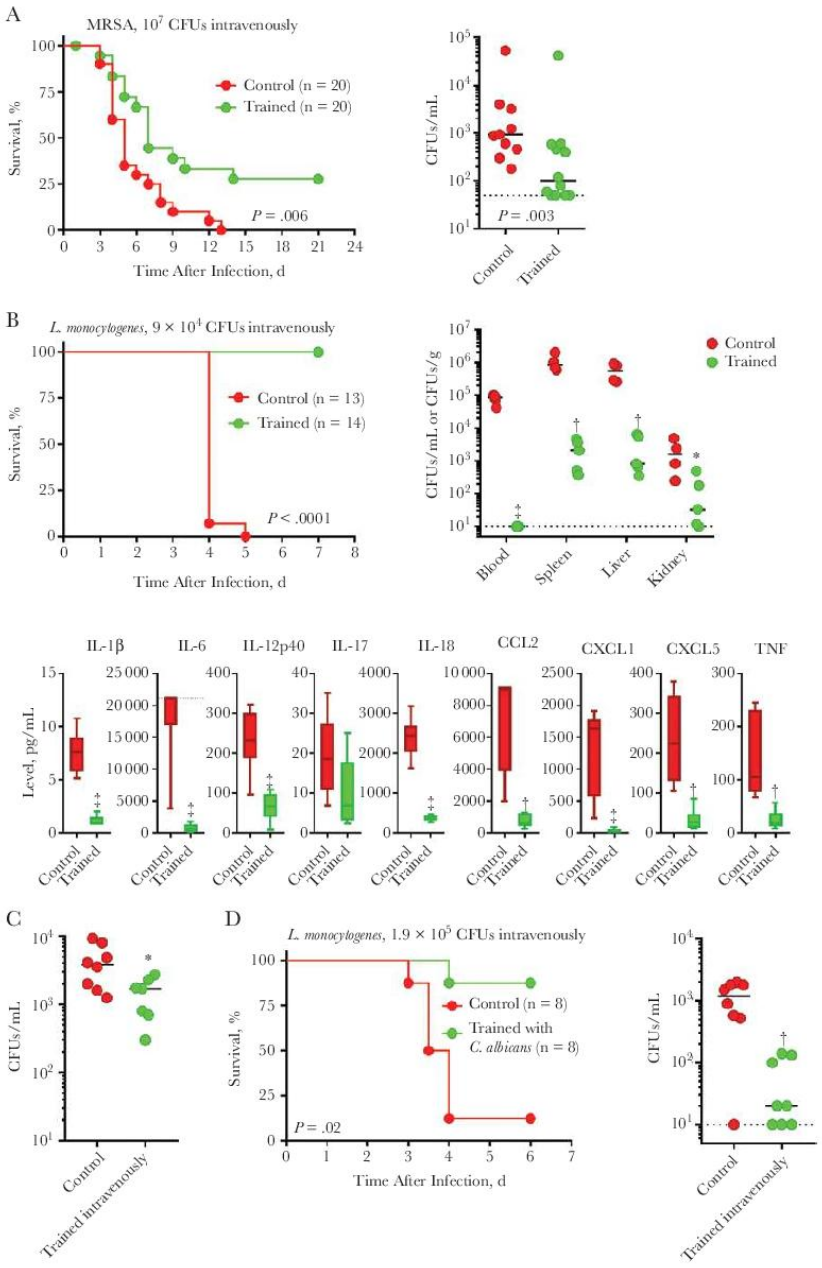

Figure 2. Trained immunity protects from systemic staphylococcal and *Listeria* infections. *A*, Control and trained mice were challenged intravenously with 10^7 colony-forming units (CFUs) of methicillin-resistant *Staphylococcus aureus* (MRSA). Survival was recorded. Blood was collected 2 days after infection to quantify bacteria. *B*, Control and trained mice were challenged intravenously with 9×10^4 CFUs of *Listeria monocytogenes*. Survival was recorded. Blood, spleen, liver, and kidney were collected 3 days after infection to quantify bacteria. Dashed line indicates lower limit of detection. Blood was collected 2 days after infection to quantify cytokines using Luminex (n = 6 mice per group). Dashed line indicates upper limit of detection. *C*, Control mice and mice trained with zymosan intravenously (0.1 mg at day −7) were infected intravenously with 10^5 CFUs of *L. monocytogenes*. Blood was collected 2 days after infection to quantify bacteria. *D*, Control mice and mice trained with heat-killed *Candida albicans* were infected intravenously with 1.9×10^5 CFUs of *L. monocytogenes*. Survival was recorded, and Blood was collected 2 days after infection to quantify bacteria. *$P \leq .05$; †$P \leq .01$; ‡$P \leq .001$. Abbreviations: IL-1β (etc), interleukin 1β (etc); TNF, tumor necrosis factor.

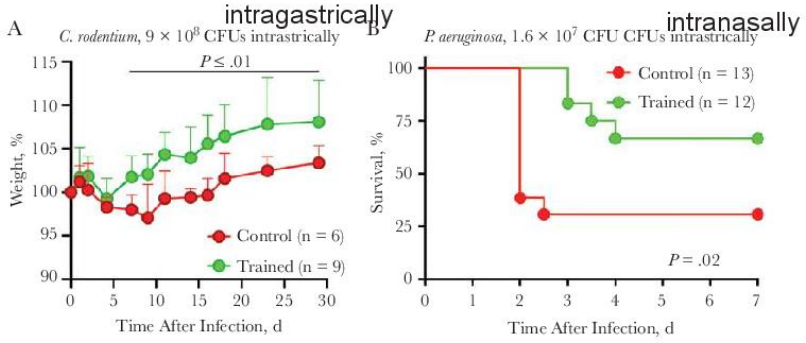

Figure 3. Trained immunity protects from enteritis and pneumonia. *A*, Weight of control and trained mice challenged intragastrically with 9×10^8 colony-forming units (CFUs) of *Citrobacter rodentium*. *B*, Survival of control and trained mice challenged intranasally with 1.6×10^7 CFUs of *Pseudomonas aeruginosa*.

Monocytes/Macrophages and IL-1 Signaling Are Involved in Protecting From Listeriosis

We analyzed the impact of depleting monocytes/macrophages by treating mice with clodronate liposomes during induction of training. Clodronate treatment fully abolished the protection conferred by training (Figure 5A). Because PMNs were also increased during training, we tested the impact of PMN depletion using anti-Ly6G mAb. In both trained and control mice, the depletion of PMNs modified neither survival nor bacteremia (Figure 5B). Two days after infection, PMNs were still fully depleted from the blood of anti-Ly6G mAb-treated mice, whether trained mice or control mice (Figure 5C). These observations suggested that monocytes are central effector cells for the protection against listeriosis conferred by trained immunity.

We tested the contribution of IL-1β/IL-1 signaling, because IL-1β has been proposed to play a role in trained immunity [37]. IL-1β was detected at higher concentrations in blood from trained mice (Figure 6A). Blood from trained mice showed a trend toward producing higher levels of IL-1β on exposure to *L. monocytogenes*, and BMDMs trained in vitro with zymosan produced higher levels of IL-1β in response to *L. monocytogenes* (Figure 6A). Interestingly, chromatin immunoprecipitation assays performed on monocytes isolated 3 weeks after the induction of training revealed increased marks associated with active chromatin (H3K4me1 and H3K4me3) and trained immunity [16, 23, 24]. (Figure 6D)

Because MyD88 is the adaptor signaling molecule downstream interleukin 1R, we quantified leukocytes as a surrogate of IL-1 signaling-mediated training in MyD88$^{-/-}$ mice. None of the changes observed in trained wild-type mice were detected in trained MyD88$^{-/-}$ mice, which behaved similarly to untrained MyD88$^{-/-}$ mice (Supplementary Figure 4). The role of IL-1 signaling was tested in mice treated daily for 4 days with recombinant IL-1 receptor antagonist (anakinra) during the induction of trained immunity (Figure 6C). Severity score and weight loss ($P < .01$ and $P < .001$, respectively) were increased in anakinra-treated mice. Accordingly, 3 of 8 anakinra-treated trained mice died of listeriosis, whereas all trained mice survived infection ($P = .06$). The partial effect on mortality rate was related to a partial (1.4-fold) reduction in Ly6C^{high} monocytes ($P = .02$) (Figure 6D). These data supported the assumption that IL-1 signaling is involved in the antilisterial activity conferred by trained immunity.

Trained Immunity Protects From Peritonitis and Listeriosis for ≥5 Weeks

The length of protection conferred by trained immunity against lethal bacterial infections is unknown. To start filling that gap, we analyzed mice trained up to 5 weeks earlier (Figure 7A). In the peritoneal cavity, leukocytes steadily increased from 2 to 5 weeks after training, reaching a maximum after 5 weeks (Figure 7B). The decreased number of LPMs remained drastic at all time points, whereas the number of SPMs increased 2–5 weeks after training. PMNs reached a maximum value 2 week after training and remained stable for up to 5 weeks. In line with these observations, mice trained 5 weeks earlier were protected from *E. coli* peritonitis, as shown by reduced weight loss and bacterial dissemination into the blood compared with untrained mice (Figure 7C). Blood cytokine levels increased mainly 1–2 weeks after training, and returned to baseline levels 5 weeks after challenge (Figure 7D). Moreover, blood leukocyte counts were back to normal 5 weeks after training (Figure 7D). Impressively, mice trained 1, 2, 4, or 5 weeks earlier were all protected from listeriosis, in term of both survival and bacteremia (Figure 7D).

DISCUSSION

We report the first broad analysis of the impact of trained immunity on bacterial infections. Trained immunity protected mice from a large panel of clinically relevant bacterial pathogens inoculated systemically and locally to induce peritonitis, enteritis, and pneumonia.

In all models, bacterial dissemination was controlled. In the peritonitis model, this results from a massive accumulation of

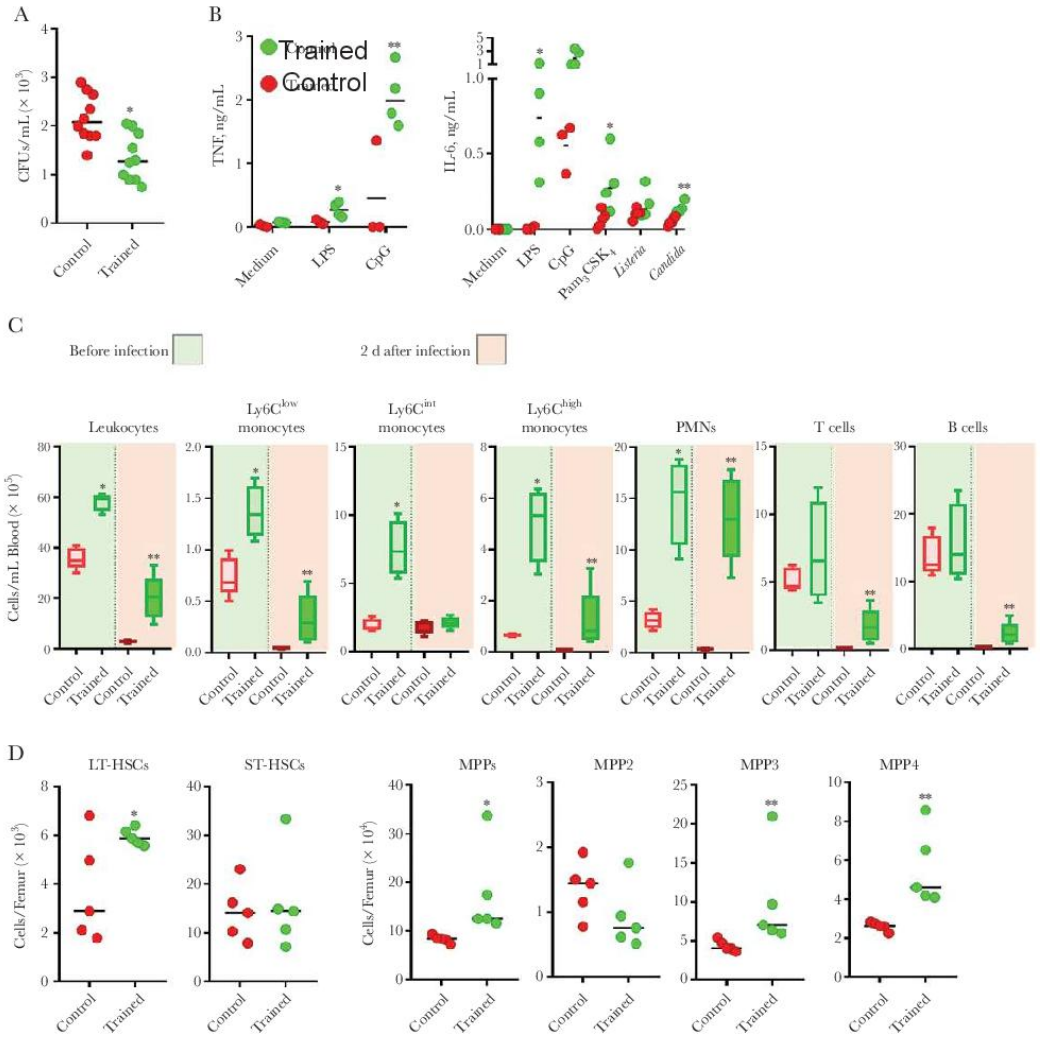

Figure 4. Trained immunity increases blood antimicrobial activity and stimulates myelopoiesis. *A*, Bacteria in whole blood from control and trained mice exposed ex vivo for 2 hours to 1.2×10^3 colony-forming units (CFUs)/mL of *Listeria monocytogenes*. *B*, Tumor necrosis factor (TNF) and interleukin 6 (IL-6) production by whole blood from control and trained mice exposed ex vivo for 6 hours (TNF) or 24 hours (IL-6) to 10 ng/mL lipopolysaccharide (LPS), 10 µmol/L CpG, 100 ng/mL Pam_3CSK_4, 10^7 heat-killed *L. monocytogenes*, and 10 mg/mL heat-killed *Candida albicans*. *C*, Leukocytes in blood collected from control and trained mice before infection (*green background*) and 2 days after an intravenous challenge with 1.1×10^5 CFUs of *L. monocytogenes* (*salmon background*) (n = 5 mice per group). *D*, Long-term and short-term hematopoietic stem cells (LT-HSCs and ST-HSCs), multipotent progenitors (MPPs), MPP2, MPP3, and MPP4 in the bone marrow of control and trained mice. *$P \leq .05$; †$P \leq .01$. Abbreviation: PMNs, polymorphonuclear neutrophils.

PMNs and SPMs in the peritoneal cavity. At baseline, SPMs represent <10% of peritoneal macrophages, which are composed mainly of self-maintaining LPMs. Local inflammation triggers the migration of LPMs to the omentum, where these cells produce growth factors and chemokines that stimulate myelopoiesis and induce the influx of PMNs and inflammatory monocytes, which are precursors of SPMs [38]. Hence, LPMs and SPMs play key roles as initiator and effector cells of trained immunity when training and infection occur in the peritoneum.

Stimulation of myelopoiesis greatly increased blood leukocyte counts, above all those of PMNs and Ly6C^{high} inflammatory monocytes exhibiting a primed/activated phenotype. Changes in peripheral blood gave an indubitable advantage to trained mice during systemic infections, as demonstrated in models of listeriosis and staphylococcal infection. The picture

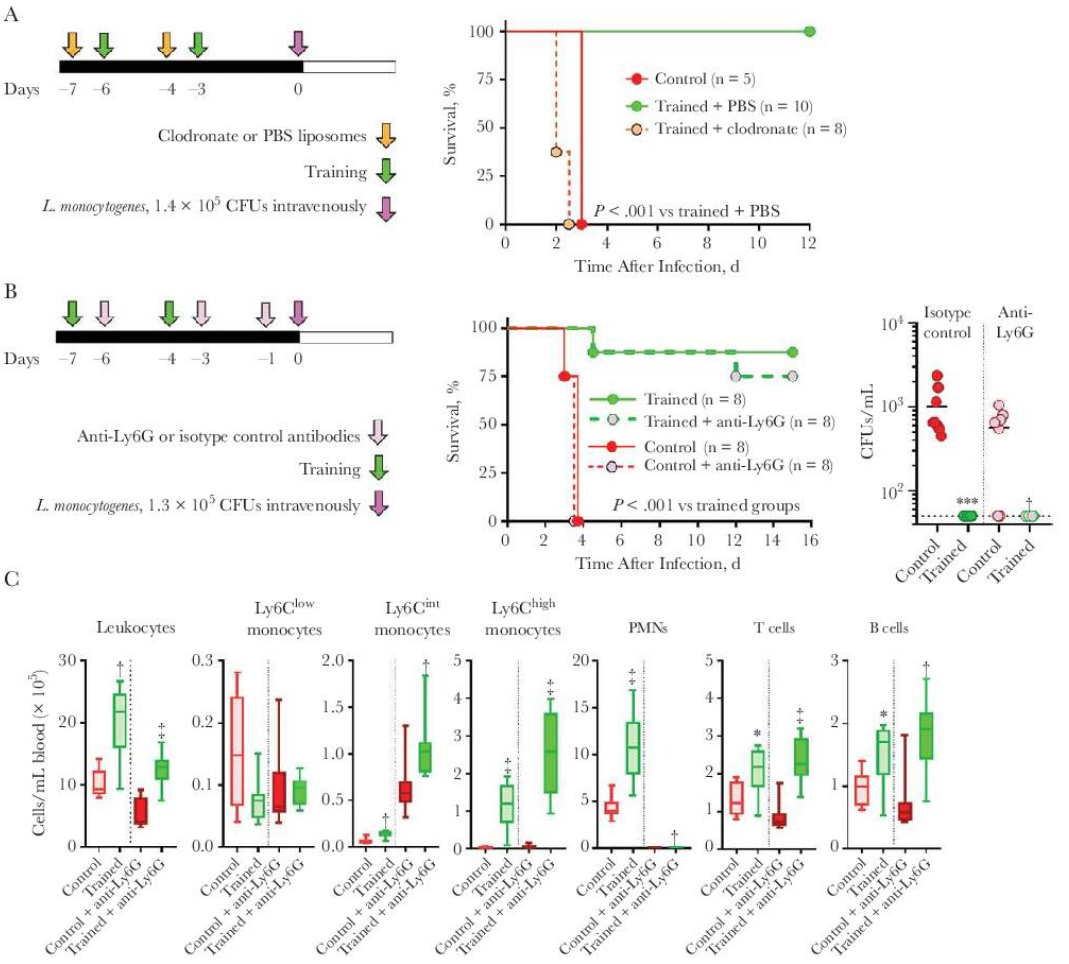

Figure 5. Monocytes/macrophages are essential to protect trained mice from listeriosis. *A*, Experimental model to study the role of monocytes/macrophages in trained immunity (*left*) and survival of mice treated with clodronate or phosphate-buffered saline (PBS) liposomes and challenged intravenously with 1.4×10^5 colony-forming units (CFUs) of *Listeria monocytogenes* (*right*). *B*, Experimental model to study the role of polymorphonuclear neutrophils (PMNs) in trained immunity (*left*). Survival of mice treated with anti-Ly6G or isotype control antibody and challenged intravenously with 1.3×10^5 CFUs of *L. monocytogenes* (*middle*) and bacteria in blood collected 18 hours after infection (right). *C*, Leukocytes in blood collected 2 days after infection with *L. monocytogenes* (n = 8 mice per group.) *$P \leq .01$; †$P \leq .01$; ‡$P \leq .001$.

was rather unexpected in the model of listeriosis, because almost all trained mice survived a challenge equivalent to 10–20 times the LD_{100}. Results of cell depletion experiments suggested that monocytes/macrophages were the main drivers of protection. This is in agreement with the fact that inflammatory monocytes were essential whereas PMNs were dispensable for clearing bacteria during the early and late phases of systemic infection by *L. monocytogenes* [39]. Circulating inflammatory monocytes migrated to foci of infection in liver and spleen to give rise to TNF- and inducible nitric oxide synthase–producing dendritic cells and monocyte-derived macrophages that replenished Kupffer cells dying through necroptosis. This helped enhance antibacterial immunity and restore tissue integrity [40, 41].

Trained immunity protected mice from enteritis and pneumonia, pointing to distant effects. Intraperitoneal injections of LPS induced epigenetic reprogramming of brain resident macrophages and modulated neuropathology in models of Alzheimer disease and stroke [42]. The broad effects of trained immunity during infections may result from the action of soluble mediators that directly or indirectly stimulate the response of intestinal, airway, and lung parenchyma cells or resident immune cells. For example, adenoviral infection improved the activity of self-renewing memory alveolar macrophages

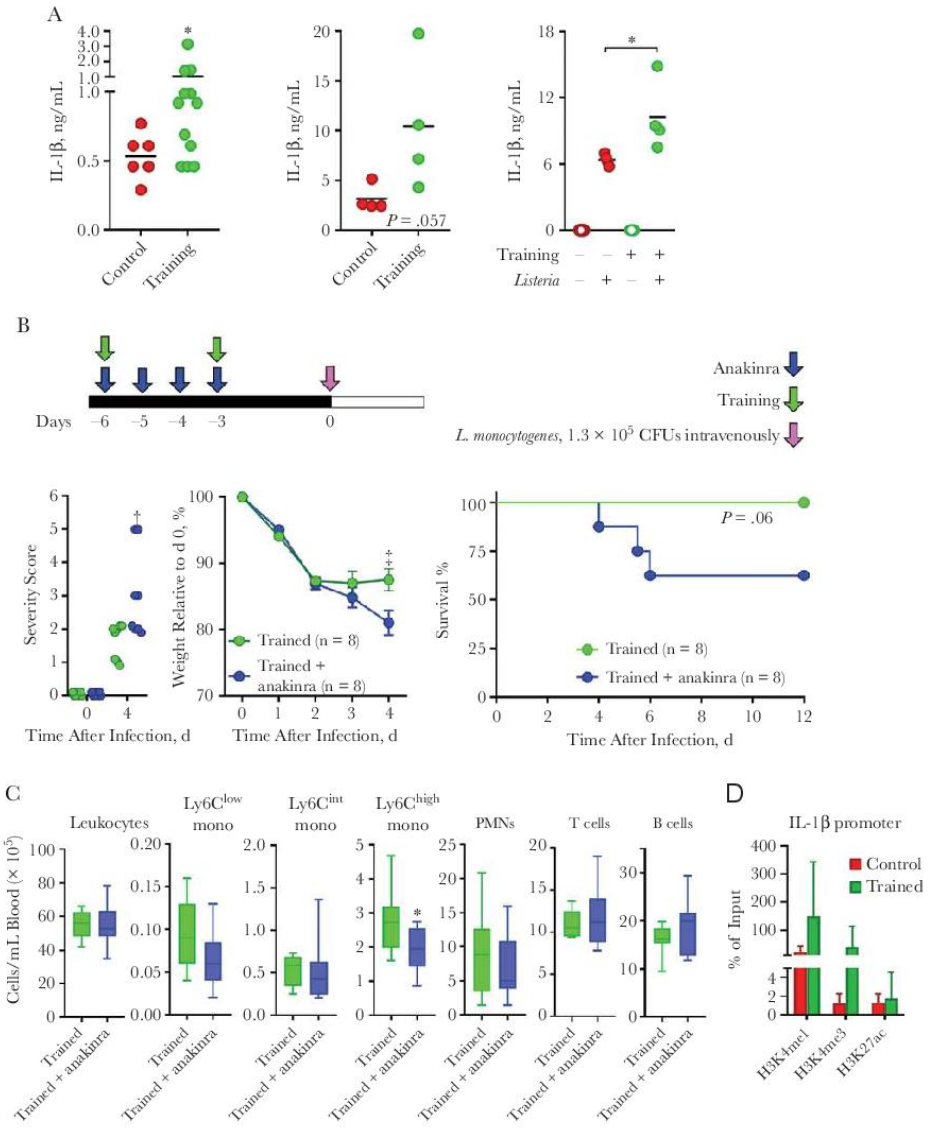

Figure 6. Interleukin 1 (IL-1) signaling participates to protect trained mice from listeriosis. *A*, Interleukin 1β (IL-1β) concentrations in blood collected from control and trained mice at day 0 (*left*), in whole blood from control and trained mice exposed ex vivo for 6 hours to 10^8 heat-killed *Listeria monocytogenes* (*middle*), and in cell culture supernatants of control and trained bone marrow–derived macrophages cultured for 24 hours with or without 1 live *L. monocytogenes* per cell (*right*). *B*, Experimental model to study the role of IL-1 signaling in trained immunity (*top*). Severity score, weight loss, and survival (*bottom*) of mice trained with or without anakinra treatment and challenged intravenously with 1.3×10^5 colony-forming units (CFUs) of *L. monocytogenes* (n = 8 mice per group). *C*, Leukocytes in blood collected just before infection from mice trained with or without anakinra (n = 9–10 mice per group). *D*, H3K4me1, H3K4me and H3K27ac signal at the *Il1b* promoter of bone marrow monocytes isolated from mice trained or not 3 weeks earlier were quantified by chromatin immunoprecipitation followed by real-time polymerase chain reaction and expressed as percentage of input (n = 4 mice per group). *$P \leq .05$; †$P \leq .01$; ‡$P \leq .001$. Abbreviation: PMNs, polymorphonuclear neutrophils.

that promoted neutrophilia and protected from *Streptococcus pneumoniae* lung infection [43].

AQ28 Bone marrow hematopoietic stem and progenitor cells adapted to acute and chronic peripheral inflammation and infection through cell-extrinsic and cell-intrinsic mechanisms, increasing proliferation and skewing toward the myeloid lineage to provide activated innate immune cells [44–46]. Our training protocol increased the number of hematopoietic stem

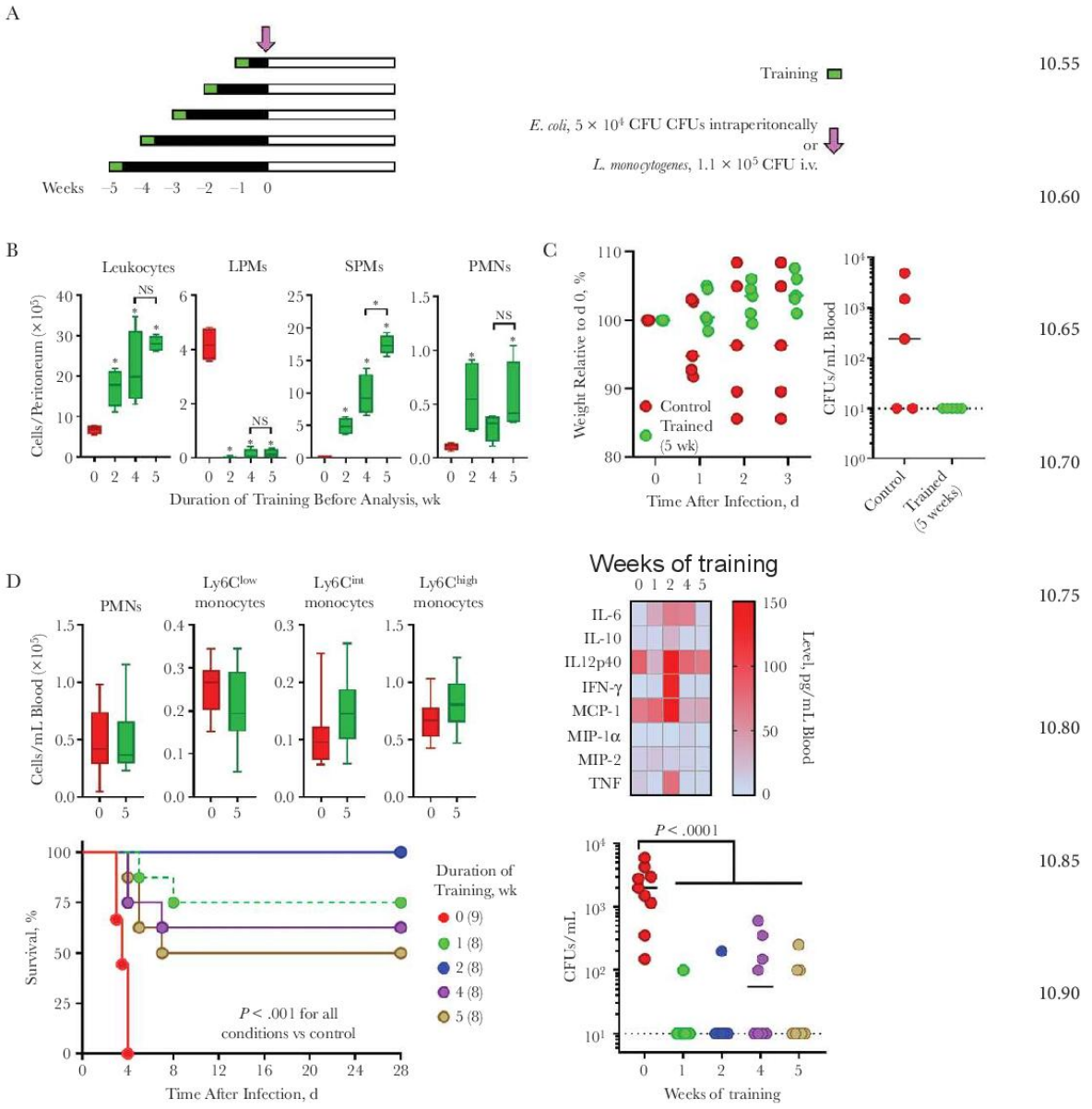

Figure 7. Trained immunity protects from peritonitis and listeriosis for at least 5 weeks. *A*, Experimental model to study the effects of training over time. *B*, Leukocytes in the peritoneal cavity of mice trained 0, 2, 4, and 5 weeks earlier (n = 5 mice per group). *C*, Weight loss and bacteria in blood collected 2 days after an intraperitoneal challenge with 5×10^4 colony-forming units (CFUs) of *Escherichia coli*. *D*, Leukocytes (n = 8–10) and cytokines (n = 6) in blood of mice trained 0, 1, 2, 4 and 5 weeks earlier (*top*). Survival and bacteria in blood collected 2 days after infection of mice challenged intravenously with 1.1×10^5 CFUs of *Listeria monocytogenes*). *$P \leq .05$. Abbreviations: IFN, interferon; IL-6 (etc), interleukin 6 (etc); LPMs, large peritoneal macrophages; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PMNs, polymorphonuclear neutrophils; SPMs, small peritoneal macrophages; TNF, tumor necrosis factor.

cells and MPPs, accounting for higher leukocyte counts before and during infection. In the same vein, the adoptive transfer in naive mice of long-term hematopoietic stem cells or bone marrow cells collected from mice trained with β-glucan and BCG vaccine increased the proportion of blood $Gr1^+CD11b^+$ myeloid cells and protected from pulmonary tuberculosis [25,

44]. We are performing experiments to delineate the length of protection conferred by trained immunity. This information will be valuable for preclinical and clinical development of trained immunity-based therapeutics.

The diversity of the models of infection tested supports wide effects of trained immunity. Work will be required to establish whether trained immunity protects from additional bacterial, fungal and viral infections. Trained immunity is most typically induced with β-glucan, which promotes T-helper (Th) 1/Th17 proinflammatory responses essential to fight bacteria, viruses, and fungi. However, training might be tipped toward Th2 immune responses beneficial during parasitic infections [47].

IL-1β has gained attention as a possible hub regulating trained immunity [24, 37, 44–46]. Uninfected trained mice expressed increased blood levels of IL-1β, and treatment with anakinra compromised trained immunity, indicating that IL-1 signaling played a role. Trained peripheral blood mononuclear cells produced higher levels of IL-1β, and IL-1β itself fueled human monocytes to produce higher levels of cytokines on stimulation [17]. In vivo, BCG vaccine–induced IL-1β production was correlated with the capacity to control viremia in healthy subjects challenged with yellow fever vaccine [24]. IL-1 family members can affect trained immunity through manifold mechanisms [37]. Interestingly, training mice with β-glucan sustained IL-1β signaling that promoted glycolytic activity and proliferation of hematopoietic stem and progenitor cells [44]. This aspect is highly relevant for listeriosis, which affects blood leukocytes and constrains vigorous myelopoiesis. Inflammasomes control IL-1β secretion and are likely to be involved in trained immunity. Supporting this hypothesis, feeding $Ldr^{-/-}$ atherosclerotic mice a Western diet induced an oxidized low-density lipoprotein/NLRP3/IL-1 axis, leading to the establishment of trained immunity [45].

Induction of trained immunity is an attractive approach to increase vaccine efficacy and resistance to pathogens. Training with β-glucan counteracted endotoxin-mediated immune tolerance associated with poor outcome in sepsis [3, 4, 21, 48]. However, immunotherapies may have doubled-edge sword effects. Low-grade inflammation sustained by trained immunity may be involved in the pathophysiology of chronic and autoinflammatory disorders [49]. Monocytes from patients with hyperimmunoglobulin D syndrome have a trained phenotype [22] and Western diet feeding induced trained immunity [45]. Interfering with the sensing of training inducers, IL-1 signaling, and inflammation, metabolic and epigenetic changes may be exploited to avoid pathogenic processes linked to trained immunity. For instance, trained immunity induced by helminth products provided an anti-inflammatory environment, attenuating the development of experimental autoimmune encephalomyelitis [50].

In summary, induction of trained immunity remodeled bone marrow and blood cellular compartments, providing efficient barriers against bacterial infections. Protection was remarkably broad when considering the pathogens and sites of infection tested. These data support the development of trained immunity-based strategies to improve the efficacy of vaccines and host defenses against infections, and they may give clues about the pathological processes underlying inflammatory and autoimmune disorders.

Notes

Author contributions. E. C., T. H., and D. L. R. performed the in vitro experiments. E. C., T. H., C. T., F. A., and D. L. R. performed the in vivo experiments. T. R. conceived the project. M. G. N. provided protocols and discussed the project. E. C. and T. R. designed the experiments and wrote the article. All authors discussed the results and revised the article.

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3.8 Characterization of *in vivo* and *in vitro* induced trained immunity: impact on the phenotype of innate immune cell populations and hematopoiesis

Tytti Heinonen, Eleonora Ciarlo, Didier Le Roy, Thierry Roger

Infectious Diseases Service, Department of Medicine, Lausanne University Hospital and University of Lausanne, CH-1066 Epalinges, Switzerland

Short title: In vitro and in vivo models of trained immunity

Corresponding author: Thierry Roger, Infectious Diseases Service, Lausanne University Hospital and University of Lausanne, CLED.04.407, Chemin des Boveresses 155, CH-1066 Epalinges, Switzerland

Tel. +41-21-314-1038; Mobile. +41-79-556-8069; E-mail: Thierry.Roger@chuv.ch

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Abstract

Innate immune cells form heterologous cell populations with high plasticity. β-glucan, a component of fungal cells wall, can prime innate immune cells to increase their responsiveness to a secondary challenge, a phenomenon called trained immunity. While the induction of trained immunity reprograms the metabolism, epigenetic and functional landscapes of numerous cell types, it is unclear whether the trained phenotype observed *in vivo* results from an increased frequency or increased reactivity of innate immune cells. We compared the cytokine production and the metabolism of M-CSF and GM-CSF induced bone-marrow (BM) derived macrophages (BMDMs). BMDMs were either trained *in vitro* or derived from the BM of trained mice. BMDMs obtained from trained mice increased cytokine production and glycolysis, while BMDMs trained *in vitro* poorly acquired a trained phenotype. In mice trained 9 weeks earlier, blood PMNs, monocytes, as well as bone marrow myeloid progenitors and extramedullary hematopoiesis were increased. The blood from trained mice limited the growth of bacteria and produced increased levels of cytokines even when blood was collected as long as 28 weeks after training induction. Together, these data suggest that increased myeloid cell number due to enhanced hematopoiesis and increased reactivity of innate immune cells account for the long-lasting phenotype of trained immunity.

Introduction

The innate immune system is the first line of defense against pathogens. Innate immune responses have to be tightly regulated to eradicate or contain invasive pathogens without causing collateral damage to the host. Immunological memory is a very efficient way to protect the host from infections and forms the basis of vaccine efficacy (1). Immunological memory has long been considered a specificity restricted to the adaptive immune system carried by T and B lymphocytes, but this concept has been recently challenged (2). Indeed, preclinical and clinical observations indicate that the innate immune system recalls a previous challenge to mount a robust response to a subsequent infection, a phenomenon called "trained immunity" (3). Contrary to adaptive immune memory, trained immunity is not antigen specific, suggestive of broad effects.

Trained immunity is induced by microbial products and has been studied mainly using the fungal cell wall component β-glucans, the Bacillus Calmette-Guérin (BCG) vaccine and lipopolysaccharide (LPS) (4). The impact of trained immunity was first reported for monocytes and macrophages and extended to innate immune progenitor, hematopoietic stem cells (HSCs) and non-immune cells such as epithelial cells (3, 5). Trained immunity reprograms cell metabolism, epigenetics and functions. However, it is unclear whether the induction of trained immunity affects the function of discrete cells or relies on increased cell number. In initial *in vitro* experiments, human peripheral blood mononuclear cells (PBMCs) or monocytes were trained through the exposure to heat killed *Candida albicans* or β-glucan during the first 24 hours of differentiation (6). Cells tested after 7 days of culture produced increased levels of IL-6 and TNF in response to various microbial products (6). This protocol has been debated, with authors suggesting that increased cytokine production reflects an increased survival rather than an enhanced reactivity of trained macrophages (7).

We have established an *in vivo* model of training with zymosan, a yeast cell wall preparation rich in β-glucan, which conferred broad-spectrum protection against systemic infections, peritonitis, enteritis and pneumonia (8). The induction of trained immunity was impressively efficient to protect from systemic infections by *Listeria monocytogenes*, with all trained mice surviving an otherwise lethal

infection. The protection was long-lasting and required monocytes/macrophages and IL-1 signaling. The acquisition of the training phenotype was associated with increased numbers of myeloid bone marrow precursors, and of phagocytic and inflammatory monocytes and of polymorphonuclear cells (PMNs) in the blood and the peritoneal cavity of trained mice.

The amplified reactivity of specific compartment (blood, peritoneal cavity, lungs) can reflect an increased number of immune cells or an enhanced reactivity of individual cells, an aspect that has been poorly addressed. Thus, we studied the functional characteristics of immune cells subjected to trained immunity *in vivo* and *in vitro*. We established protocols to train bone marrow (BM) derived macrophages (BMDMs) during the differentiation process induced by either M-CSF or GM-CSF, and to derive BMDMs from trained mice. We also analyzed mice subjected to training 9 and 28 weeks earlier to characterize the long-lasting effects of training notably on BM and peripheral hematopoietic progenitors. Overall, our results suggest that the induction of trained immunity modulates the frequency, the metabolism and the functions of immune cell populations. Macrophages derived from trained mice have characteristics not observed in macrophages trained *in vitro* and training has long-lasting effects on blood cells. These findings are important for the characterization of the protection afforded by the induction of training.

Results

In vitro training modifies cytokine production by M-CSF and GM-CSF-derived macrophages

To train PBMCs or monocytes, cells were exposed for 24 h to β-glucan, medium was changed, and cells differentiated into macrophages for 6 days before analysis (6). This method has been argued because it does not control the number of cells used for stimulation. To avoid this problem and fulfill the requirements for the differentiation of bone marrow (BM) derived macrophages (BMDMs), we developed and *in vitro* BM-to-BMDMs training assay (**Fig. 1A**). BM cells were incubated with zymosan for 24 h in the presence of L929 supernatant (a source of M-CSF) or recombinant M-CSF or GM-CSF. After 24 h, medium was changed and cells differentiated for 6 days. The 7^{th} day, BMDMs were detached, counted and seeded at equal numbers before being exposed to LPS. GM-CSF-derived BMDMs (GM-BMDMs) produced much more IL-6 than L929-derived BMDMs and M-CSF-derived BMDMs (M-BMDMs) (**Fig. 1B**). BMDMs exposed to training conditions (trained BMDMs) produced higher levels of IL-6 than control BMDMs, regardless of the growth factor used for differentiation (**Fig. 1B**). We then focused on M-BMDMS and GM-BMDMs.

To extend the panel of mediators measured, we performed a Luminex assay based on the detection of seventeen cytokines (**Fig. 1C**). Among ten and twelve cytokines produced at measurable concentrations by M-BMDMS and GM-BMDMs, respectively, four were produced significantly less by trained than control M-BMDMs (IL-10, CCL2, CCL3, TNF) while six were produced significantly more by trained than control GM-BMDMs (G-CSF, IL-1α, IL-10, CCL2, CXCL1, CXCL10). Trained GM-BMDMs produced lower levels of IL-12p40 than control GM-BMDMs. Overall, *in vitro* training mainly increased cytokine production by GM-BMDMs while it predominantly decreased cytokine production by M-BMDMs.

In vitro training has no strong influence on the metabolism of BMDMs

Increased glycolysis is a key feature of the induction of trained immunity in PBMCs (9). To assess whether a similar metabolic reprograming exists in mouse macrophages, we measured the

extracellular acidification rate (ECAR, a readout of glycolytic activity) and oxygen consumption rate (OCR, a readout of mitochondrial respiration) by resting and LPS-stimulated control and trained BMDMs (**Fig. 1D**). As expected, GM-BMDMs were more metabolically active than M-BMDMs, showing higher glycolytic and mitochondrial activities. Training marginally affected the glycolysis, glycolytic capacity and glycolytic reserve of resting and LPS-stimulated M-BMDMs and of resting GM-BMDMs (**Fig. 1D**). Trained GM-BMDMs failed to increase glycolysis upon LPS stimulation (**Fig. 1D**). Basal OCR was slightly increased in trained M-BMDMs and significantly decreased in trained GM-BMDMs when compared to their respective controls (**Fig. 1E**). The induction of trained immunity *in vitro* in BMDMs had no strong impact on metabolic parameters, and did not increase glycolysis in trained cells.

***In vivo* training affects *in vitro* generated M-CSF and GM-CSF-derived macrophages**

Induction of trained immunity modifies the expression of mediators circulating in the body and modulates the phenotype and functionality of multiple cell types (3). For example, induction of trained immunity reprograms bone marrow hematopoietic stem and progenitor cells (HSPCs) (10, 11). Hence, training *in vitro* might exclude signals playing a key role in the induction of trained immunity *in vivo*. To take into consideration this aspect, BM collected from mice trained 7 and 3 days earlier with zymosan was used to generate M-BMDMs and GM-BMDMs (**Fig. 2A**).

At the time of collection, the BM of trained mice contained 1.4-fold more $CD45^+$ hematopoietic BM cells than the BM from non-trained mice (**Fig. 2B**). After 7 days of differentiation, the BM of trained mice gave rise to less M-BMDMs and less GM-BMDMs than the BM of control mice (**Fig. 2C**). The proportion of $CD11b^+$ cells within M-BMDMs cells was similar when BMDMs were derived from trained and non-trained mice, but significantly lower within GM-BMDMs derived from trained mice when compared to non-trained mice (**Fig. 2D**). GM-BMDMs and to a lesser extend M-BMDMs derived from trained mice expressed MHC-II at a higher density (*i.e.* higher mean fluorescence intensity, MFI) than BMDMs derived from control mice (**Fig. 2E**). Upon stimulation with LPS, M-BMDMs derived from trained mice produced increased levels of IL-6 (**Fig. 2F**) and, albeit not significantly, of TNF (**Fig. 2G**) while GM-BMDMs derived from trained and non-trained mice produced

similar levels of cytokines (**Fig. 2F-G**). These data indicate that *in vivo* and *in vitro* training differentially affects the phenotype of BMDMs.

Sirtuins regulate metabolism, epigenetic and inflammation and might influence the establishing of trained immunity (3). We have shown that deficiencies in sirtuins modify glycolysis and cytokine production by BMDMs, especially in double knockouts ((12, 13), Heinonen et al. in preparation). Therefore, we quantified mRNA levels of the 7 sirtuins (Sirt1-7) in M-BMDMs and GM-BMDMs. M-BMDMs and GM-BMDMs derived from trained and non-trained mice showed very similar expression patterns of sirtuins, though we noticed a slight increase of Sirt5 mRNA level in trained M-BMDMs and a slight decrease of Sirt2 mRNA level in GM-BMDMs (**Fig. 2H**).

***In vivo* training increases the glycolytic and oxidative metabolism of BMDMs**

Training with β-glucan reduced OCR and increased lactate production by PBMCs (9) while splenocytes from BCG vaccinated mice showed increased basal and maximal OCR and ECAR (14). To test whether training *in vivo* affected the metabolic parameters of BMDMs, we compared the OCR and ECAR from BMDMs derived from control and trained mice. BMDMs were tested in basal conditions and upon exposure to *L. monocytogenes*. While there was a consistent trend of increased OCR by M-BMDMs, no significant difference in OCR were recorded between trained and non-trained M-BMDMs and GM-BMDMs (**Fig. 2I**). The exposure to *L. monocytogenes* marginally increased maximal and spare OCR of M-BMDMs and GM-BMDMs. Regarding glycolytic parameters, M-BMDMs and to a lesser extent GM-BMDMs had higher glycolysis and glycolytic capacity at baseline and upon stimulation with *L. monocytogenes* (**Fig. 2J**). The glycolytic reserve was increased in GM-BMDMs exposed to *L. monocytogenes*. Altogether, M-BMDMs derived from trained mice increased glycolytic activity and to some extend mitochondrial respiration, an effect much less pronounced in GM-BMDMs.

The frequency and reactivity of blood leukocytes are altered up to 28 weeks after training

BCG vaccination improved human blood reactivity to microbial stimulation for several months, and the induction of trained immunity reprogramed bone marrow progenitors for up to 28 days in mice

(10, 15). We hypothesized that the induction of trained immunity impacts mouse immune system for several months, and tested blood collected from naïve mice and mice trained 9 weeks earlier.

The induction of training increased leukocyte counts (control *vs* training: 4.7 *vs* 6.0 million cells/ml; **Fig. 3A**), reflecting a robust 2.5-fold increase of PMNs and smaller 1.2-1.3-fold increase of lymphocytes, while monocytes were not affected. However, training increased the proportions of classical ($Ly6C^{high}$) and non-classical ($Ly6C^{low}$) monocytes at the expense of intermediate monocytes (**Fig. 3B**). The reactivity of whole blood was tested against a range of microbial and immunological stimuli: LPS, CpG, PHA, *L. monocytogenes*, *S. aureus* and *C. albicans*. The blood from trained mice produced significantly more IL-6 after stimulation with LPS, CpG and PHA (**Fig. 3C**). A common readout of pyroptosis, a form of inflammatory programmed cell death induced by inflammatory caspases associated with the secretion of IL-1β and IL-18 (16), is the release of lactate dehydrogenase (LDH) from cells. Blood from trained mice released significantly more LDH at baseline and after stimulation with *S. aureus*, and to some extent after stimulation with LPS, *C. albicans* and CpG (**Fig. 3D**), suggesting that training favors pyroptosis. To address further LPS-stimulated cytokine production by blood collected from control and mice trained 9 weeks earlier, we quantified 17 cytokines by Luminex. The blood from trained mice released increased levels of 12 cytokines, which reached statistical significance for G-CSF, IFNγ, IL-1α, IL-1β, IL-6, IL-10, TNF and CXCL2 (**Fig. 3E**). To more closely reflect infectious conditions, we tested the capacity of whole blood to limit the growth of *L. monocytogenes* (**Fig. 3F**). The blood from trained mice contained more efficiently the growth of *L. monocytogenes* when infected with either a low (150 CFU) or a high (1'000 CFU) inoculum of the bacteria (**Fig. 3F**). Altogether, the increased number of PMNs, the increased production of cytokines and the improved control of bacterial growth were all indicators of a trained phenotype.

Finally, given that we had a set of 8 mice trained 28 weeks earlier, we assessed whether the increased cytokine production by LPS-stimulated whole blood was a persistent mark of trained immunity. Remarkably, the blood from mice trained 28 weeks earlier released higher concentrations of 16/16 cytokines measured by Luminex, though the results reached statistical significance for 7

cytokines (IFNγ, IL-1β, IL-12p40, IL-18, TNF and CXCL2: **Fig. 3E**). This result suggests that the induction of trained immunity had a long-term effect on cytokine response.

BM and splenic hematopoiesis are increased 9 weeks after training

The increased number of blood leukocytes suggests an increased BM hematopoiesis, an effect reported to last for 4 weeks after training (10). In line, we observed that common myeloid progenitors (CMPs), granulocyte-monocyte progenitors (GMPs), neutrophils and monocytes were increased in the BM of mice trained 9 weeks earlier (8). We extended our analyses to hematopoietic progenitors (LSKs; $Lin^-cKit^+Sca1^+$), long-term (LT) hematopoietic stem cells (HSCs) ($CD48^-CD150^+$ LSKs), short-term (ST) HSCs ($CD48^-CD150^-$ LSKs) and the myeloid-biased multipotent progenitors (MMPs) MPP2 ($Flt3^-CD48^+CD150^+$ LSKs) and MPP3 ($Flt3^-CD48^+CD150^-$ LSKs), and lymphoid-biased MPP4 ($Flt3^+CD48^+CD150^-$ LSKs). There was no significant impact of progenitors, while the most marked effect was an increased number of LSKs and MPP3 (**Fig. 4A**).

More than 30 years ago, it was shown that adherent splenocytes (macrophages) from mice primed with a sublethal dose of *C. albicans* protected mice against *S. aureus* infection (17). In our model, the spleens of mice trained 9 weeks earlier were 1.8-fold larger than the spleens of control mice (**Fig. 4B**). Surprisingly however, the number of $CD45^+$ leukocytes per spleen was not affected by training (**Fig. 4C**), as the number of B cells, T cells, pDCs and cDCs (**Fig. 4D**). On the contrary, there was a strong increase of PMNs (7.4-fold) and a tendency to increase $Ly6C^{high}$ monocytes (**Fig. 4D**). The proportion of cDC1 and cDC2 among cDCs was not affected (**Fig. 4E**).

During emergency myelopoiesis, HSCs migrate through the blood to the spleen and liver to induce extramedullary hematopoiesis (18, 19). PMNs being short-lived cells, we questioned whether their increased number in the spleen could result from splenic hematopoiesis. Indeed, the numbers of HSCs and MPP2 and MPP3, but not MPP4, were all increased in the spleen of trained mice (**Fig. 4F**). This finding supports the presence of an important extramedullary hematopoiesis in the spleen of trained mice persisting at least 9 weeks post-training.

Discussion

We compared the phenotype of BMDMs trained *in vitro* with that of BMDMs derived from the BM of trained mice. We show that *in vitro* trained GM-BMDMs but not M-BMDMs increased cytokine production without increasing glycolysis. On the contrary, M-BMDMs derived from the BM of trained mice acquired a phenotype similar to that of innate immune cells isolated from trained subjects (20, 21), including increased expression of MHC-II, glycolysis and cytokine production. Of note, GM-BMDMs were less reactive than M-BMDMs as they did not increase TNF and IL-6 production, possibly because GM-CSF induces more inflammatory macrophages than M-CSF (22). Overall, the protocol used to derive "trained" mouse macrophages impacts on the global phenotype of the cells.

The cytokines initially associated to a trained phenotype were IL-1β, IL-6 and TNF (6). In our model of training *in vitro*, training increased the secretion of IL-6 by M-BMDMs and GM-BMDMs. However, M-BMDMs downregulated the expression of IL-10, CCL2, CCL3 and TNF, indicating that increased IL-6 production alone might not be sufficient to confirm a trained phenotype. M-BMDMs derived from the BM of trained mice produced increased levels of TNF and boosted their oxidative and glycolytic metabolism. This suggests that the BM is subjected *in vivo* to the influence of cells or mediators allowing the subsequent generation of a trained phenotype. Saz-Leal and colleagues trained BM cells *in vitro* similarly to our protocol, but primed BMDMs with IFNγ before stimulation to induce cytokine production (23). In line, IFNγ producing $CD8^+$ T cells were important for the induction of a trained phenotype in alveolar macrophages (20). In our whole blood stimulation experiments, IFNγ levels were elevated in trained samples, supporting a possible role in priming myeloid cells. Further studies should unravel whether IFNγ is important before, during or after priming with β-glucan and if other cytokines play a role in the initiation of a trained phenotype. A good candidate is IL-1β. Trained monocytes/macrophages produced increased levels of IL-1β upon stimulation with microbial compounds, and IL-1β directly contributed to trained immunity induced by β-glucan, BCG vaccination and Western diet (6, 10, 11, 14, 15, 24, 25). Moreover, we have shown that blocking IL-1 signaling with

recombinant IL-1 receptor antagonist (anakinra) during the induction of trained immunity reversed the protective effect against lethal infections conferred by training immunity (8).

Short-term training of BMDMs with β-glucan increased the production of GM-CSF which in turn upregulated dectin-1 (the receptor for β-glucan) and promoted cytokine production (26). Therefore, training of BMDMs *in vitro* probably puts GM-BMDMs to the concomitant influence of zymosan and GM-CSF. These cells decreased MHC-II expression (data not shown) as previously reported for *in vitro* trained mouse spleen monocytes (7). On the contrary, *ex vivo* trained BMDMs increased MHC-II expression as reported for alveolar macrophages (20) and monocytes (27) isolated from trained mice. Since MHC-II expression is important for the priming of T cells, these results support a global effect of training on the immune system. Future studies should clarify whether trained immunity also supports adaptive immune responses.

Training affects the epigenetic status of immune cells by altering histone acetylation and methylation (28). The increased NAD^+/NADH ratio observed in trained human monocytes questioned the role of sirtuins in the induction of training. Indeed, sirtuins are NAD-dependent histone deacetylases (HDACs), a family that also contains the so-called “classical” Zn-dependent HDAC1-11. Although high NAD^+ promotes sirtuin activity, the expression of SIRT1 was reduced in trained monocytes (9). In BMDMs derived from trained mice, the expression of sirtuins was marginally affected. However, the gene expression of sirtuins does not necessarily reflect their activity since sirtuins are subjected to post-translational regulation (29). Moreover, the epigenetic regulation of acetylation is highly dependent on the balance between histone acetyltransferases (HATs) and classical HDACs rather than sirtuins (30). Considering that sirtuins target multiple metabolic pathways and influence inflammatory responses, they may influence trained immunity through multiple mechanisms. It will be of great interest to analyze how much enzyme specific and pan-HDAC/sirtuin targeting drugs affect trained immunity (31).

We characterized long-lasting training in mice challenged 9 weeks and 28 weeks before with zymosan. Nine weeks after training, trained mice showed increased central and extramedullary

hematopoiesis probably accounting for the increased number of inflammatory monocytes and neutrophils observed in the blood. Under steady-state conditions, HSCs are present in spleen, but their contribution to hematopoiesis is undefined (32). Besides altered cell populations, whole blood of trained mice produced higher levels of cytokines and controlled better the growth of *L. monocytogenes*. Therefore, we have identified several parameters that can be used to monitor the trained status of mice based on the analysis of a small amount of blood and not requiring animal sacrifice. This will allow us to assess more closely and more deeply the evolution of the trained phenotype over time.

Whole blood from 9-week trained mice produced increased levels of cytokines previously linked to training (IL-1β, IL-6, TNF, IFNγ, G-CSF and CCL2), but also IL-1α, IL-10, CXCL1 and CXCL10. The levels of these cytokines were increased after stimulation of whole blood from children vaccinated with BCG (33). Moreover, CCL2 production increased in human monocytes trained *in vitro* with oxidized low density lipoproteins (oxLDL), an inducer of atherosclerotic foam cells, or β-glucan (34). Amazingly, a pilot experiment performed using blood from mice trained 28 weeks earlier revealed an enhanced LPS-induced cytokine production. This observation opens the venue for additional studies about the maintenance of the trained phenotype *in vivo*.

Elevated concentrations of G-CSF were detected in the BM of trained mice and were linked to enhanced myelopoiesis (10). Extramedullary hematopoiesis is triggered by physiological stress such as pregnancy and diseases. Interestingly, *E. coli* infection triggered NLR and TLR signaling and induced the expression of G-CSF that mobilized HSCs and progenitor cells to the spleen. Progenitors gave rise to neutrophils and monocytes that contributed to limit secondary infection (35). As we observed with zymosan, injection of Pam_3CSK_4 lipopeptide into mice promoted BM and extramedullary hematopoiesis with an important niche in the spleen (37). Training increased spleen size without affecting the number of splenic leukocytes supporting an effect on other cell types. In a model of zymosan-induced generalized inflammation (ZIGI), injection of zymosan reduced BM erythropoiesis with a concomitant increase in spleen erythropoiesis (36). Moreover, migration of erythropoietic

progenitors from the BM to the spleen increased the proportion of $CD45^+$ cells in the BM and the size of the spleen. Whether erythropoiesis and red blood cells play a role in trained immunity has not been investigated. Since chronic diseases are linked to anemia in patients (37) and training has a long-lasting effect on immune responses, the blood parameters of trained mice are likely to be affected.

Overall, we show that training affects the functionality and the number of innate immune cells with long-lasting effects. The differences in the phenotype and functionality observed between BMDMs trained *in vitro* and derived from the BM of trained mice are critical parameters to take into consideration when studying macrophage training *in vitro*. Since low-grade inflammation is involved in the pathophysiology of chronic and autoinflammatory disorders and inflammation is considered as the basis of most age-related diseases (38), the link between induction of trained immunity and the occurrence of inflammatory-age related diseases is an exciting future field of research (39). While trained immunity may be used to enhance vaccine efficiency, counterbalancing the activity of training inducers, IL-1 signaling, metabolic and epigenetic adaptations may all be exploited to avoid the occurrence of trained immunity-related pathogenic processes.

Materials and methods

Ethics statement. Animal experiments were approved by the Service des Affaires Vétérinaires, Direction Générale de l'Agriculture, de la Viticulture et des Affaires Vétérinaires (DGAV), état de Vaud (Epalinges, Switzerland) under authorizations n° 876.9 and 3287 and performed according to Swiss and ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines).

Cells and reagents. Naïve mice and mice injected i.p. with 1 mg of zymosan (Sigma-Aldrich, St-louis, MO) 7 and 3 days before sacrifice were used to collect BM from femurs and tibias (40). BM cells were differentiated into BMDMs by culture for 6 days in RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Biochrom GmbH, Berlin, Germany), 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) and growth factors: 30% (v/v) L929 cell supernatant, 50 IU/ml recombinant mouse macrophage colony-stimulating factor (M-CSF, ImmunoTools, Friesoythe, Germany) or 50 IU/ml granulocyte-macrophage CSF (GM-CSF, ImmunoTools). For training *in vitro*, 10 µg/ml of depleted zymosan (Invivogen, Toulouse, France) was added to culture medium during 24 h. Then, cells were collected, centrifuged at 400g for 5 min, resuspended in fresh medium containing growth factors and cultured for 6 days to obtain BMDMs. BMDMs were detached using versene (Thermo Fisher Scientific, Waltham, MA), enumerated and seeded in 96-well or 24-well plates at a density of 2 x 10^6 cells/ml in medium without growth factors. BMDMs were stimulated with TLR ligands, bacteria and *C. albicans*.

Salmonella minnesota ultra pure lipopolysaccharide (LPS) was from List Biologicals Laboratories (Campbell, CA), *Phaseolus vulgaris* phytohemagglutinin-L (PHA) from Sigma-Aldrich (St. Louis, MO) and CpG ODN 1826 (CpG) from Microsynth (Balgach, Switzerland). *Listeria monocytogenes* 10403S (*L. monocytogenes*) and methicillin-resistant *Staphylococcus aureus* AW7 (*S. aureus*) were grown in brain heart infusion broth (Thermo Fisher Scientific). *Candida albicans* 5102 (*C. albicans*) was cultured in yeast extract-peptone-dextrose (BD Biosciences, Franklin Lakes, NJ). Microorganisms were washed with PBS and heat-inactivated for 2 h at 56°C (*S. aureus*, *C. albicans*) or 70°C (*L. monocytogenes*). Blood was collected from the submandibular vein in EDTA coated tubes (Sarstedt,

Nümbrech, Germany) using 5 mm GoldenRod animal lancets (Braintree Scientific, Braintree, MA). Twenty microliters of blood were incubated with 80 µl of stimulus for 6 h or 24 h for cytokine assay, with 130 µl of live bacteria for killing or directly stained for flow cytometry.

RNA analyses. RNA was extracted and reverse transcribed into cDNA used to quantify gene expression as described (41) using gene-specific primers (12).

Flow cytometry. Spleens were disrupted in 40 µm cell strainers (Corning, Corning, NY). Spleen and BM were incubated with red blood cell (RBC) lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA) on ice, washed and resuspended in cell staining medium (CSM: 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS). Cells were incubated with 2.4G2 (cell culture supernatant of confluent cells, ATCC-HB-197TM), stained and fixed with 2% PFA (Thermo Fisher Scientific). Twenty microliters of blood were incubated with 50 µl of antibodies in CSM, diluted in RBC lysis buffer, fixed with 2% PFA and resuspend in CSM. Data was recorded using an Attune NxT Flow Cytometer (Thermo Fisher Scientific) and analyzed using FlowJo_V10_CL software (FlowJo LLC, Ashland OR). Antibodies are listed in **Table S1** and gating strategies have been described (8).

Metabolic measurements. BMDMs were plated in Seahorse XFe96 plates at 40'000 cells per well. Glycolytic capacity and mitochondrial metabolism were analyzed using the Glycolysis Stress Test Kit and Mito Stress Test kit (Agilent, Santa Clara, CA) as described (13).

Cytokine and LDH measurements. Cytokine concentrations in cell-culture supernatants and blood were determined using IL-6 and TNF DuoSet ELISA kits (R&D systems, Minneapolis, MN) and a Mouse Custom ProcartaPlex 17-plex (G-CSF, IFNγ, IL-1α, IL-1β, IL-3, IL-6, IL-10, IL-12p40, IL-17A, IL-18, CCL2/MCP-1, CCL3/MIP-1α, CXCL1/KC, CXCL2/MIP-2, CXCL5/ENA-78, CXCL10/IP-10, TNF) (Thermo Fisher Scientific) using a bioplex 200 system (Bio-Rad, Hercules, CA). LDH was measured with the Cyto Tox-ONE assay (Promega, Fitchburg, WI) according to manufacturer's protocol.

Bacterial growth assay. Blood was incubated for 2 h with 150 or 1000 colony forming units (CFU) of *L. monocytogenes* in 96-well plates. Plates were shaked for 1 min at 400 rpm and serial dilutions were

plated on Columbia III Agar with 5% Sheep blood medium (BD Biosciences). Plates were incubated for 18 h at 37°C and colonies were enumerated.

Statistical analyses. Groups were compared by variance analysis followed by two-tailed unpaired non-parametric Mann-Whitney statistical test. For repeated measures, area under the curve (AUC) was calculated and used for group comparison. Analyses were performed using PRISM version 8.0.1 (GraphPad Software). P values were two sided and values < 0.05 were considered to be statistically significant. *, $P < 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

Authorship

TH, EC and DLR performed experiments. TR and TH conceived the project, designed the experiments and wrote the paper. All the authors revised the paper.

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Conflict of Interest Disclosure

The authors declare no competing financial interests.

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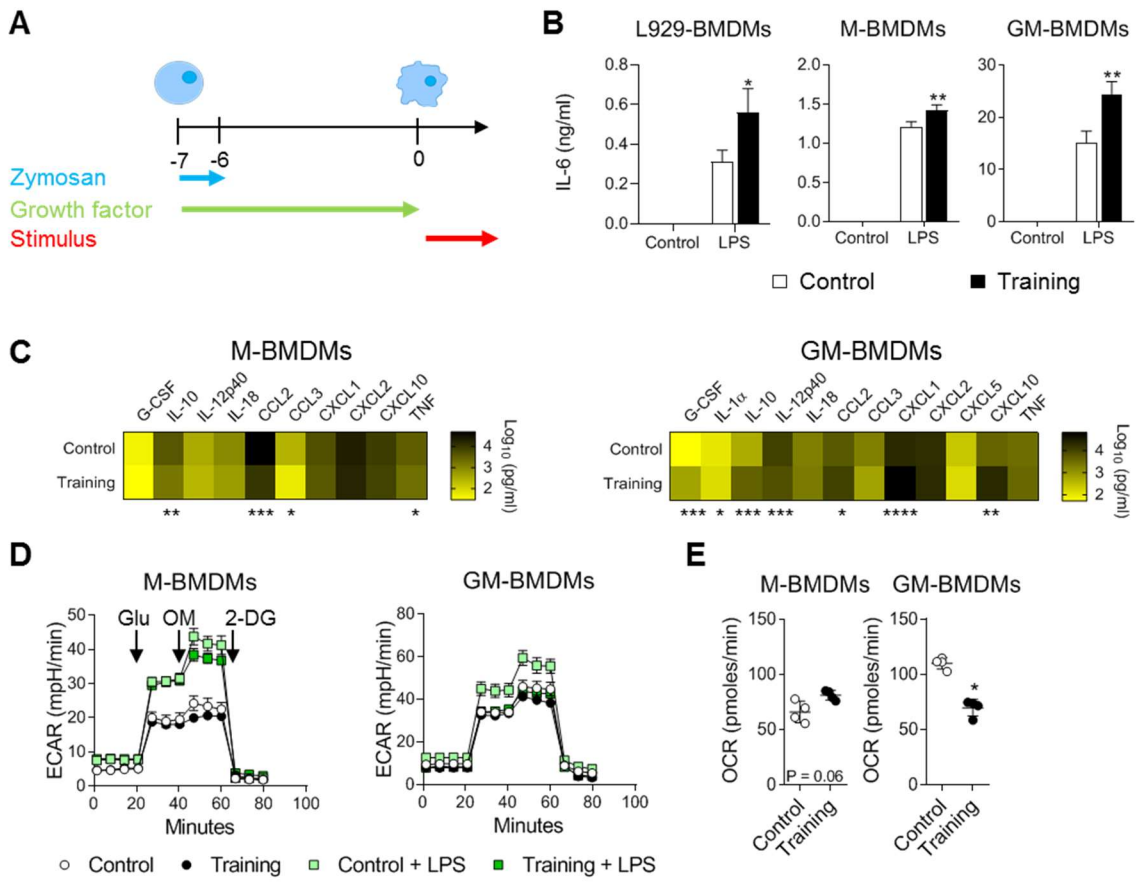

Figure 1: *In vitro* training modifies cytokine production by M-CSF and GM-CSF-derived BMDMs. (**A**) *In vitro* experimental training scheme. BM cells were cultured with 10 µg/ml zymosan for 24 h in the presence of growth factors. Medium was replaced by fresh medium containing growth factors and cells were cultured for an additional 6 days. Cells were detached, enumerated and plated at a fixed number for stimulation. (**B, C**) Cytokine concentrations in cell culture supernatants of trained and non-trained BMDMs differentiated with L929 supernatant, M-CSF (M-BMDMs) or GM-CSF (GM-BMDMs) and stimulated for 24 h with 10 ng/ml LPS were quantified by ELISA (**B**) and Luminex (**C**). (**D-E**) Metabolic parameters of control and trained M-BMDMs and GM-CSF. Glucose (Glu), oligomycin (OM) and 2-deoxyglucose (2-DG) were sequentially added and extracellular acidification rate (ECAR) was measured by Seahorse (**D**). Basal oxygen consumption rate (OCR) was measured by Seahorse (**E**). Data are mean ± SD of 3 to 6 mice analyzed in triplicate. *, $P < 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

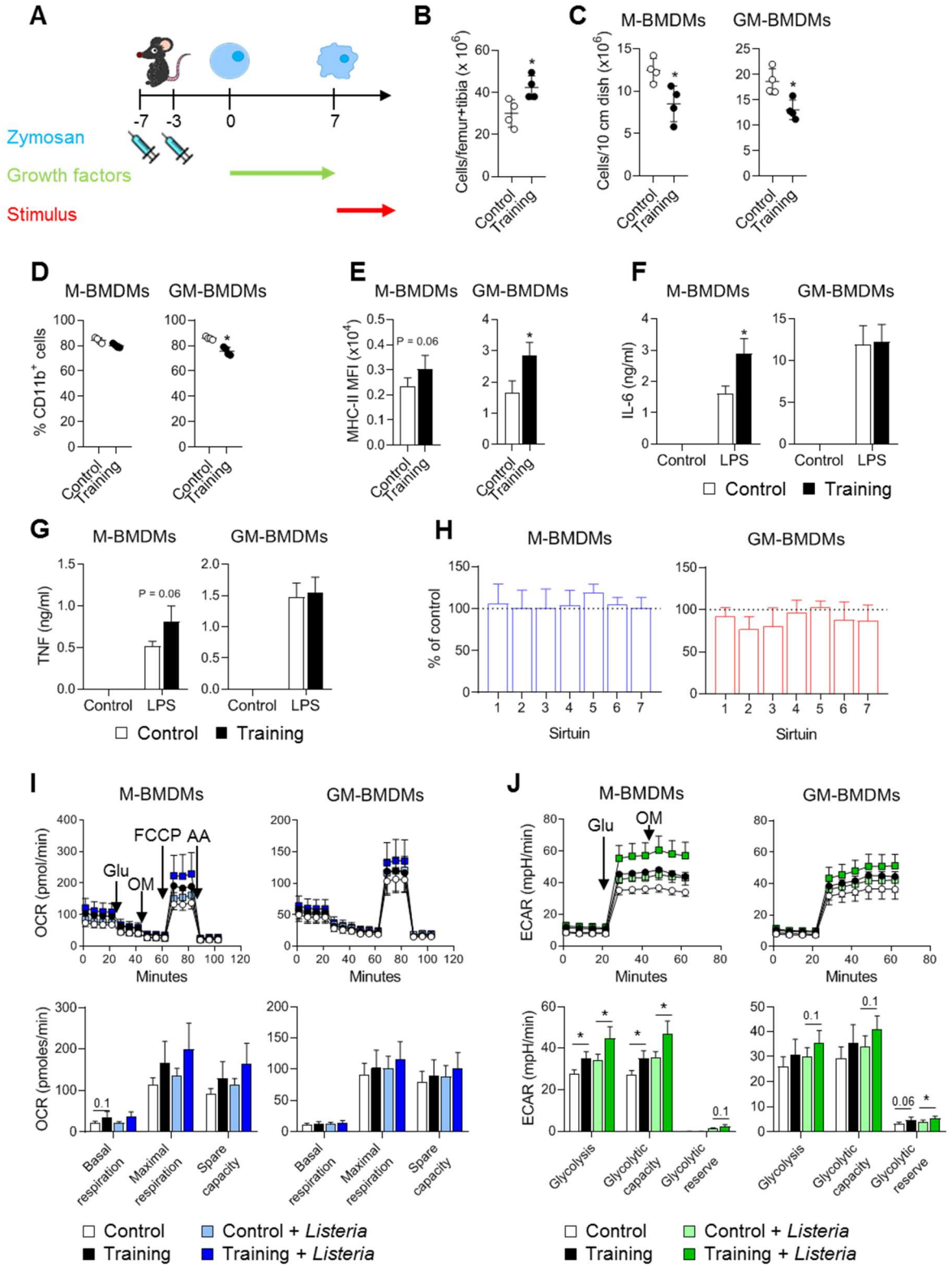

Figure 2: *In vivo* training affects *ex vitro* generated M-CSF and GM-CSF-derived macrophages. (**A**) Experimental setup to obtain BMDMs from trained mice. Mice were challenged intraperitoneally (i.p.) with 1 mg zymosan 7 and 3 days before sacrifice. BM was collected from control mice and trained mice and used to derive BMDMs in the presence of growth factors. (**B**) Number of cells recovered in the BM collected from femur and tibia of trained and non-trained mice. (**C**) Number of adherent cells recovered after 7 days of differentiation of BM with M-CSF (M-BMDMs) and GM-CSF (GM-BMDMs).

Figure 2 (continued): (**D, E**) Percentage of $CD11b^+$ cells (**D**) and MFI of MHC-II (**E**) were measured by flow cytometry. (**F, G**) M-BMDMs and GM-BMDMs were stimulated for 24 h with 10 ng/ml LPS and the concentrations of IL-6 (**F**) and TNF (**G**) in cell culture supernatants were measured by ELISA. (**H**) The mRNA levels of sirtuins were quantified by RT-qPCR, normalized to the actin mRNA levels, and expressed as relative to the levels in untrained (control) BMDMs. (**I, J**) Metabolic parameters of M-BMDMs and GM-BMDMs. Glucose (Glu), oligomycin (OM), FCCP and antimycin A (AA) were sequentially added to cells. OCR (**I**) and ECAR (**J**) were measured using Seahorse. Data are mean ± SD of 4 mice analyzed in triplicate or quadruplicate. *: $P < 0.05$.

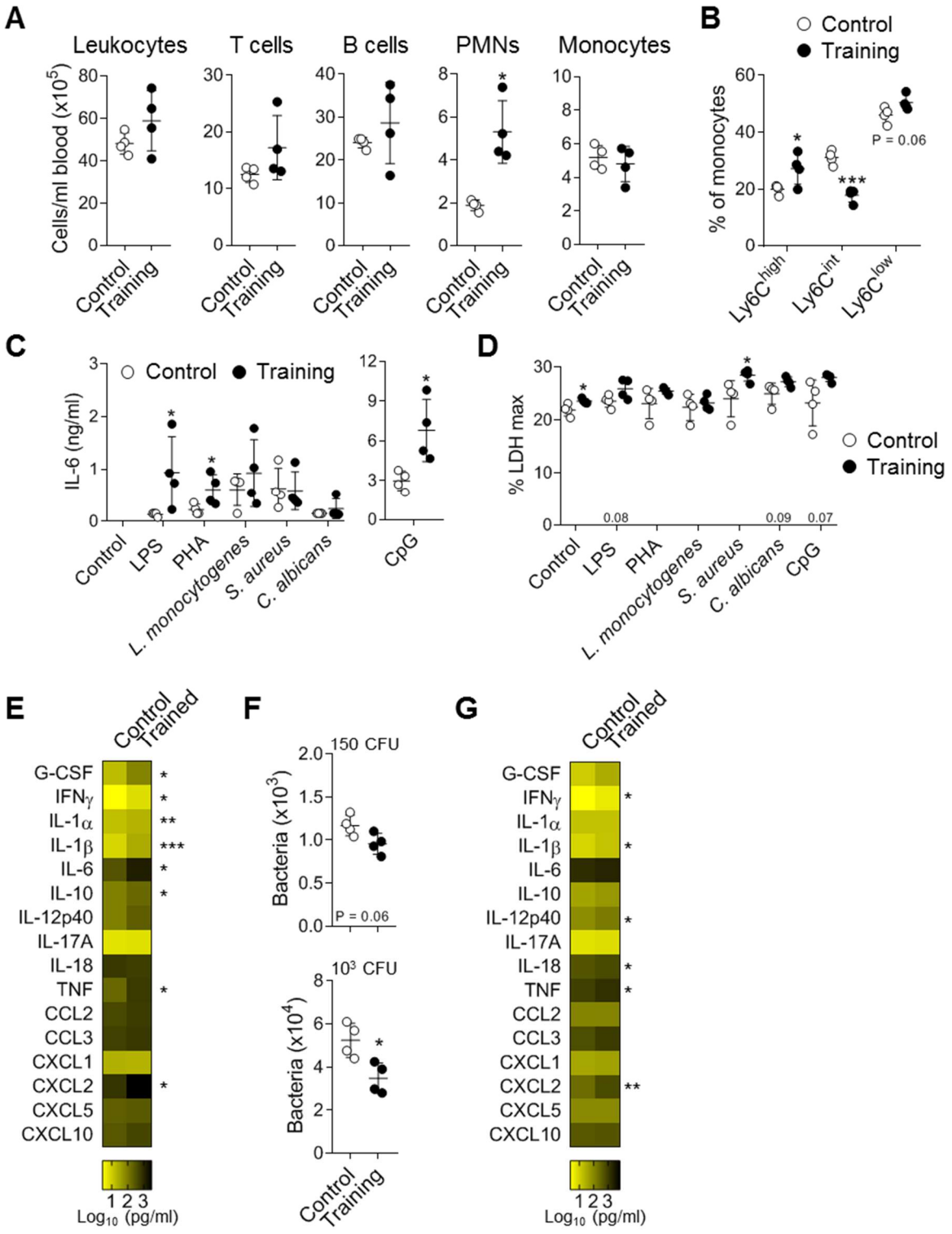

Figure 3: The frequency and reactivity of blood leukocytes are altered up to 28 weeks after training. Mice were trained with two injections of 1 mg zymosan given 4 days apart. Blood was collected 9 weeks later (**A-F**) or 28 weeks later (**G**). (**A**) Blood concentrations of leukocytes ($CD45^+$), T cells ($CD3^+$), B cells ($CD19^+$), PMNs ($Ly6G^+$) and monocytes ($CD11b^+Ly6G^-$) measured by flow cytometry. (**B**) Percentage of $Ly6C^{high}$, $Ly6C^{int}$ and $Ly6C^{low}$ monocytes among total blood monocytes.

Figure 3 (continued): (**C-E**) Blood was stimulated with 10 ng/ml LPS, 1 µg/ml PHA, 10 µM CpG and 10^8 CFU/ml heat-killed *L. monocytogenes, S. aureus* and *C. albicans* for 6 h. The concentrations of IL-6 (**C**) and LDH (**D**) in cell culture supernatants were quantified by ELISA and with the CytoxOne kit, respectively. Cytokines were measured by Luminex (**E**). (**F**) Blood was incubated for 2 h with 150 or 10^3 CFU *L. monocytogenes*, and bacteria were enumerated. (**G**) Blood collected from control mice and mice trained 28-weeks earlier was stimulated with 10 ng/ml LPS for 6 h. Cytokines were quantified by Luminex. Data are mean ± SD of four (9 weeks) or 7-8 (28 weeks) mice. Each dot represents a mouse. *, $P < 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

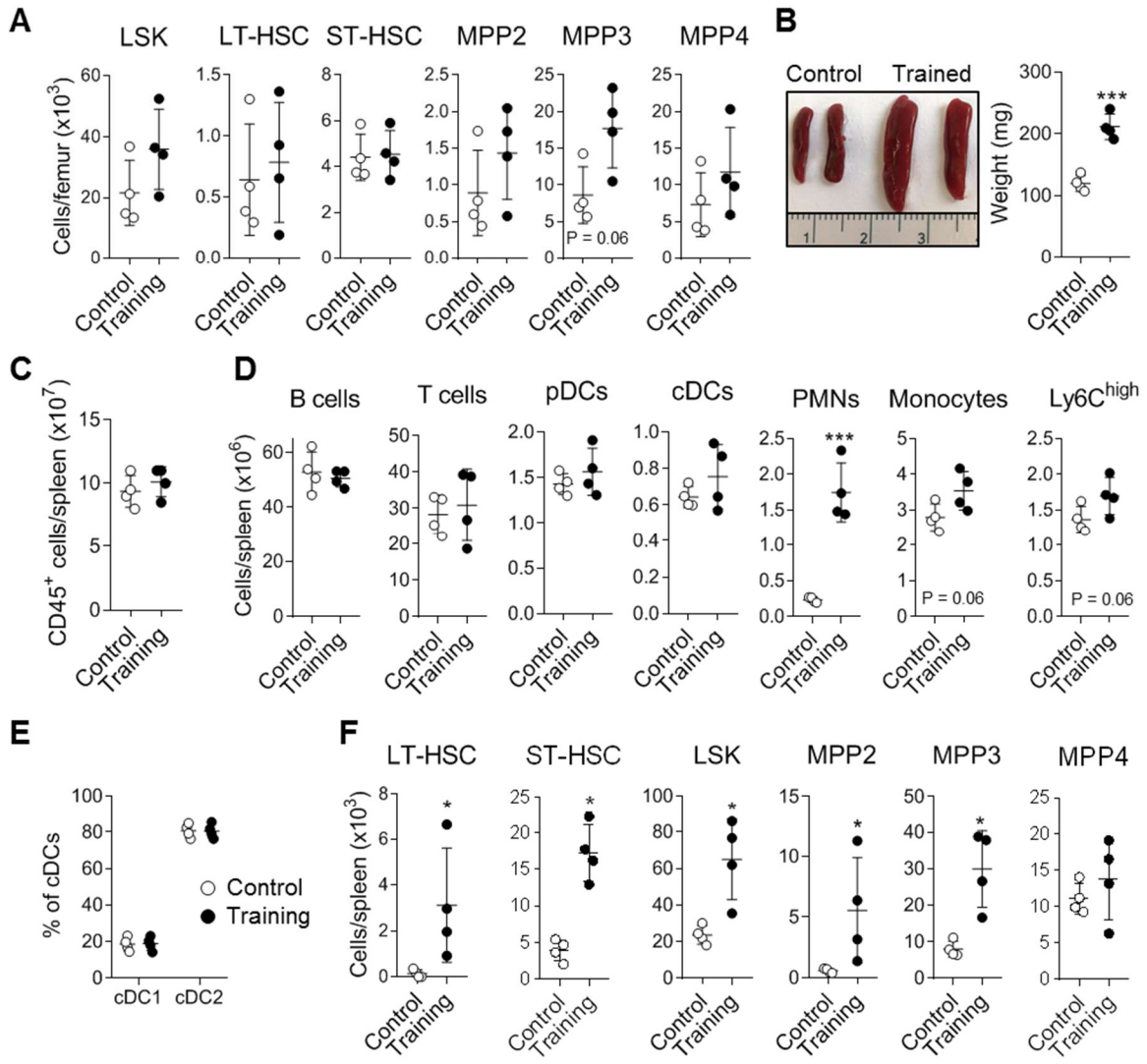

Figure 4: Training promotes myelopoiesis in the BM and spleen 9 weeks after training. BM (**A**) and spleen (**B-F**) were collected from control mice and mice trained 9 weeks earlier. Cell populations were analyzed by flow cytometry. (**A**) Number of HSCs and progenitor cells in the BM. (**B**) Images of the spleens from two control and two trained mice (left) and spleen weight (right). (**C, D**) Number of $CD45^+$ leukocytes (**C**), B cells ($B220^+CD11c^-$), T cells ($CD3^+$), pDCs ($CD11c^+$ $B220^+$), cDCs ($CD11c^+$ $B220^-$), PMNs ($Ly6G^+$), monocytes ($CD11b^+$ $Ly6G^-$) and inflammatory monocytes ($Ly6C^{high}$) (**D**) per spleen. (**E**) Percentage of cDC1 ($CD11b^-$) and cDC2 ($CD11b^+$) among total cDCs. (**F**) Number of HSC and progenitor cells in the spleen. Each dot represents a mouse. *, $P < 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Table S1: Antibodies used for flow cytometry

Target	Clone name	Coupling	Brand	Reference
B220	RA3-6B2	PE-Cy7	BioLegend	103222
B220	RA-6B2	Brilliant Violet 570	BioLegend	103237
CD117	2B8	APC	BioLegend	105812
CD11b	M1/70	APC-eFluor780	eBioscience	47-0112
CD11b	M1/70	PE-Cy7	BioLegend	101216
CD11b	M1/70	PercCP-Cy5.5	eBioscience	45-0112
CD11c	N418	APC	BioLegend	117310
CD11c	N418	PE	BioLegend	117307
CD135	A2F10	PE	eBioscience	12-1351
CD150	TC15-12F12.2	PerCP-Cy5.5	BioLegend	115922
CD19	1D3	PE-Cy7	eBioscience	25-0193
CD3e	17A2	APC	eBioscience	17-0032
CD3e	17A2	PE-Cy7	BioLegend	100220
CD4	GK1.5	APC-Cy7	BioLegend	100414
CD41	MWReg30	eFluor450	eBioscience	48-0411
CD45	30-F11	FITC	BD Biosciences	553079
CD48	HM48-1	APC-Cy7	BioLegend	103432
CD8a	53-6.7	FITC	BioLegend	100706
Ly6C	HK1.4	PerCP-Cy5.5	eBioscience	45-5932
Ly6C/G	RB6-8C5	PE-Cy7	BioLegend	108416
Ly6G	1A8	eFluor450	eBioscience	48-9668
Ly-76	TER119	PE-Cy7	BioLegend	116222
MHCII	M5/114.15.2	PE	eBioscience	12-5321
Sca-1	D7	FITC	eBioscience	11-5981
Viability dyes				
Zombie			BioLegend	77168
Fixable Aqua			ThermoFisher	L34957
Fixable Violet			ThermoFisher	L34955

4 GENERAL DISCUSSION AND PERSPECTIVES

4.1 Diet, microbiota, short chain fatty acids and trained immunity

Nearly ten years ago, studies on human cohorts unravelled the diversity and complexity of human body microbiomes [434]. Since then, it has become clear that food dictates the diversity and the composition of the gut microbiota [435]. Gut bacteria interact with immune cells affecting not only immune cell polarization but also hematopoiesis [436]. Accordingly, germ free mice have a defective myelopoiesis [437]. SCFAs produced by the gut microbiota interact with immune cells through GPCRs or by diffusing inside cells, acting as inhibitors of HDAC (HDACi) and reducing inflammation [180]. Like other HDACi, the SCFA propionate reduced the production of inflammatory cytokines by macrophages and DCs. The lower production of IL-12p40 by DCs has been linked to decreased antigen presentation and T cell activation [438].

The anti-inflammatory effect of SCFAs questions their impact on host responses to infections. In mice, supplementation with propionate in drinking water did not increase susceptibility to infection and bacterial burden supporting a mechanism compensating for the reduced inflammatory phenotype. In line, butyrate decreased cytokine production but increased the secretion of antimicrobial peptides by macrophages to reduce bacterial burden [186]. Since multiple SCFAs exhibit anti-inflammatory properties, targeting bacterial enzymes in pathways involved in specific SCFA synthesis would be useful to delineate the role of individual SCFAs in inflammatory responses *in vivo* [439]. Western diet (WD), contrary to high fiber diet, induced a trained phenotype in mice characterized by increased concentrations of cytokines in the blood of WD-fed mice and in *ex vivo* stimulated cells [431]. Moreover, WD promoted the proliferation of bone marrow HSCs, MPPs and GMPs, enhancing the number of circulating neutrophils and inflammatory monocytes. In opposite, SCFA supplementation increased the proportion of macrophage and DC precursors (MDPs) and patrolling monocytes [440]. Both SCFAs and WD increased the proportion of immunosuppressive

Tregs defined as Foxp3^{+} T cells [187, 441]. Single-cell analyses disclosed that Tregs form a heterogeneous cell population with a range of possible activation and differentiation states [442]. Refining the Treg subpopulations induced by SCFAs or trained immunity (WD or another training agent) could reveal regulatory mechanisms of training and define new targets to promote specific subtypes of Tregs.

4.2 Sirtuins and immune responses

Sirtuins regulate metabolic and inflammatory pathways and are linked to overall cellular homeostasis. The observation that sirtuins increased the lifespan of invertebrates prompted research on the relationship between sirtuins and mammalian lifespan and the development of sirtuin activating compounds (STACs) [214]. Sirtuin overexpression in transgenic mice and sirtuin activation by STACs protected from inflammation and replicated the well-known beneficial effects of caloric restriction, but failed to increase the lifespan of complex organisms. The role of SIRT2, SIRT3 and SIRT5 in immune responses is mainly based on observations in chronic inflammatory diseases. In our studies, we did not observe any strong effect of single sirtuin deletion on innate immune responses and host defenses. Yet it is still possible that sirtuins have a role in infections revealed only under stress conditions. For example, inhibition of SIRT2 in ob/ob mice was protective against CLP [358] while SIRT3 deficiency increased hepatic and neuronal inflammation in HFD fed mice [355, 443]. SIRT5 maintained cardiac homeostasis under stress conditions but showed no effect under steady state [444]. These observations advocate that sirtuin modulators should be tested for their effect on host susceptibility to infections when used in the treatment of inflammatory diseases associated with age or stress.

The activation of sirtuins is a double-edged sword since sirtuins were reported to promote inflammation-related disorders including cancer [231] and neurodegeneration [334]. The conserved structures in sirtuins imply that STACs and sirtuin inhibitors inevitably modulate the function or activity of multiple sirtuins [219]. Therefore, to evaluate the overall impact of a sirtuin modulator, it is

important to understand the crosstalk and the antagonistic or additive effects between sirtuins. The deletion of SIRT3 and SIRT5 were reported to increase the overall mitochondrial acetylome and succinylome respectively [235, 272]. While LPS stimulation increased protein succinylation [445], the impact of succinylation on cellular homeostasis is not understood. Interestingly, the main source of succinate during macrophage activation is glutamine metabolism, a pathway repressed by SIRT4 [269] suggesting some crosstalk between SIRT4 and SIRT5.

Mice deficient in two sirtuins (SIRT2/3$^{-/-}$ and SIRT3/5$^{-/-}$ mouse lines) showed phenotypes unseen in the corresponding single sirtuin deficient mice. While SIRT2$^{-/-}$ macrophages increased glycolysis, SIRT2/3$^{-/-}$ macrophages decreased glycolysis. This effect in SIRT2/3$^{-/-}$ macrophages could result from the increased expression of SIRT6 known to downregulate glucose metabolism through HIF-1α destabilization [446]. These compensatory mechanisms between sirtuins (**Figure 11**) and possibly other enzymes could explain some of the contradictory results obtained upon silencing, knocking in or knocking out sirtuins, and using drugs targeting sirtuins to study the role of a specific sirtuin. They also highlight the importance of assessing the expression and function of all the sirtuins when using modulators of sirtuins.

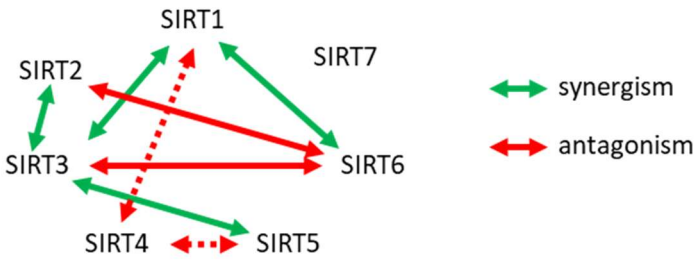

Figure 11: Interaction between sirtuin in metabolism and inflammatory responses. Interactions were observed (solid line) or predicted (dotted line) based on our experiments or findings in the literature using *in vitro* experiments.

4.3 Sirtuins and classical HDACs in trained immunity

Innate immune cells were described as cells carrying a limited number of defined functions with little plasticity. In the recent years, it has become clear that the classically-defined populations of innate immune cells are heterogenous and comprise subpopulations of cells able to modulate their functions in response to external stimuli [447]. The field of trained immunity is bringing new insight on the immune, metabolic and epigenetic reprogramming of innate immune cells inducing long-lasting increased immune responses. Among epigenetic marks, training altered histone methylation at gene promoters (H3K4me3) and distal elements (H3K4me1) associated with histone acetylation (H3K27Ac) which are all indicative of active gene transcription [385]. Histone H3 acetylation is regulated by the balance between HDACs (classical HDACs and nuclear sirtuins) and HATs. The observation that HDACs/sirtuins and training target the same epigenetic marks suggests a possible role for HDACs/sirtuins in training mechanisms. Several HDAC and sirtuin inhibitors increased overall histone H3 and H4 acetylation while reducing immune responses of macrophages and DCs [131]. Histones can be acetylated at numerous lysines [99] and measuring overall acetylation does not inform about the specific sites of acetylation. Moreover, HDAC inhibition by TSA induced demethylation of repetitive sequences [448] while SIRT1 was reported to interact with histone methyltransferases removing acetyl groups to enable methylation [449]. These studies support an indirect impact for HDACs on histone methylation levels and underline the need for epigenetic analyses considering multiple histone modifications. Evaluation of HDAC expression and their activity in trained cells and organisms would indicate the possible effect of HDAC modulation on training.

Sirtuins and trained immunity impact on numerous cellular processes with equivalent or antagonistic effects. SIRT1 and SIRT6 cooperate to switch metabolism from glycolysis to fatty acid oxidation (FAO) during inflammation resolution [450], an effect counteracted by SIRT4 [451]. SIRT1 expression was reduced in trained human monocytes and resveratrol reduced the production of TNF but not IL-6 by β-glucan trained cells [386]. We showed that BMDMs derived from the BM of trained

mice compared to non-trained mice marginally altered the expression of sirtuin mRNA. However, the expression of sirtuin mRNA does not necessarily reflect sirtuin protein expression and activity [452]. SIRT2/3$^{-/-}$ and SIRT3/5$^{-/-}$ macrophages produced increased levels of cytokines supporting an antagonistic effect for some sirtuins on training. To further investigate the role of sirtuins in training, their expression and activity should be assessed in trained cells and in the organs of trained mice. Moreover, supplementation of trained mice with sirtuin activators or inhibitors could elucidate a possible role for sirtuins in the mechanisms regulating training. Finally, we will perform irradiation-induced bone marrow chimeric mice to determine the contribution of wild-type and sirtuin knockout hematopoietic versus nonhematopoietic compartments in the establishment of a trained phenotype.

Training affects histone methylation and acetylation, but no report focused on the impact of training on general levels of post-transcriptional modifications in cells. Despite being members of the family of HDACs, sirtuins not only catalyze the removal of acetyl groups, but also other functional groups including acyl, crotonyl, malonyl, myristoyl and succinyl [202]. Still, the biological relevance of most of these modifications remains unknown. Acetylation generally represses enzymatic activity. Succinylation, the main modification catalyzed by SIRT5, frequently targets the same lysine as acetylation [453]. During LPS stimulation, malonylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) induces its dissociation from the promoter of inflammatory cytokines enabling their transcription [454]. SIRT5 is the only enzyme reported with a demalonylase activity. Future studies on the effect of protein translational modifications on protein functionality should unravel novel mechanisms regulating cellular processes. Moreover, measuring by proteomics the impact of training on these modifications could identify new enzymes regulating training and general immune cell functionality and plasticity. Combined with whole genome epigenetic and protein-RNA interaction studies, this should give a picture of the overall regulatory mechanisms altered by the induction of trained immunity (**Figure 12**).

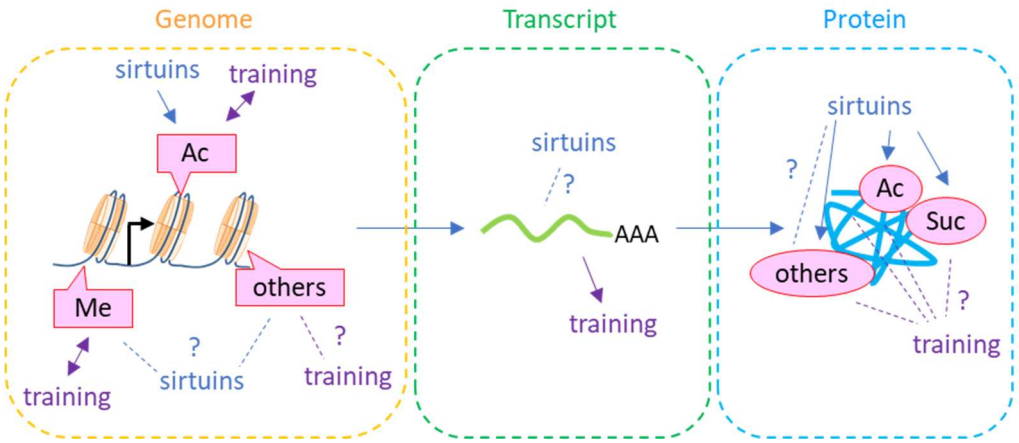

Figure 12: Regulation of and by sirtuins and training at gene, transcript and protein levels. Sirtuins and training modulate and are modulated at the genomic, transcriptional and translational levels. Dotted lines indicate unknown or poorly characterized effects. At the transcript level, only an effect of long non-coding RNA (lncRNA) on the regulation of training has been reported. Ac: acetylation, Me: methylation, Suc: succinylation, others: any other post-translational modification.

4.4 Trained immunity in pulmonary inflammation and infections

The lungs are constantly exposed to air transporting oxygen crucial for cellular respiration but also inert molecules and potentially harmful microbes. Because the function of the lungs is vital and collateral damage can be fatal, the lungs contain specialized epithelial and immune cells expressing a wide range of PRRs and interacting with the lung microbiota at homeostasis [455]. Pulmonary immune cells comprise mainly macrophages (alveolar, interstitial and monocyte derived), DCs (pDCs, cDCs), granulocytes and lymphoid cells (T cells, B cells, NK cells and innate lymphoid cells) [456]. SCFAs influence distally the lung microbiota composition, the responsiveness of lung immune cells, and reduce allergic asthma [194, 195]. In a model of pneumonia induced by *Klebsiella pneumoniae*, the HDACi valproic acid increased the susceptibility of mice [141] while the SIRT1 and SIRT2 inhibitor cambinol was protective [230]. In our models of pneumonia, neither the supplementation with propionate nor the deficiency in SIRT2, SIRT3 or SIRT5 affected the survival of mice. Therefore, the impact of SCFAs on lung immunity is likely independent of their HDACi activity.

Pulmonary infections and chronic inflammation are among the leading causes of death worldwide [457]. Treatments are in development either to boost or to contain the pulmonary immune system in order to fight infection or reduce non-resolving harmful inflammation. Local and systemic training affected the response of pulmonary immune cells and the overall immune status. Intranasal instillation of *C. albicans* or *Lactobacillus* protected mice against *P. aeruginosa* and pneumovirus infections [403, 405] reducing microbial loads and lung lesions. The intranasal priming with adenovirus induced a trained phenotype in alveolar macrophages (AMs) that was independent of bone marrow progenitors and afforded protection against streptococcal pneumonia [458]. Interestingly, the murine herpesvirus protected against allergic asthma by replacing resident AMs by monocytes derived macrophages (MDMs) [458]. In both cases, pulmonary macrophages (AMs or MDMs) showed heightened expression of MHC-II but no increase in phagocytosis. These observations suggest that the nature, the dissemination and possible latency of the training agent dictates the cellular composition and functionality of the trained organ. While intranasal priming with herpesvirus protected against intravenous infection with *L. monocytogenes* [404], its influence on pulmonary infections is unknown. Similarly, we showed that intraperitoneal training with zymosan protected against pulmonary infections supporting an immunomodulatory effect on lung immunity. The phenotype of pulmonary macrophages and other immune cells in our training model still needs to be determined. We are planning to fill this gap by performing single-cell RNA-Seq analyses and single cell high profiling using cytometry by time of flight (CyTOF) in lungs of trained and control mice. The effect of training agents on inflammation questions whether the protection against allergic asthma and pathogens are mutually exclusive and whether a training agent could be protective in both pathological conditions. Therefore, would like to extend our range of *in vivo* models in zymosan-trained mice to include allergic diseases.

4.5 Length and magnitude of trained immunity

Understanding the long-term effects of training requires to unravel the initiating mechanisms and the range of cells affected. Training with β-glucan signals through dectin-1 inducing phenotypic and functional alterations in target cells. Macrophages and DCs are the main cells expressing dectin-1 [459]. While the effect of training on the phenotype and functionality of macrophages is well described, no report focused on the effects of training on DCs. DCs represent a heterologous family with roles in direct pathogen eradication, cytokine secretion to modulate cell recruitment and antigen presentation to T and B cells [460]. DCs can induce both inflammation and tolerance, thereby playing a role in balancing immune responses. The possible role of DCs in trained immunity is supported by observations that *in vitro* cultured DCs can uptake zymosan upregulating the expression of maturation markers (CD40, CD80, CD86 and HLA-DR) [461] and that the activation of DCs reprograms their metabolism similarly to macrophage [462]. *In vivo*, *L. monocytogenes* localizes in splenic DCs and macrophages immediately after infection, supporting a role for these cells in the early eradication of the pathogen [463]. In our model of training, we observed a strong increase of the size of the spleen and no dissemination of *L. monocytogenes* in the blood in trained mice. The number of pDCs and cDCs in the spleen of trained and non-trained mice nine weeks after training was very similar, which suggests that training does not affect the frequency of DCs on the long term without indicating the effect on the functionality of DCs. We are planning to analyze the frequency and reactivity of DC subsets isolated from the spleen and other organs including the lymph nodes, to characterize the overall effect of training on DCs. Further analyses using single cell transcriptomics could give clues about cells and cell-cell interactions [464] affected by training and disclose whether DCs orchestrate the overall immune state induced by training.

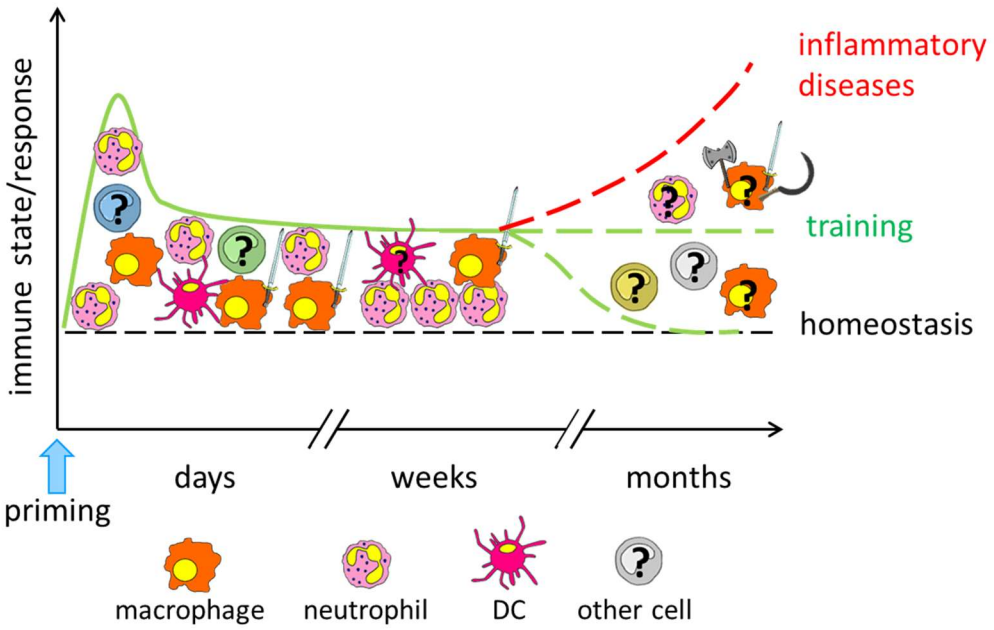

Figure 13: **Long-lasting effect of trained immunity.** Training increases the reactivity and the number of macrophages, neutrophils, and possibly DCs and other immune cells. The effect was reported to last for several weeks. Whether the immune state of trained subjects goes back to homeostasis or promotes the development of inflammatory diseases is not known.

Several studies in endemic countries suggest that vaccination of children with BCG protects against heterologous infections [408]. Supporting this observation, monocytes from BCG vaccinated subjects produced higher levels of inflammatory cytokines up to a year after vaccination [387]. Moreover, BCG and β-glucan induced-training were reported to reprogram bone marrow myeloid progenitors [413, 422], supporting the long-lasting effect of training. The trained phenotype induced by adenovirus or BCG lasted for 16 weeks [413, 458] while, in our models, zymosan was protective for 9 weeks. At that time, trained mice had increased myelopoiesis in the bone marrow and spleen with a higher number of circulating myeloid cells. Since activated cells produce cytokines promoting myeloid cell generation and recruitment, it is unclear which cells are fueling the trained phenotype and which cells or cellular components to target to return to homeostasis. Moreover, whether the immune system ever returns to homeostatic level or promotes the development of inflammatory diseases is unknown (**Figure 13**). Studies analyzing the evolution of the trained phenotype over time would be crucial to answer these questions.

The increased inflammatory state induced by training interrogates whether trained mice are more susceptible to autoinflammatory and autoimmune diseases and whether these conditions are maintained by mechanisms linked to training (**Figure 13**). Among others, rheumatoid arthritis, systemic lupus erythematosus, type 1 diabetes mellitus and periodic fever syndromes are diseases with inflammatory, epigenetic and metabolic rewiring comparable to trained immunity [465]. Autoinflammatory and autoimmune diseases are characterized by a chronic inflammatory state involving innate immune cells and sustained by adaptive immune cells [466]. The link between training and autoimmune conditions implies an activation of adaptive immune cells by antigen-presenting cells (APCs), possibly mediated by macrophages and DCs. Autoimmune conditions are usually connected to an abnormal activation of macrophages with a predominant M1 activation profile [467]. In several cases, M1 macrophages simultaneously express M2 markers supporting an aberrant (or not classical) macrophage polarization. Recent studies also support a role for DCs in autoimmune diseases [468]. DCs are important for T cell activation and autoreactive T cell suppression. Interestingly, both an increased number and the ablation of DCs prompted the development of autoimmune diseases [468]. Since several autoimmune and autoinflammatory diseases involve epigenetic modifications, a comprehensive study comparing these modifications with training induced changes would bring light on the role of training in these pathological conditions. Besides, long-term follow-up of trained mice would indicate whether they are more prone to develop autoinflammatory or autoimmune disease. Together, these findings could uncover novel targets for the treatment of autoimmune and inflammatory disorders.

4.6 Therapeutic perspectives of trained immunity

The field of trained immunity opens new perspectives in the development of vaccines and treatments to enhance or impede the inflammatory response of innate immune cells [428]. Whereas conventional vaccines rely on the generation of antigen-specific T and B cells, vaccines combined to training agents

aim at enhancing the immune status to increase protection against target pathogens and/or heterologous infections [469]. Although the BCG vaccine has been the most studied vaccine in the field of trained immunity, several other vaccines including smallpox, measles and polio vaccines afforded heterologous protection [470]. These heterologous effects of vaccination are interesting in endemic countries to afford the broadest possible protection against infections. Besides, including training agents such as β-glucan or flagellin as adjuvants to poorly efficient vaccines could boost the efficiency of vaccination by promoting the maturation and antigen-presentation by DCs to generate memory T cells [469]. BCG vaccination was associated with severe complications in patients with primary immunodeficiency diseases [471] but not in healthy subjects. Infants are the most prone to benefit from training agent containing vaccines since their immune system is able to modulate upon vaccination with minimal collateral damage due to less responsive NK and T cells [472]. Therefore, the development and the usage of trained immunity-based vaccines should consider not only the training agent but also the immune status of the recipient.

Sepsis is characterized by both an excessive inflammatory response and a state of immunosuppression occurring concomitantly [473]. Even after recovering, sepsis patients show long-term impairments due to the inability of the organism to recover homeostasis. The diagnosis and treatment of sepsis are challenging with few, if any, biomarkers and targets available. Training and tolerance being models of overwhelming inflammation and immunosuppression of sepsis, uncovering the mechanisms underlying these immune states could reveal new biomarkers to diagnose sepsis and enable the development of targeted therapies. In human monocytes, β-glucan and LPS differentially affected epigenetic marks [474]. Importantly, β-glucan could reverse the LPS-induced epigenetic landscape and tolerant phenotype supporting the potential of targeting histone modifications to treat sepsis. However, sepsis is a multifactorial disease influenced by both the host and the pathogen and all patients with sepsis might not react similarly to the same treatment. In our experiments, the protection afforded by zymosan-induced training increased survival by 30-70% in models of infection with *S. pneumoniae*, *P. aeruginosa* and *S. aureus* and 80-100% in *L. monocytogenes* systemic infection

and *E. coli* peritonitis. The current limitation on the effect of training in infections resides in its assessment only in acute infections questioning the effect of training in sublethal, chronic or endotoxemic models. As mentioned above, single cell studies combining transcriptome and mass cytometry analyses should give clues about the mechanisms regulating immune responses in training and possibly explain how trained mice control an infection and simultaneously avoid excessive lethal inflammation (**Figure 14**).

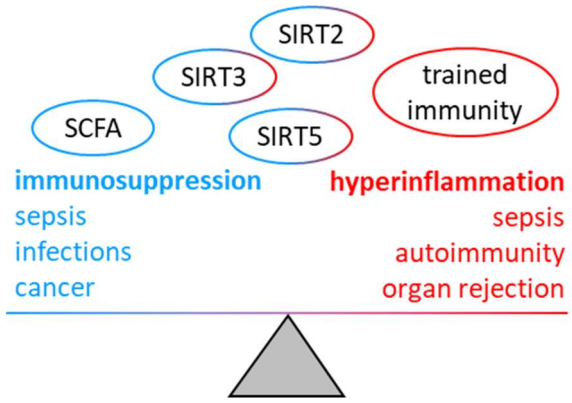

Figure 14: Balance between excessive and impaired immune responses. The increased immune response induced by training as well as the anti-inflammatory effect of SCFAs and, to some extent sirtuins, may be compensated by other uncharacterized mechanisms avoiding excessive imbalance from homeostasis.

Altogether, the findings in this work support that propionate, sirtuins and trained immunity modulate innate immune responses. However, the effect observed *in vitro* or focusing on a single sirtuin might diverge from the phenotype *in vivo* and neglect compensatory mechanisms. Our results support the ongoing development of high-throughput analyses to study the overall impact of genetic deletions and treatments and to identify novel targets for the treatment of inflammatory and infectious diseases.

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6 APPENDIX

Profound and Extended Alterations of Innate and Adaptive Immunity Highlighted by High-dimensional Mass Cytometry in Patients Infected with HCV

Jacobus Herderschee[*], Tytti Heinonen[*], Craig Fenwick[†], Khalid Ohmiti[†], Giuseppe Pantaleo[†,‡], Darius Moradpour[+], Matthias Cavassini[*], Thierry Roger[*$], Thierry Calandra[*$], and the Swiss HIV Cohort Study

[*]Infectious Diseases Service, [†]Division of Immunology and Allergy, and [+]Division of Gastroenterology and Hepatology, Department of Medicine, Lausanne University Hospital, Lausanne, Switzerland; [‡]Swiss Vaccine Research Institute, Lausanne, Switzerland.

[$]These authors contributed equally to this work.

Short title: CyTOF reveals prolonged immune dysfunction before and after HCV clearance

Corresponding author: Thierry Calandra

Tel. +41-21-314-1037; E-mail: Thierry.Calandra@chuv.ch

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Abstract

Background & Aims: HCV infections are associated with substantial morbidity and mortality worldwide. HCV chronicity is due in part to the subversive effects of HCV on the immune system. While IFN-based therapy hindered initial studies on the bona fide immune modulating properties of HCV, we took advantage of direct-acting antiviral therapies and single cell mass cytometry to explore the full spectrum of HCV-mediated effects on the immune system.

Methods: Blood was collected from 10 healthy controls and from 30 patients chronically infected with HCV with or without HIV infection before, during and 12 weeks after the end of anti-HCV treatment (SVR12). The frequency of 22 blood cell populations and the expressions of co-stimulatory and checkpoint molecules and cytokines were analysed by single cell mass cytometry.

Results: Infection with HCV or HCV/HIV had a major impact on innate and adaptive immunity with striking quantitative changes observed in 14 (64%) blood cell populations. Before therapy, the frequencies of intermediate and non-classical monocytes, type 2 conventional DCs (cDC2) and $CD56^{dim}$ NK cells were markedly reduced (35% to 50%, $P<0.05$), while those of activated double negative, CD4 and CD8 T cells were prominently increased (2 to 3-fold, $P<0.05$). Upon stimulation with microbial products *ex vivo*, innate immune cells of patients produced higher levels of cytokines than those of controls. Nearly all (13 of 14, 92%) of the detected changes among immune cells and most of cytokine rises persisted up to SVR12. HCV and HCV/HIV did not influence the expression of co-stimulatory or checkpoint molecules.

Conclusion: High-dimensional analytic technology revealed that patients with HCV or HCV/HIV infections exhibit profound and persistent alterations of innate and adaptive immune cells.

My contribution to this work: I performed blood stimulation and storage.