Mechanisms of epigenetic regulation relevant to innate responses against pathogens

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MECHANISMS OF EPIGENETIC REGULATION RELEVANT TO INNATE RESPONSES AGAINST PATHOGENS

Tesis Doctoral

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"*Para mí no hay emoción comparable a la que produce la actividad creadora, tanto en ciencia como en arte, literatura u otras ocupaciones del intelecto humano. Mi mensaje, dirigido sobre todo a la juventud, es que, si sienten inclinación por la ciencia, la sigan, pues no dejará de proporcionarles satisfacciones inigualables. Cierto es que abundan los momentos de desaliento y frustración, pero estos se olvidan pronto, mientras que las satisfacciones no se olvidan jamás*".

Severo Ochoa

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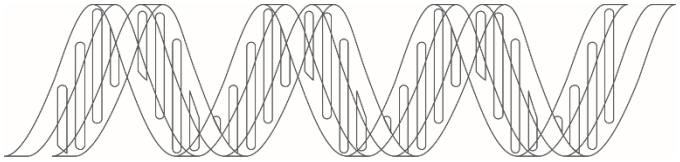
ABBREVIATIONS

5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5hmU	5-hydroxymethyluracil
5mC	5-methylcytosine
AID	Activation-induced cytidine deaminase
AIM2	Absent in melanoma 2
ALRs	AIM2-like receptors
AP-1	Activator protein-1
ASC	Apoptosis-associated speck-like protein
BCG	Bacillus Calmette–Guérin
BER	Base excision repair
CAPS	Cryopyrin-associated syndromes
CBA	Cytometric bead array
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CDP	Common dendritic cell progenitor
CLP	Common lymphoid progenitor
CLRs	C-type lectin receptors
CMP	Common myeloid progenitor
cMoP	Common monocyte progenitor
CREB	cAMP response element binding
CpG	CpG site
DAMPs	Danger-associated molecular patterns
DNMT	DNA methyltransferase
DVP	Differentially variable CpG position
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal regulated kinase
FCAS	Familial cold autoinflammatory syndrome
FDR	False discovery rate
FMF	Familial Mediterranean fever

GO	Gene ontology
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HLA	Human leukocyte antigen
HMGB-1	High-mobility group protein B1
HMT	Histone methyltransferase
HSC	Haematopoietic stem cell
IFN	Interferon
IKK	IκB kinases
IL	Interleukin
ILC	Innate lymphoid cell
IRAK	IL-1R-associated kinases
IRF	Interferon regulatory factors
JAK	Janus kinase
JmjC	Jumonji domain-containing proteins
JNK	Jun N-terminal kinase
lncRNA	Long non-coding RNA
LRR	Leucine-rich repeat
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDP	Monocyte-macrophage DC progenitor
miRNA	Micro-RNA
MHC	Major histocompatibility complex
MPP	Multipotent progenitor
MyD88	Myeloid differentiation primary-response protein 88
MWS	Muckle-Wells syndrome
NF-kB	Nuclear factor-kappa B
NK	Natural killer
NLR	Nucleotide-binding oligomerization (NOD)-like receptor
NOMID	Neonatal-onset multisystem inflammatory disease
P3C	Pam3CSK4
PAMPs	Pathogen-associated molecular patterns

PBMC	Peripheral blood mononuclear cell
PCA	Principal component analysis
PCDH	Protocadherin
PIAS	Protein inhibitors of activated STATs
poly-HEMA	Poly-2-hydroxyethyl methacrylate
PRR	Pattern recognition receptor
PTP	Protein tyrosine phosphatase
RLR	RIG (retinoic-acid-inducible gene 1)-I-like receptor
ROS	Reactive oxygen species
SAR	Systemic acquired resistance
SIGGIR	Single immunoglobulin interleukin-1-related receptor
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
TDG	Thymine DNA glycosylase
TET	Ten-eleven translocation
TF	Transcription factor
TGF	Transforming growth factor
TIRAP	TIR-domain-containing adaptor protein
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	Tumour necrosis factor receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing IFNβ
TSS	Transcription start site
TYK2	Tyrosine kinase 2



INTRODUCTION

1. INTRODUCTION

1.1 The immune system

1.1.1 Innate and adaptive immunity

The immune system comprises the host's defence mechanisms against bacterial, fungal, viral or parasite infections. It includes a complex network of many biological molecules, cell types and processes that neutralize the invading pathogens and protect the body. Classically, the immune system has been classified in relation to the two different types of responses as innate and adaptive (Figure 1) [1].

On one hand, innate immunity has generally been considered a rapid and non-specific response against pathogens. The components of innate immunity include physical and chemical barriers (skin and mucosal barriers), innate immune cells, such as macrophages, neutrophils and dendritic cells, and blood proteins, like complement components, leukotrienes, prostaglandins and other cytokines and chemokines [2]. Monocytes and macrophages are potent phagocytic cells whereas dendritic cells are mainly responsible for antigen presentation; crucial processes involved in microorganism clearance. Furthermore, this innate response does not develop immunological memory. However, during the last twenty years, this dogma has dramatically changed. The discovery of the pattern recognition receptors has introduced the concept of semi-specific recognition in these innate cells. In addition, in plants and invertebrates, which lack the adaptive system, memory processes have been observed during second infections. This emergent concept, defined as innate immune memory, has also been identified in vertebrates, in which epigenetic modifications have been determined as key mechanisms involved in the acquisition of this memory [3].

On the other hand, adaptive immunity arises later in time, both during development and in response to insults, and can build up immunological memory through different lymphocyte populations. This memory is referred as the ability to respond faster and more efficiently after repeated exposures. Subsets of B and T lymphocytes mediate this adaptive response; through gene rearrangement and clonal expansion, in order to protect against secondary infections. Somatic recombination, also known as V(D)J recombination, generates a high repertoire of B and T clones given high diversity and variability to respond to insults [1,2].

Innate system	Adaptive system
Immediate response	Slow response
Wide specificity	High specific
Non-memory	Immunological memory

Figure 1. Scheme of the main differences between innate and adaptive immunity.

1.1.2 Immune cells and their functions

1.1.2.1 Haematopoiesis

Haematopoiesis is the generation of all blood cellular components, including white and red blood cells and platelets. It is a highly dynamic process, with respect to time and space, during vertebrate development. Haematopoietic stem cells (HSCs) are common precursors for the different blood cell types and have two main characteristics, self-renewal and pluripotency [4]. HSCs are in specialized niches in the bone marrow that maintain and regulate stem cells in a proper microenvironment [5,6]. During haematopoiesis, HSCs are differentiated into multipotent progenitors (MPPs), that lose self-renewal capacity. Subsequently, MPPs give rise to the two divergent differentiation branches: the lymphoid and myeloid lineages, with progressively decreasing differentiation potential. B and T lymphocytes, innate lymphoid cells and natural killer cells come from a common lymphoid progenitor (CLP) whereas myeloid cells, such as monocytes, macrophages, dendritic cells and granulocytes, arise from a common myeloid progenitor (CMP) [7–10].

1.1.2.2 Myeloid and lymphoid cells

Sometimes physical barriers of defence (epithelial and mucosal layers) are not enough to contain the infection or damage and the immune system makes use of immune cells that derive from HSCs.

The myeloid lineage is the main source of innate immune cells which includes macrophages, dendritic cells, granulocytes and mast cells, among others. The main functions of these innate cells are phagocytosis, antigen presentation and production of cytokines and inflammatory mediators. Macrophages and neutrophils are phagocytic cells involved in pathogen elimination and are potent secretors of cytokines that regulate the immune response. Together with dendritic cells, macrophages are also antigen presentation cells, initiating T cell-mediated response. Mast cells, along with basophils, increase vascular permeability and induce inflammatory response; and eosinophils protect from parasite infections [11–13].

Recently, innate lymphoid cells (ILCs) have been characterized as a group of lymphoid cells with crucial roles in innate immunity, inflammation and tissue repair. Natural Killer (NK) cells, which belong to ILCs, release IFN-gamma and secrete perforins, being crucial for cytotoxic activity [14,15].

T and B lymphocytes are the main drivers of the adaptive response. B cells secrete antibodies, which bind antigens with high affinity and specificity to eliminate the pathogen. Just as B cells are responsible for humoral immunity, T cells control cell-mediated immunity. T lymphocytes activate macrophages to kill microbes and help B lymphocytes to eliminate infected cells [12,16]. As mentioned above, T and B cells present immunological memory which confers long-term protection against re-infection due to the production of memory cells (Figure 2).

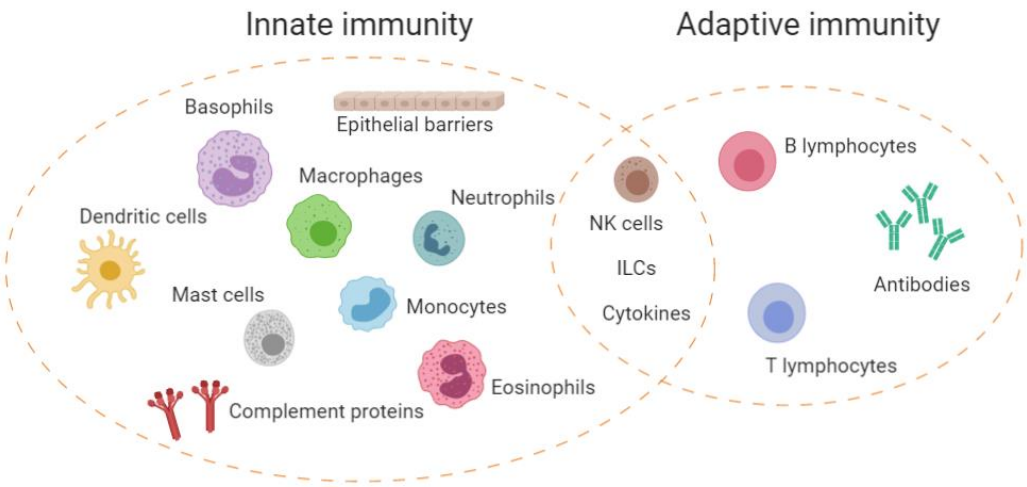


Figure 2. Innate and adaptive immune components. Innate response is the first barrier against infectious pathogens. Innate effector cells phagocytose, release cytokines and chemokines and activate the adaptive response. T and B lymphocytes are responsible for adaptive immunity and kill infected or damaged cells.

1.1.2.3 Monocytes

Monocytes, macrophages and dendritic cells are members of the mononuclear phagocytic system, which is fundamental for immune responses. Phagocytic cells are very dynamic and plastic, and they act as a first defence line against pathogens as well as activating the adaptive response. Following stimulation or damage, monocytes, which represent the primary type of mononuclear phagocytes, rapidly migrate to tissues and there, they mature to macrophages or dendritic cells [17]. Monocytes derive from common precursors located in bone marrow and spleen. CMPs differentiate to monocyte-macrophage DC progenitor (MDP) and finally differentiate into common monocyte progenitor (cMoP) to develop phagocytic cells. Another common dendritic cell progenitor

(CDP) has been characterized to develop into classical dendritic cells and plasmacytoid dendritic cells but not monocytes and macrophages [11,18,19].

Circulating monocytes, that constitute approximately 10% of human leukocytes, are heterogeneous and are classified in three different subtypes: classical, intermediate and non-classical. Classical monocytes ($CD14^{++}$, $CD16^{-}$), express high levels of CCR2, are highly phagocytic, and migrate to sites of inflammation to act as precursors of mononuclear phagocytes. Intermediate monocytes ($CD14^{++}$, $CD16^{+}$) also display high CD14 expression but express low levels of CD16 and release pro-inflammatory cytokines. Finally, non-classical ($CD14^{low}$, $CD16^{++}$) monocytes, that present high expression of CX3CR1, are involved in patrolling vessels, surveillance and homeostasis [20–22]. Monocyte heterogeneity can contribute to disease development. For instance, expansion of the intermediate monocyte subpopulation has been observed in atherosclerosis, rheumatoid arthritis and sepsis [23–26].

Monocytes differentiate into macrophages in peripheral tissues during inflammation and steady-state conditions. Macrophages also represent a heterogeneous population that can phagocytose pathogens to induce and resolve inflammation. Despite the diversity of macrophages in tissues (peritoneal, intestinal, lung, spleen, microglia or Kupffer cells), most tissue-resident macrophages have embryonic progenitors in adult steady-state, with only one exception, yolk-sac-derived tissue macrophages [19,21,27,28]. Macrophage polarization depends on multiple signals from their microenvironment. *In vitro*, LPS (or bacteria) and IFN-γ polarize macrophages to the M1 classical phenotype, the pro-inflammatory type. These macrophages are characterized by releasing high levels of IL-12 and IL-23, and reactive oxygen and nitrogen intermediaries, and are responsible for killing intracellular pathogens. M1 macrophages signal through the TLR4 and IFNR pathways to activate transcription factors (TF), including activator protein-1 (AP-1), nuclear factor-kappa B (NF-kB), interferon regulatory factor 3 (IRF3) and signal transducer and activator of transcription (STAT)1, which lead to pro-inflammatory gene expression. On the other hand, the M2 phenotype is developed *in vitro* as a response to IL-4 or IL-13. M2 macrophages have more phagocytic capacity and high expression of IL-10 cytokine, and respond to helminths, allergic reactions and tissue repair. M2 macrophages activate the TF STAT6 and regulate expression of anti-inflammatory or immunosuppressive genes, such as *SOCS3, IL-10, PGE2* or *A20* [29–32].

1.1.3 Innate immune response

1.1.3.1 Inflammation

Inflammation is a biological response of the immune system that results from the exposure to a wide range of factors, including pathogens, damaged cells and toxic compounds. The classical manifestations of inflammation comprise redness, heat, pain, swelling and loss of function [33,34]. Generally, inflammatory responses are beneficial and provide host defence against pathogens, tissue-repair response and homeostatic restoration. However, inflammation can sometimes be dysregulated and it becomes detrimental, which occurs in many diseases including sepsis, atherosclerosis, type II diabetes, autoinflammatory diseases, neurodegenerative disorders or cancer [35,36].

The typical inflammatory response includes inflammatory inducers, sensors that detect them, mediators of inflammation and effector molecules [35]. Pathogens and damaged signals are detected by immune receptors to initiate inflammatory signalling. These signals induce mediators of the acute inflammatory response that include pro-inflammatory cytokines, such as IL-6, IL-1 and IL-12, and chemokines, complement system, eicosanoids (prostaglandins, leukotrienes), oxygen-derived free radicals or vasoactive amines, among others. These mediators act on target tissues to induce inflammation.

Following inflammation, it is important for the body to return to homeostasis. Several mechanisms are involved in this resolution [37]. The anti-inflammatory cytokine IL-10, which activates STAT3, suppresses inflammatory gene expression. TNF receptor (TNFR) and IL-1 receptor (IL-1R) act as a decoy receptor, limiting the effects of inflammation, due to their inability to signal. Receptors antagonist also inhibit this response. Prostaglandins like PGE2, lipid mediators such as resolvins and protectins and acute-phase proteins can also exert anti-inflammatory properties. Finally, negative regulators of toll-like receptor signalling, as IRAK-M and A20, also neutralize inflammation [33,38,39].

Immune cells cooperate, by interacting cellular receptors and external signals such as cytokines, to appropriately be recruited to the site of inflammation. Adhesion molecules, which include integrins, selectins and cadherins, play a crucial role in this cellular communication. They are expressed constitutively, or are up-regulated under certain signals, to direct cell migration [40]. In addition, chemokines are also important in controlling leukocyte migration. Some chemokines are constitutively presented to recruit macrophages and dendritic cells to peripheral tissues and others are expressed to

manage the recruitment to inflamed tissues. Monocytes have adhesion and chemokine receptors that mediate migration to sites of tissue damage and infection, for example, CCR2 is necessary for a proper immune response after inflammation [41]. Once monocytes arrive at inflammatory sites, they differentiate to macrophages or dendritic cells. In general, macrophages display phagocytic activity to eliminate pathogens whereas dendritic cells migrate to lymph nodes and act as antigen-presenting cells.

Furthermore, it is important to highlight that during inflammation and infection, hundreds of myeloid cells are generated via myelopoiesis as part of the immune defence. HSCs express several receptors which are responsible to recognize molecules and patterns associated with pathogens that induce their differentiation into myeloid lineage cells to initiate the immune response [42].

1.1.3.2 Patterns and receptors in innate immunity

Innate immune cells are activated following the recognition of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) via the pattern recognition receptors (PRRs), as part of the host defence. This microbial recognition is key for the initiation of innate immune response that is necessary for killing invaded pathogens. In addition, PRR signalling also activates dendritic cell maturation. Dendritic cells participate in antigen-presentation to induce adaptive response, the second line of defence, in which they phagocytose pathogens and process them into antigen peptides to be presented by major histocompatibility complex (MHC) class I or II molecules. In the lymph nodes, dendritic cells present pathogen-derived antigens to T cells, resulting in their activation.

PAMPs are conserved molecular patterns that are shared by most microorganisms. PAMPs can activate different functions: endocytosis and antigen presentation, activation of pro-inflammatory cascades as NF-kB signalling or opsonization. NF-kB is crucial in the inflammatory response to regulate the expression of pro-inflammatory cytokines and chemokines and anti-apoptotic genes that may protect from cell death [43]. DAMPs are molecules that are released by dying cells after stress or damage to enhance inflammatory response. Hyaluronan fragments, S100 proteins, high-mobility group protein B1 (HMGB-1) or heat-shock proteins are all endogenous signals triggering inflammation as well as repair responses.

PAMPs and DAMPs act through PRRs. PRRs are germline-encoded immune receptors that are mainly expressed by myeloid cells, but they are also expressed in various non-immune cells. Engagement of PRRs triggers inflammatory signalling

pathways that are involved in cytokine and chemokine release and lymphocytes recruitment and activation, which are all essential processes in immunity. Several types of PRRs have been characterized which are mainly classified as follows [44]:

- Toll-like receptors (TLRs), the most well-characterized receptors, recognize specific microbial-derived components after invasion. These receptors have an extracellular domain that contains leucine-rich repeat (LRR) motifs and a cytoplasmic region composed by Toll/IL-1 receptor (TIR) domain. There are 13 members in mammals, each of them recognizes distinct microbial and host-derived molecules to activate TLR pathways. For instance, TLR4 recognizes LPS on the surface of gram-negative bacteria, TLR2 binds lipoteichoic acid and peptidoglycan from gram-positive bacteria, TLR3 interacts with dsRNA from virus, TLR5 recognizes flagellin, and TLR9 binds CpG-containing DNA from bacteria. TLRs can be localized on the cell surface as TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 or can be expressed on intracellular vesicles (TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, TLR13). All receptors elicit an inflammatory signalling program leading to NF-kB and AP-1 activation [45–48].
- Nucleotide-binding oligomerization (NOD)-like receptors (NLRs) are cytosolic proteins that respond to various PAMPs and DAMPs to trigger the inflammatory response. NLRs detect microbial infections and form the core of inflammasomes. Inflammasomes are key components in the innate immune pathway. Once inflammasomes are assembled, caspase-1 is activated to process pro-IL-1β and pro-IL-18 into their mature forms and induce pyroptosis, a form of cell death, protecting against bacterial infections.
- C-type lectin receptors (CLRs) are membrane proteins which contain one or more C-type lectin-like domains and activate inflammatory response via ITAM domains. SYK tyrosine kinase is activated inducing nuclear factor of activated T cells (NFAT) and NF-kB activation through CARD9. The main receptors are dectin-1 and dectin-2 that recognize β-glucan from fungi.
- RIG (retinoic-acid-inducible gene 1)-I-like receptors (RLRs) recognize RNA molecules delivered after infections and regulate NF-kB, IRF3 and IRF7 TFs culminating in pro-inflammatory cytokine expression and interferon (IFN)-related genes.
- AIM2-like receptors (ALRs) bind cytosolic dsDNA during infection and activate the formation of absent in melanoma 2 (AIM2) inflammasome which induces IL-1β maturation and cell death [44,49,50].

1.1.3.3 TLR signalling

The most important activated signal routes through TLRs are mitogen-activated protein kinases (MAPKs), IκB kinases (IKK complex) and interferon regulatory factors (IRFs) [51,52]. The engagement of TLRs triggers cell signalling pathways that lead to the recruitment of adaptor molecules, including myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor protein inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM), protein kinase (IL-1R-associated kinases, IRAKs) and members of the tumour necrosis factor receptor-associated factor (TRAF) family [51]. Upon stimulation, IRAKs are recruited through interaction with adaptor molecules. Subsequently, IRAK complex is activated and readily associates with TRAF6. Activated TRAF6 forms a complex with TAK1, TAB1, TAB2 and TAB3 to activate both MAPK and NF-kB signalling pathways.

MAPKs and IKKs are activated via MyD88-dependent mechanisms. Firstly, MAPKs, which include among others Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 [53], phosphorylate and activate several TFs such as AP-1 and cAMP response element-binding (CREB). Secondly, IKKs participate in the activation of the TF NF-kB and subsequently all their gene targets. IKK complex phosphorylates IκBα inhibitor of NF-kB, leading to their ubiquitylation and subsequent proteasome-degradation with the concomitant release and translocation to the nucleus of NF-kB. Finally, several TLRs, such as TLR4, also activate IRF signalling, a MyD88-independent pathway, leading to the expression of IFN-inducible genes, and mediate late-phase activation of NF-kB and MAPK signalling (Figure 3) [54–58].

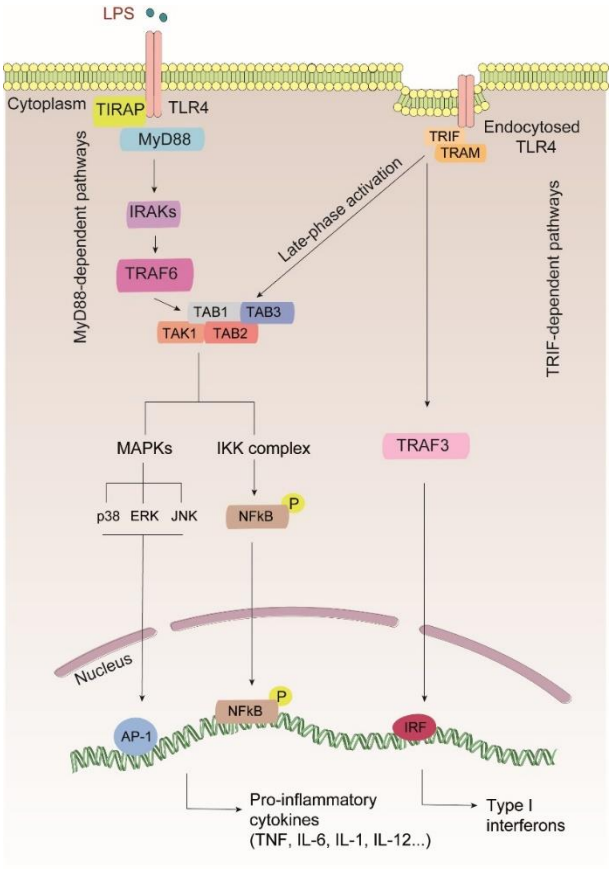


Figure 3. Scheme depicting TLR4 signalling pathway. LPS binds to TLR4 receptor, which activates MAPK and IKK signalling routes. MAPKs, which include p38, ERK or JNK among others, activate several transcription factors. IKK complex allows NF-kB translocation to the nucleus, activating their gene targets. Through a TRIF-dependent pathway, IRF is activated and induces IFN-related genes expression. Furthermore, a late-phase of NF-kB is triggered by this pathway.

Molecules that negatively regulate TLR responses are also necessary for immune homeostasis and many of them are produced after TLR stimulation. First, MyD88s, an alternative spliced variant, is not able to bind IRAK-4 and therefore inhibits NF-kB signalling. Second, the suppressor of cytokine signalling 1, SOCS1, inhibits IFNR pathway. Third, IRAK-M lacks kinase activity and reduces the production of pro-inflammatory cytokines and fourth, Toll interacting-protein (Tollip) reduces IRAK-1 phosphorylation and activation, preventing NF-kB activation. Additionally, A20, also known as TNFα-induced protein 3 (TNFAIP3), deubiquitinates and deactivates TRAF6, thereby inhibiting downstream signalling. Finally, SIGGIR (single immunoglobulin interleukin-1-related receptor) is a membrane-bound protein that interacts with IRAK-1 and TRAF6 to negatively modulate TLR signalling [47,51,59].

Although TLR signalling is crucial for host defence, it is important to highlight that aberrant activation of these pathways or mutations in TLR signalling molecules have

been related to TLR-mediated inflammatory diseases including sepsis, atherosclerosis, asthma, diabetes and autoimmune diseases [60–62].

1.1.3.4 Inflammasomes

Inflammasomes mediate crucial signalling for innate immunity during microbial infection and cellular damage. Inflammasomes are cytosolic multimeric complexes that consist of a sensor molecule (including members of the NLR or AIM2), an adaptor molecule (apoptosis-associated speck-like protein (ASC)) and the effector molecule pro-caspase-1. Through microbial pathogens and multiple stimuli recognition (potassium efflux, reactive oxygen species, monosodium urate crystals, ATP, silica, asbestos, etc), inflammasomes are assembled as a key signalling pathway to control innate immune response [63,64].

The most widely studied is the NLRP3 inflammasome. Inflammasome formation is required for caspase-1 activation. Once caspase-1 is activated, it cleaves pro-IL-1β and pro-IL-18 into their mature forms. These cytokines stimulate inflammatory pathways by binding the IL-1 and IL-18 receptors that contain TIR domains and trigger signalling via MyD88 similarly to TLRs. In addition, inflammasome assembly leads to an inflammatory cell death called pyroptosis. For inflammasome activation, two signals are required: a priming step that involves NF-kB through TLR signalling and induces transcription of NLRP3 and pro-cytokines; and a second activation step that is a result of diverse mechanisms including ROS release, mitochondrial DNA, lysosomal damage or potassium efflux through ion channels [63,65–67] (Figure 4). However, in human monocytes, an alternative inflammasome activation has been described. In these cells, caspase-1 activation, and consequent IL-1β release, only requires LPS stimulation and is independent of K+ efflux or pyroptosis [68–70]. Finally, a non-canonical activation pathway by intracellular LPS has been described in mice to involve caspase-11 (or caspase-4 and caspase-5 in humans) [71].

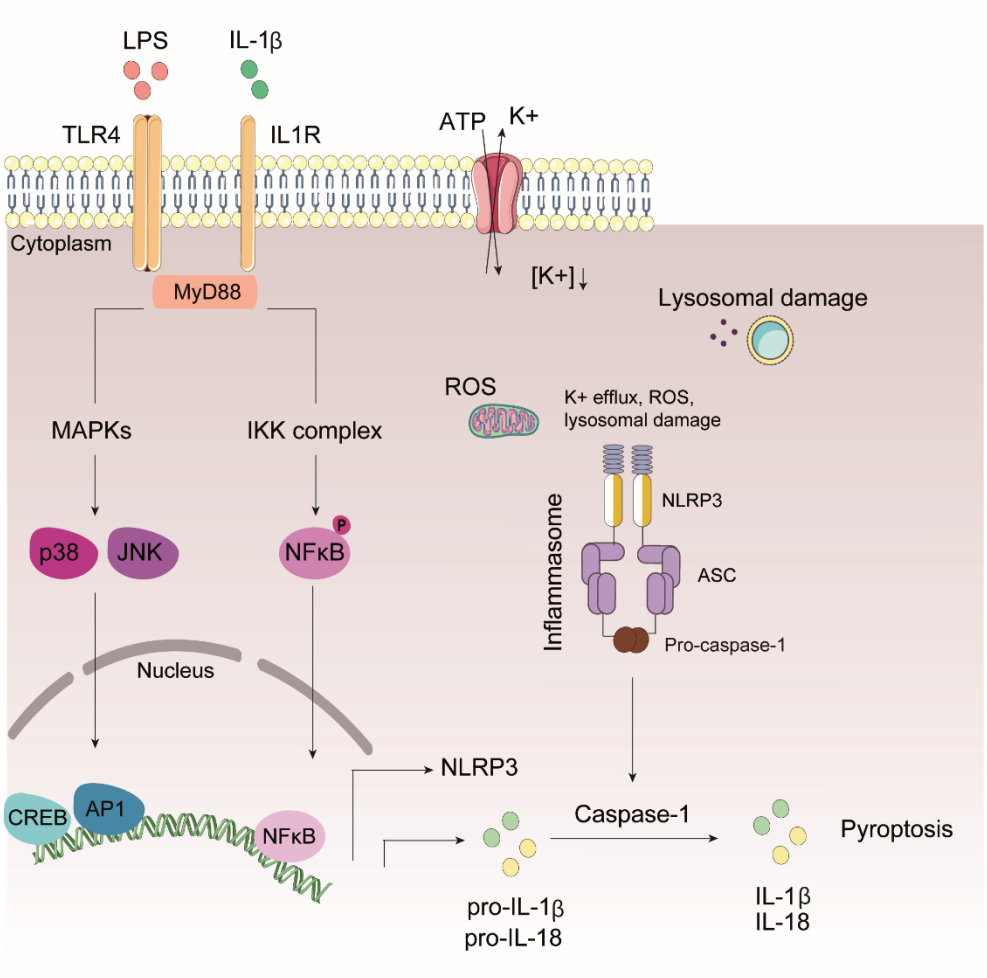


Figure 4. Scheme representing key signalling during inflammasome activation. Firstly, NLRP3 and pro-cytokines are produced through NF-kB pathway. Then, caspase-1, which is activated after inflammasome assembly, mediates the cleavage of IL-1β and IL-18 into their bioactive forms and these cytokines initiate inflammation. Release of ROS, K+ efflux or lysosomal rupture are mechanisms involved in inflammasome activation step.

An exacerbated NLRP3 activation leads to the development of inflammatory diseases (autoinflammatory pathologies, gout, atherosclerosis, type II diabetes, neurodegenerative disorders…). For this reason, there are mechanisms implicated in the negative regulation of this response. CD4+ effector lymphocytes inhibit inflammasome activation and type I IFNs can suppress IL-1β and IL-18 production. Autophagy also regulates mitochondrial damage and ROS release and, in this way, prevents excessive inflammasome activation [72,73]. In addition, NF-kB inhibitors also repress inflammasome signalling [74].

Autoinflammatory disorders are a group of innate immune pathologies characterised by systemic or organ-specific inflammation and recurrent fevers without autoreactive T cells, high autoantibody titers or any detectable pathogens. Familial Mediterranean Fever (FMF) and cryopyrin-associated syndromes (CAPS) are two

monogenic autoinflammatory diseases, resulting from mutations in inflammasome components. The impact of genetic mutations of the inflammasome highlights the importance of the tight regulation of its components on innate responses. FMF is an autosomal recessive disorder characterized by recurrent fever attacks and it is the most prevalent autoinflammatory disease. FMF patients present mutations in the gene encoding pyrin (*MEFV*), which regulates caspase-1 activation. On the other hand, CAPS are monogenic autoinflammatory diseases which comprise a spectrum of heterogeneous phenotypes that include, in increasing order of severity, familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and neonatal-onset multisystem inflammatory disease (NOMID). CAPS patients present *NLPR3* gene mutations with exaggerated IL-1β release. Due to this gain of function, patients have episodic fevers with systemic inflammation as well as swelling of joints, hearing loss and neurological complications in the most serious cases [75–78]. Fortunately, targeted IL-1 inhibition is a good therapeutic approach, in which the recombinant IL-1 receptor antagonist (Anakinra), the fusion protein of humanized IL-1 receptor (Rilonacept), and the anti-IL-1β human monoclonal antibody (Canakinumab) are all effective drugs for treating CAPS patients [79].

1.1.3.5 JAK/STAT signalling

Another key signalling pathway in innate immune response is the Janus kinase and signal transducer and activator of transcription (JAK/STAT) route that controls and regulates cytokine gene expression and thus inflammatory response. Furthermore, this pathway is crucial for host defence, activating antigen presentation and T and B cells.

In mammals, there are four members of the JAK family: JAK1, JAK2, JAK3 and TYK2. Following the binding of ligand (comprised of cytokines and growth factors) to their corresponding receptors, JAKs are activated, and this leads to the recruitment, via SH2- interactions, and phosphorylation of STAT proteins. The phosphorylated STAT dimers are then translocated to the nucleus where they bind to specific DNA sequences and activate gene transcription (Figure 5). The STAT TF family include seven members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. STATs can form homo- and hetero-dimers among them depending on diverse cytokine activation [80–82].

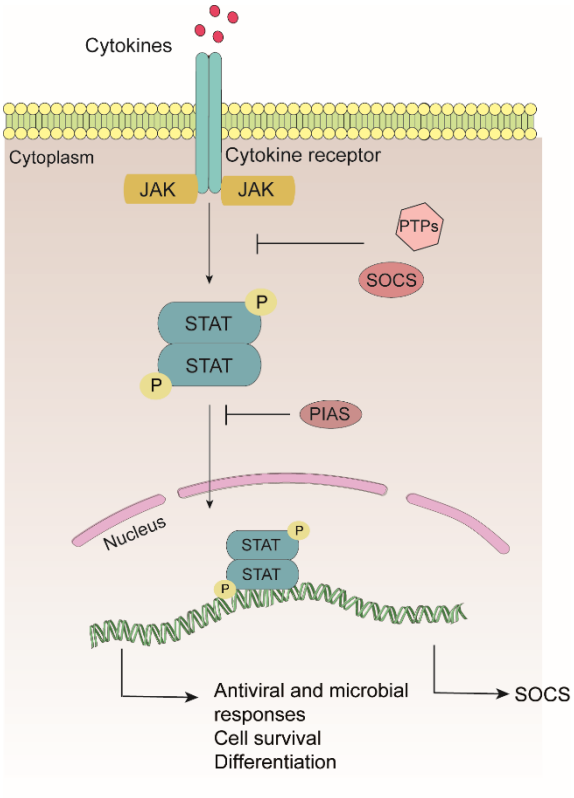


Figure 5. JAK/STAT signalling scheme. Cytokine binding to receptors triggers JAK activation, followed by the recruitment and phosphorylation of STAT proteins. Activated STAT dimers migrate to the nucleus and activate or repress gene transcription. Several molecules (SOCS, PIAS and PTPs) regulate negatively the pathway.

A crosstalk between TLR and JAK/STAT signalling has been described during the immune response. For instance, IFNα/β are produced via TLR4 signalling, which subsequently activate the JAK/STAT pathway via their receptor. IL-6, which is expressed after TLR stimulation, also activate STAT1 and STAT3. In addition, IL-10 receptor signals through STAT3 and is deemed crucial for limiting inflammation [83,84]. STATs can also drive epigenetic mechanisms in immune cells by physically interacting with p300 and EZH2, as well as controlling the expression of genes encoding chromatin remodelling enzymes. For instance, in primed macrophages, STAT1 enhances histone acetylation which increases the recruitment of TLR-induced TFs and cytokine gene transcription [85].

Like all inflammatory signalling, JAK/STAT activation is tightly regulated. Members of the suppressors of cytokine signalling (SOCS) family, which are expressed after LPS stimulation, are involved in their inhibition by different mechanisms (JAK inhibition, ubiquitin-proteasome degradation, etc). For instance, SOCS1 negatively regulates JAK/STAT signalling and TLR cascade, and, similarly, SOCS3 inhibits NF-kB signalling and suppresses IL-6-STAT activation [86]. In addition, post-translational modifications as phosphorylation, acetylation or sumoylation of STAT proteins also

regulate their activation. Protein inhibitors of activated STATs (PIAS) bind to STAT dimers and suppress DNA binding activity. Finally, protein tyrosine phosphatases (PTPs) inactivate JAK and STATs by tyrosine dephosphorylation [81,87].

A dysregulation of this signalling is linked with many immune diseases including immunodeficiencies, asthma, psoriasis and rheumatoid arthritis, and thus, rigorous research into JAK inhibition as therapeutic approach are currently carried out [88,89].

1.2 Gene regulation of immune system

1.2.1 Epigenetic regulation of gene expression

Epigenetics is defined as the set of mechanisms that register, signal or perpetuate altered activity states, without changing the DNA sequence [90]. In general, epigenetics mainly involves post-translational modification of amino acid residues in the histone N-terminal ends and methylation of cytosines (and other nucleosides) in the DNA. Non-coding RNAs-mediated mechanisms are commonly considered a type of epigenetic regulation. Epigenetic modifications have an essential role in the regulation of gene expression. The set of epigenetic modifications, the epigenome, is specific of a given cell type, like the transcriptome, allowing genetically identical cells to have different identities and functions. DNA methylation, histone modifications and non-coding RNAs therefore determine cell fate and on immune cellular response by shaping gene expression programs.

1.2.1.1 DNA methylation

In mammals, DNA methylation generally refers to the covalent addition of a methyl group to a cytosine (5mC) in its 5' position and it mainly takes place in CpG dinucleotide sequences. DNA methyltransferases (DNMTs) are the family of enzymes that catalyse this incorporation of a methyl group to a cytosine base. During replication, DNMT1 is responsible for the maintenance of methylation in the CpGs of the new DNA strands. DNMT3A and DNMT3B are involved in the establishment of the *de novo* methylation (Figure 6A) [91]. The reversion or loss of methyl groups is known as DNA demethylation. DNA demethylation can be passive or active. Passive demethylation is the result of the inefficient maintenance of methylation during DNA replication. In contrast, active demethylation depends on specific enzymatic activities and occurs in a multi-step manner. In the first step, Ten-eleven translocation (TET) enzymes catalyse the subsequent oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). 5fC and 5caC can then be excised by thymine DNA glycosylase (TDG) leading to an abasic site. Finally, the activity of the

DNA repair machinery restores the incorporation of an unmethylated cytosine to the abasic site. In parallel, other group of studies have shown that activation-induced deaminase (AID)/APOBEC could mediate the deamination of 5-hmC to 5-hmU to also render unmethylated cytosines (Figure 6B) [92–95]. However, the potential role of AID in active demethylation remains controversial [96].

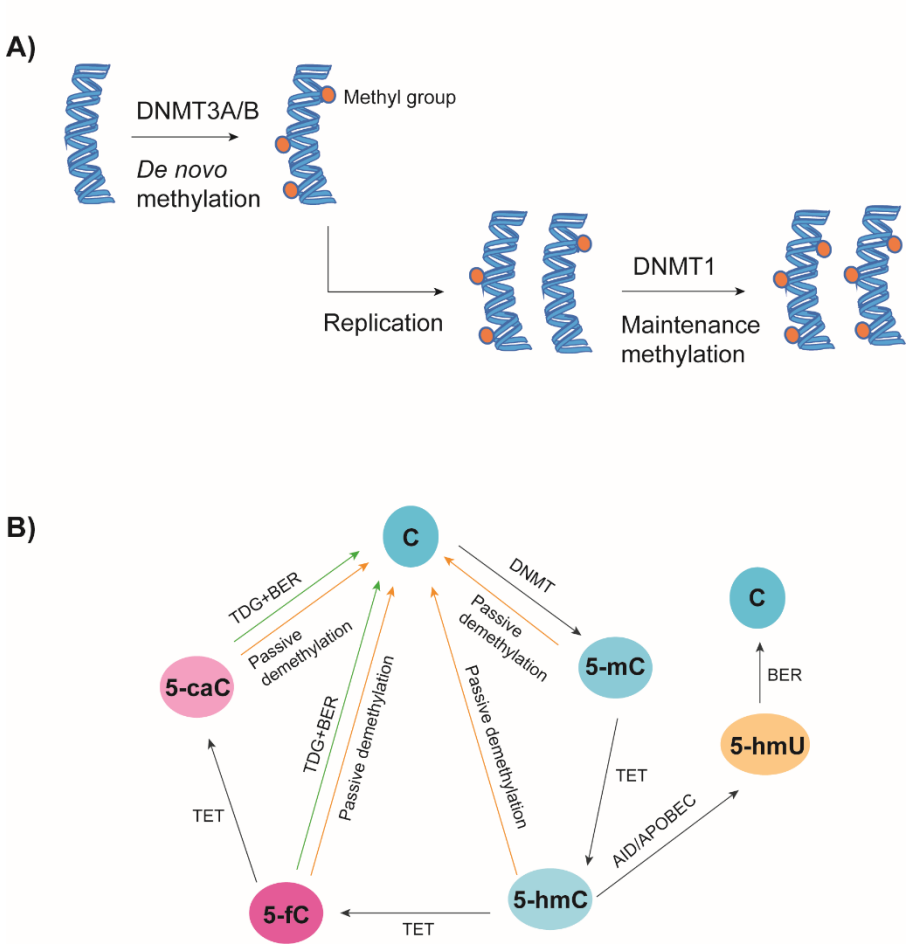


Figure 6. DNA methylation mechanisms. (A) DNA methylation is the addition of methyl groups to cytosines catalysed by DNA methyltransferases (DNMTs). DNMT3A and DNMT3B are involved in *de novo* DNA methylation. During replication, DNMT1 is recruited and is implicated in maintenance DNA methylation. (B) The inefficient or impaired maintenance of DNA methylation across replication cycles results in passive DNA demethylation. TET enzymes are responsible to oxidize 5-mC to 5-hmC, 5-fC and 5-caC. Active demethylation is achieved through TDG-mediated removal of oxidized form of cytosines followed by base excision repair (BER). It has also been proposed that AID/APOBEC could mediate the deamination of 5-hmC to 5-hmU, which would render unmethylated cytosines also through BER.

DNA methylation is one of the mechanisms that regulate gene expression and it is also important in the genomic imprinting or the inactivation of X chromosome [97]. 5mC can directly influence gene expression through direct mechanisms by inhibiting/interfering with TF binding. It can also mediate the recruitment of methyl-CpG binding domain (MBD) proteins which form part of nuclear complexes with histone

modification enzymes and/or chromatin remodelling enzymes. These complexes alter the histone modification status and/or chromatin accessibility and therefore regulate gene transcription [98]. In gene promoters, DNA methylation is generally associated with a transcriptionally repressed chromatin state. However, DNA methylation effects can be diverse depending on the genomic location [99,100].

Mammalian DNA methylation is a dynamic process necessary for proper development and defects are critical in the context of diseases [101]. For instance, during embryonic development, DNA methylation increases rapidly in the blastocyst stage to mark a differential methylation pattern between cells to the inner cell mass and those of the trophectoderm [102]. In disease, genome-wide methylation changes have been known for decades in the context of cancer and imprinting disorders [103,104]. *TET2* mutations occur in about 15% of patients with myeloid malignancies [105]. *DNMT3A* mutations are also frequent in myeloid leukaemia patients [106]. There are intensive studies to describe their implications in neurological, metabolic, cardiovascular and immune-related disorders.

During haematopoiesis, chromatin state is dynamic [107]. DNA methylation has an important role in HSC differentiation and lineage-specific progenitors through modulating the activity of enhancers and promoters [92,108]. For instance, global DNA methylation analyses have shown distinct trends between the myeloid and lymphoid lineage commitment, with more prominent losses of DNA methylation during myeloid cell differentiation [109]. Different leukocytes subsets have distinct DNA methylomes [110]. In addition, loss of DNMT3A and DNMT3B results in an increase of HSC self-renewal and impairs cell differentiation by altering gene expression [111,112]. Another study showed that DNMT1 is indispensable for early haematopoiesis; HSCs from mice with reduced DNMT1 levels can differentiate into myeloerythroid lineages with impaired B lymphopoiesis [113]. TET2 is also a key enzyme in the acquisition of myeloid commitment [114,115]. During C/EBPα-mediated transdifferentiation of pre-B cells into macrophages, TET2 is activated and de-represses myeloid genes [116]. Furthermore, in a triple knockout TET mouse, embryonic stem cell differentiation and development are severely compromised [117]. Finally, the enzyme AID, that it may be involved in DNA demethylation, plays an important role during B cell development and maturation [118].

DNA methylation is also important in immune cell activation. Many myeloid TFs can recruit DNMTs and TET enzymes and therefore modify gene transcription during the innate response. For instance, PU.1 binds TET2 and DNMT3B to target differentially methylation during osteoclast differentiation [119]. Furthermore, during macrophage

differentiation, lower expression of DNMT3B promotes the alternatively activated M2 macrophage [120], dendritic cell development and activation are associated with significant DNA demethylation [121] and TET2 is required for the conversion of 5mC into 5hmC in human monocytes demethylation [122]. In addition, the DNA methylation and demethylation machinery are associated with histone modifications in immune cells. For instance, DNMT3B and HDAC9 act together during antiviral response [123]. Another example is the recruitment of HDAC2 by TET2 to specifically repress IL-6 contributing to resolve inflammation [124]. Associations with inflammation are also exemplified by the finding that TET2 deletion in tumour-tissue myeloid cells shifts from immunosuppressive function to a pro-inflammatory gene expression program [125].

1.2.1.2 Histone modifications

In eukaryotic cells, nuclear DNA is physically associated with a family of highly basic proteins named histones and this DNA-protein complex represents the main component of the structure known as chromatin. The basic repeating subunit of the chromatin is the nucleosome, which consists of a histone octamer wrapped by approximately 147 DNA base pairs. The amino-terminal ends of histones are subjected to hundreds of covalent post-translational modifications including Lys and Arg methylation, Ser and Thr phosphorylation, Lys acetylation, Lys ubiquitylation and sumoylation [126].

Histone acetylation is the addition of an acetyl group to specific Lys residues of the histones, mainly at their N-terminal ends. This modification is carried out by histone acetyltransferases (HATs). Acetylation of histone H3 and H4 correlates with chromatin accessibility and is associated with active transcription in promoters and enhancers whereas low levels of acetylation are linked to silent heterochromatin. The removal of the acetyl group to histone residues is catalysed by histone deacetylases (HDACs) (Figure 7) [126,127].

Methylation of histone Lys or Arg residues is carried out by different families of histone methyltransferases (HMTs) enzymes. Methylation also affects gene transcription, promoting activation or repression, depending on which residue is modified. For instance, trimethylation of each K4, K36 and K79 of histone H3 is associated with active gene transcription. By contrast, methylation of K9, K27 of histone H3 and K20 of histone H4 are linked to a more closed chromatin structure and correlate with silenced regions (Figure 7). Histone demethylation is the removal of methyl groups in modified histones via histone demethylases as Jumonji domain-containing proteins (JmjC) [126,128].

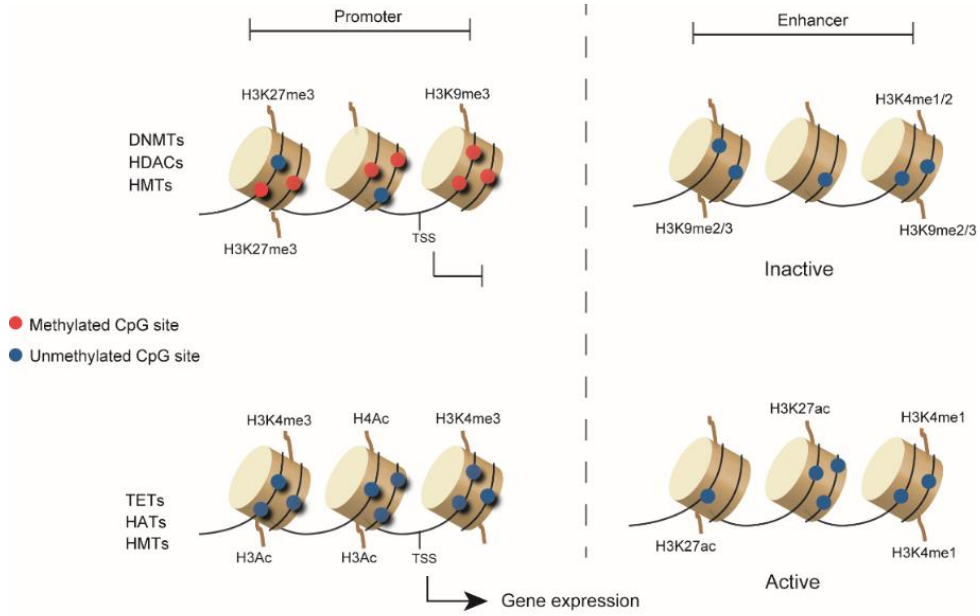


Figure 7. Epigenetic modifications in promoters (left) and enhancers (right). DNA methylation in promoters is associated with gene repression. Histone acetylation is related to active transcription. In addition, promoters have high amounts of H3K4me3 mark and enhancers are marked with H3K4me1 [129].

During haematopoietic differentiation, histone modifiers are important in lineage choice and cell development [7,130]. Epigenomic studies of HSCs have shown that the genomic binding sites of important TFs for haematopoiesis are enriched in the activating histone H3K4me3 [131]. Other study described that the histone demethylase KDM2B regulates haematopoietic cell development, in which loss of KDM2B severely impairs lymphoid differentiation [132]. Moreover, class II histone deacetylase HDAC7 is crucial for proper B cell development and has a transcriptional repression role of specific myeloid genes [133,134]. Finally, HDAC1 and HDAC2 are critical for the development of erythrocytes and megakaryocytes [135].

Epigenetic control is also crucial in myeloid cell activation through TLR- and inflammation-related pathways [136,137]. One of the first studies in this field demonstrated that phosphorylation of H3S10 has a role in NF-kB recruitment and gene induction [138]. Later studies revealed that the HAT p300 binds to enhancers controlling LPS-stimulated gene expression and these enhancers are also enriched in binding sites for PU.1, NF-kB or IRFs [139]. Moreover, HDAC3 inhibits M2 polarization through IL-4-related genes repression [140] and HDAC3 is also required for IFN-β expression [141]. In addition, after LPS stimulation, pro-inflammatory cytokines production is partially dependent on histone acetylation [142].

Several studies on HMTs have also revealed an important role of this epigenetic modification during myeloid cell activation as shown in the following examples. EZH2, a

key component of Polycomb repressive complex 2 mediating H3K27 trimethylation, regulates TLR-induced pro-inflammatory gene expression. Therefore, EZH2 deficiency reduces the inflammatory response in macrophages [143]. During DC maturation, EZH1, a specific H3K27 methyltransferase, promotes TLR-triggered inflammatory cytokine production by inhibiting Tollip, a negative regulator [144]. The H3K4 methyltransferase MLL1 is involved in the regulation of LPS and TNF-stimulated downstream genes, particularly CXCL10 [145,146]. Ash1l, a methyltransferase, suppresses IL-6 and TNF production by regulating A20 expression conferring protection to sepsis [147] and the lysine methyltransferase KMT2B, also known as MLL4, regulates the levels of H3K4me3 during LPS response [148]. In mouse macrophages, Smyd2, which is a histone methyltransferase, specifically facilitates H3K36 methylation at pro-inflammatory gene promoters to suppress their production [149]. Demethylase KDM6A is recently reported to regulate IL-6 and IFN transcription during virus infection [150]. Finally, in macrophages, after TLR stimulation, the histone demethylase Jmjd3 is induced via NF-kB [151]. Jmjd3, which decreases H3K27 methylation, is recruited to the transcription start sites of LPS-related genes and regulate M2 macrophage polarization [152].

1.2.1.3 Interplay between transcription factors and epigenetic modifications in immune cells

Immune cells respond to external stimuli through different functional and transcriptional programs. The immune system has developed wide and multi-level mechanisms to regulate haematopoiesis, immune cell terminal differentiation and activation and inflammatory responses. Transcriptional activity can be tightly regulated by modulating the accessibility or affinity of TFs to the DNA in the context of chromatin. This modulation can be exerted by epigenetic modifications that can turn transcriptionally silent heterochromatin into active euchromatin and vice versa. Also, TFs can play a role in recruiting epigenetic enzymes and participate in the establishment of active or silent chromatin. Epigenetic modifications, namely histone post-translational modifications and DNA methylation, are key in regulating gene expression by the recruitment of nuclear protein complexes that facilitate or repress gene transcription [153,154]. For instance, DNA methylation in promoters generally mediates transcriptional repression [97]. These mechanisms provide the versatility that innate cells require to quickly respond to external stimuli in a stimulus-specific manner [155,156].

A complex network of TFs acts to regulate immune response at different stages, with the involvement of epigenetic enzymes. TFs bind to target DNA or interact with other TFs to regulate transcription and play a role in cell stimulation and cell differentiation.

Many TFs have been described to be important for innate cell function and regulation [157]. Firstly, TLR, TNFR and IL-1R stimulation activates NF-kB and AP-1 TFs via IKK and MAPK signalling respectively; as mentioned above, TLR pathway also activates IRF proteins. Secondly, through IFN-receptors or cytokine receptors, STATs are phosphorylated and translocated into the nucleus, activating several target genes. In addition, STATs are pivotal in macrophage polarization; STAT1 is involved in M1 macrophage activity whereas STAT6 drives differentiation into the M2 phenotype. INF-γ and IL-4 exert mutual epigenomic and transcriptional changes during this process [158]. Third, nuclear TFs, such as glucocorticoid receptors, LXRs and PPARs, are negative regulators of inflammation [159,160]. Finally, others TFs that are important in monocyte-macrophage development and function are PU.1, C/EBP, IRF8 and RUNX [160]. The relevance of TF like C/EBPα in conferring myeloid identity is highlighted by the fact that its ectopic expression in B cells induces transdifferentiation into functional macrophages [161]. PU.1 is required for macrophage development [28] and IRF8 is expressed in the monocyte-macrophage branch but it is not necessary for neutrophil differentiation [156].

Most TFs directly bind to DNA at promoters or enhancers to modulate gene transcription. Promoter regions are localized near to the transcription start site (TSS) where gene transcription is initiated, while enhancers are regulatory sequences located at a significant distance (even mega base pairs) upstream or downstream to TSS. Promoters and enhancers have distinct chromatin signatures. Whereas active promoters are marked with high amounts of trimethylation of H3K4, enhancers are characterized by monomethylation of H3K4 [162,163]. In addition, chromatin modifications at enhancers show little overlap between cell types [164].

Following stimulation, TFs bind to the promoters and enhancers of specific target genes and initiate modification events that result in active or silence gene expression. Epigenetic enzymes through complex interactions with TFs and other transcriptional machinery can also activate or repress gene expression. For instance, upon LPS re-stimulation, tolerant promoters, which include those for inflammatory genes, remain deacetylated and inaccessible for transcription whereas non-tolerant promoters are more acetylated and preserve H3K4me3, given an open chromatin structure [165]. Moreover, a new class of enhancers, called latent enhancers, has been defined, which maintain H3K4me1 mark after stimulation, and therefore response faster to second challenges [166]. Another example is during viral infection, where STAT1 and STAT6 promote chromatin reorganization; they have an antagonist role on each other's downstream signalling in activated NK cells [167].

Finally, the complex crosstalk between epigenetic immune regulation and metabolic pathways has been studied, playing a key role in the innate response, host defence and inflammation. Metabolites derived from important metabolic routes including glycolysis, Krebs cycle, fatty acid metabolism or pentose phosphate pathway impact the activity and availability of substrates of epigenetic enzymes, that, in turn, regulate gene transcription [168,169]. Intermediaries of these metabolic pathways serve as essential cofactors or substrates for epigenetic modifying enzymes. For instance, acetyl-CoA is a cofactor for HATs, nicotinamide adenine dinucleotide controls class III HDACs, known as sirtuins, and flavin adenine dinucleotide regulates JmjC histone demethylases. In addition, S-adenosylmethionine serves as a methyl donor substrate for DNA and histone methyltransferases and TET enzymes rely on α-ketoglutarate, an intermediate of Krebs cycle [170,171]. Other intermediates of the Krebs cycle also regulate transcription. Fumarate inhibits histone demethylase KDM5 family and increases histone methylation which facilitates more open chromatin and succinate stabilizes HIF-1α, enhancing IL-1β transcription after LPS activation [172,173].

1.2.2 Non-coding RNAs

Non-coding RNAs regulate the expression of their target genes at the transcriptional and post-translational levels. Non-coding RNAs are crucial regulators in immune cell development and function, in chronic inflammation as well as in numerous pathologies including cancer, autoinflammatory diseases and diabetes [174–176]. Examples of non-coding RNAs include micro-RNA (miRNA) and long non-coding RNA (lncRNA), which do not translate into proteins [177].

miRNAs control immune response [156,178,179]. The first level of control is during haematopoiesis; human CD34+ cells have high expression of more than 30 different miRNAs [180]. In addition, in myelopoiesis context, miR-223 is important in the regulation of granulocyte differentiation [181] and miR-146a restricts massive myeloproliferation [182]. In this way, miRNAs may play a role in haematological diseases. Numerous studies have shown miRNAs regulation in acute myeloid leukaemia or in chronic myelogenous leukaemia [183].

PRRs induce the expression of multiple non-coding RNAs being important in the control of immune response after stimulation [184–186]. For instance, miR-146a is expressed after LPS stimulation and is responsible to decrease TRAF6 and IRAK1 expression [187] and miR-155 controls SOCS1 expression in T cells [188]. Furthermore, it has been described that lincRNA-Cox2, a long non-coding RNA, mediates inflammatory gene expression to control the immune response [189]. Recently, it has been suggested that

TET2 promotes myelopoiesis during pathogen infection by decreasing 5-mC in *Socs3* mRNA transcripts [190].

Non-coding RNAs are important regulators of innate immune memory [191]. During endotoxin tolerance and systemic inflammation, in tolerant THP-1 cells, miR-221, miR-579 and miR-125b have altered expression and can modify TNFα post-translational regulation [192]. In addition, miR-146a and miR-223 have significantly lower expression in serum from septic patients [193]. More recently, it has been observed an increase in miR-221 and miR-222 expression in septic patients and this enhanced expression correlates with immunosuppression [194]. Furthermore, the lncRNA, UMLILO, participates in the regulation of trained immunity [195].

In autoinflammatory diseases, miRNAs can also act regulating the innate immune response. In CAPS patients, a significant downregulation of miR-29c and miR-103-2 expression in parallel with an increase of miR-9-1, miR-199a-2, miR-203 and miR-320a has been found in skin lesions of these subjects [196]. In addition, an upregulation of miR-4520a has been described in FMF patients [197].

1.3 Innate immune memory

Organisms are constantly exposed to pathogens and danger signals and the immune system must always be alert to these threats. Innate immunity is the first line of host defence against insults and constitutes the basis of adaptive immune development.

As aforementioned, innate responses have been classically defined as immediate, non-specific and without immunological memory. However, intense research has changed this dogma. The discovery of the PRRs revealed a semi-specific recognition of pathogens by innate immune cells. In addition, many evidences suggest that innate immune cells are also capable of developing immunological memory [198–200].

In plants and invertebrates, which lack the adaptive system, memory processes have been studied [201]. Plants have capacity to respond more efficiently to reinfection by a phenomenon denominated systemic acquired resistance (SAR) [202]. SAR represents the first line of memory evidence in the innate system; studies of the mechanisms that mediate this process have provided epigenetic modifications as crucial, in particular, acetylation of H3K9 and trimethylation of H3K4 [203–205]. In invertebrates, the immune response also depends exclusively on innate mechanisms. Several studies have described life-long protection against secondary attacks by pathogens in the absence of lymphocyte-mediated response. For instance, a pre-challenge of mealworm beetles with LPS induces protection against fungi [206] and flies infected with *Streptococcus pneumonia*

or *Beauveria bassiana* are protected for reinfection with the same microorganisms [207]. In addition, innate memory has been observed in *Anopheles gambiae* that were infected with *Plasmodium falciparum* [208].

The first studies in mammals revealed that NK cells confer long-memory to the host in a T/B cell independent manner. Those studies showed that the protection against cytomegalovirus is dependent on virus-specific Ly49H receptor expression on NK memory cells from mice [209]. Moreover, after Bacillus Calmette–Guérin (BGC) vaccination, human NK cells present trained phenotype producing increase levels of pro-inflammatory cytokines [210]. During the last 10 years, many studies have been published in relation to innate memory in mammalian cells, especially, in NK cells, monocytes and monocyte-derived macrophages.

Innate immune memory is an important property of host defence. Innate memory is described as the ability to respond in a more vigorously (trained) or attenuated (tolerance) manner to subsequent encounters with the pathogens and confers protection against secondary infections through mechanisms that do not imply T and B cells [211]. Innate memory may also be involved in pathologies [3]. For instance, a tolerant state or immunoparalysis has been described during sepsis. By contrast, trained immunity can produce hyperinflammation in atherosclerosis or autoinflammatory diseases [212]; but it is also the mechanism that gives long-term non-specific effects of vaccines [213]. Trained immunity is defined as enhanced non-specific protection against infections after prior exposure to certain microbial components as β-glucan. Trained cells exhibit a heightened production of pro-inflammatory cytokines upon secondary stimulations. On the other hand, upon priming with some molecules as LPS or Pam3CSK4, tolerance induces a transient state of hypo-responsiveness of cells, producing fewer pro-inflammatory cytokines after second encounters (Figure 8) [214].

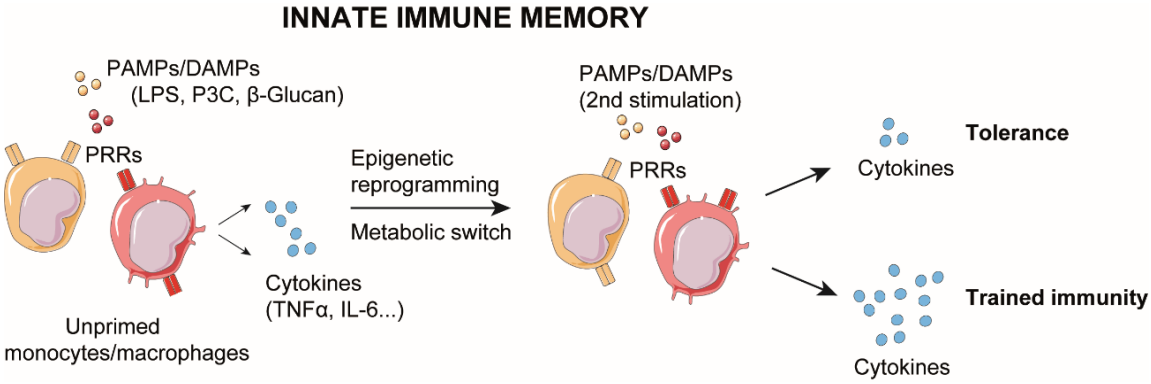


Figure 8. Innate immune memory processes. Unprimed innate cells are stimulated via PRR signalling; after second stimulations, these cells can respond with enhanced (trained) or reduced (tolerance) immune response. The main effects described during innate memory are altered cytokine release, epigenetic reprogramming and metabolic switch.

1.3.1 Epigenetic modifications during innate immune memory

Epigenetic modifications shape immune cell development, immune activation as well as innate immune memory. Innate memory induces different functional and transcriptional programs, which are regulated by epigenetic changes. For example, LPS confers long-lasting immune effects reducing inflammatory cytokines production (tolerant promoters) upon re-stimulation but also enhancing antimicrobial response (non-tolerant promoters) by maintaining acetylated H4 and methylated H3K4 only in non-tolerant promoters [165,215]. In addition, the TF ATF7 decreases the histone repressive H3K9me2 mark controlling innate memory [216].

After BCG vaccination, the worldwide used vaccine against tuberculosis, monocytes exhibit enhanced levels of cytokines (trained phenotype). Thus, immunization with BCG confers non-specific protection against unrelated pathogens for at least 3 months after vaccination; and this protection is associated with H3K4me3 changes mediated through NOD2 [217]. Even one year after BCG vaccination, it has been observed non-specific training effects on receptor expression and pro-inflammatory cytokine production [218]. In addition, genome-wide studies have revealed histone modification reprogramming of human monocytes after BCG vaccination, given a crucial role to IL-1β cytokine during the acquisition of the trained phenotype [219]. It has also been reported the occurrence of changes in DNA methylation patterns after BCG vaccination [220].

After exposure to β-glucans from Candida albicans, monocytes show epigenetic reprogramming, in particular, stable changes in H3K4 trimethylation, through a dectin-1/Raf-1 dependent pathway, with enhanced production of cytokines like IL-6 and TNFα, upon secondary stimulation with the same glucans in mice deficient in functional T and B cells [221]. In another study, Saeed and colleagues did a genome-wide analysis and they

showed that macrophage training and LPS tolerance are associated with epigenetic alterations in H3K4me3, H3K27Ac, H3K4me1 and DNase I sensitivity, allowing for enhanced or reduced response upon a second challenge respectively (Figure 9). In addition, they identified the cAMP signalling pathway as an important mechanism of training cells [222]. Importantly, β-glucan can partially reverse functional, transcriptional and epigenetic changes of LPS-induced tolerance monocytes [223]. Moreover, β-glucan, through epigenetic and metabolic reprogramming, restores the expression of succinate dehydrogenase in tolerant monocytes, to revert the state of immunoparalysis [224].

A large body of literature exists describing the association of innate immune memory with profound intracellular metabolic alterations. Cheng et al. showed that trained immunity is associated with a shift from oxidative phosphorylation toward glycolysis through an Akt/mTOR/HIF-1α pathway. In relation to this, β-glucan-trained monocytes exhibited reduced baseline oxygen consumption, and increased glucose consumption, lactate production and NAD+/NADH ratio [225]. BCG training also increases glycolysis, lactate production and oxygen consumption and these metabolic changes contribute to long-term epigenetic reprogramming [226]. Another supporting evidence of the relationship between metabolism and innate memory is that glycolysis, glutaminolysis and cholesterol metabolism are crucial in trained cells for the increased cytokine production and for epigenetic modifications [172]. In addition, mevalonate, a crucial metabolite in cholesterol synthesis, induces trained immunity in human monocytes. Monocytes from Hyper IgD syndrome patients, who accumulate mevalonate due to mevalonate kinase deficiency, have a trained phenotype with higher cytokine production [227].

Recently, it has been studied the potential effects of innate memory at the level of hematopoietic and myeloid progenitor cells. Since myeloid cells have short-lived, the question is whether innate immune memory is maintained and propagated over a long period. In this way, trained immunity modulates cell metabolism of myeloid cell progenitors in the bone marrow and increases myelopoiesis [228]. Similar observations have been reported after BCG training. BCG-trained HSCs differentiate to macrophages that have enhanced protection against *Mycobacterium tuberculosis* infection [229]. In addition, Christ et al. have demonstrated that high-fat diet induces long-lasting immune reprogramming in myeloid precursor cells, and IL-1 plays a crucial role in this response [230].

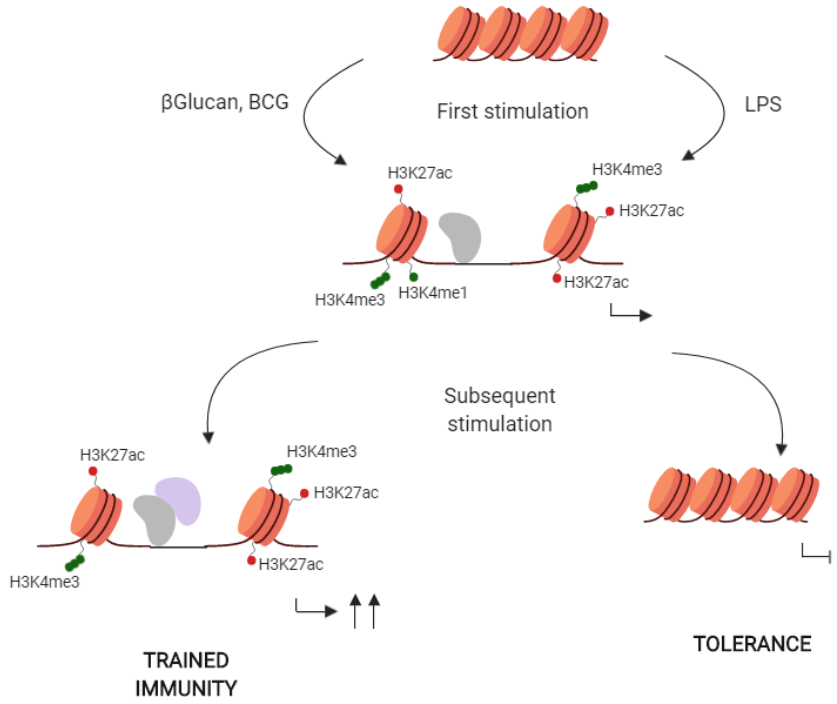


Figure 9. Epigenetic modifications during innate immune memory. PRR stimulation of immune cells leads to K4 trimethylation of histone H3 (H3K4me3) and global acetylation on promoters of pro-inflammatory cytokines whose expression is increased. Following subsequent stimulation, trained cells (left) have increased gene expression associated with H3K4me3 and H3K27ac marks. By contrast, in tolerized cells, gene expression is silent (methylation is represented by green circles and acetylation by red circles).

1.4 Epigenetic dysregulation and endotoxin tolerance in sepsis

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection [231] and it represents the most frequent cause of mortality in most intensive care units. Despite the high mortality rate described during sepsis, there are not currently approved drugs to specifically target the disease. Sepsis leads to imbalance cytokine production affecting vascular permeability, cardiac function and metabolic homeostasis that result in tissue damage, multiple organ failure and even death [231,232].

It has been recognized two different phases, that can overlap, during the disease: an initial phase characterized by an exaggerated systemic release of cytokines, known as “cytokine storm”, in which also coagulation and complement systems are activated; and a later phase, that shows an immune paralysis state, to compensate this imbalance response, by enhanced expression of negative regulators of TLR signalling, secretion of anti-inflammatory cytokines and immune-cell apoptosis among others (Figure 10) [233–235]. Monocytes can potentially contribute to both phases, releasing inflammatory cytokines during “cytokine storm” and later adopting an immunosuppressive phenotype at which they are unable to respond to secondary infections.

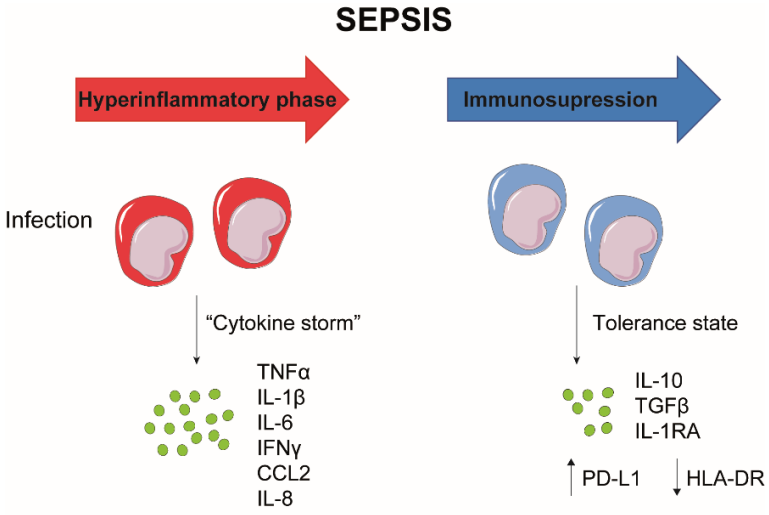


Figure 10. Scheme representing human sepsis phases. Firstly, after infection, immune cells release an exaggerated amount of pro-inflammatory cytokines, a phase known as "cytokine storm" (in red). Later, cells present an immunosuppressive state (in blue) characterized by IL-10, TGFβ and IL-1RA expression and reduced levels of HLA-DR.

Sepsis initiates a complex immune response that fluctuates over the course of the disease affecting both innate and adaptive functions. Immune effector cells become altered during sepsis promoting secondary and/or nosocomial infections development. For instance, apoptosis is increased in dendritic cells, NK cells and B/T lymphocytes. Neutrophil dysfunction is also observed with reduced phagocytosis and ROS release. Antigen presentation is impaired due to a decrease in human leukocyte antigen (HLA) expression in macrophages and dendritic cells. Finally, cytokine secretion is highly affected [236,237].

Therefore, in sepsis, cells present an altered immune response characterized by reduced inflammatory cytokine production after subsequent encounters with pathogens, also known as endotoxin tolerance. During endotoxin tolerance, pro-inflammatory cytokine expression, such as TNFα, IL-6 and IL-1β, is reduced whereas anti-inflammatory (IL-10, IL-1RA, TGFβ) cytokines are up-regulated. Moreover, tolerant monocytes have up-regulation of IRAK-M, TREM-1 and CD64 while MHC class II molecules are down-regulated [238,239]. All these anti-inflammatory mechanisms are needed to counteract the initial effects, however, patients become susceptible to opportunistic pathogens and the long-term immunosuppression observed in septic patients is related to a high mortality rate in later years [240,241]. Monocytes from septic patients exhibit many characteristics of this endotoxin tolerance state. For instance, septic monocytes show a reduced capacity to produce IL-1α, IL-1β, IL-6, and TNFα after *ex-vivo* stimulation [242,243]. Moreover, gene expression changes in components of the inflammasome have been found in septic monocytes [244,245] and enhanced ROS and NO

levels have been also described in these patients [246]. Recently, it has been described an impaired NLRP3 inflammasome activation in septic leukocytes by P2X7 receptor, which is associated with a high rate of mortality [247].

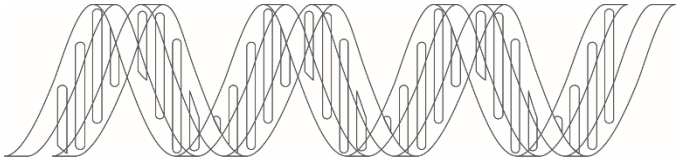
Sepsis activates inflammatory signalling pathways including NF-kB, AP-1 and IRF routes. In this regard, immune signalling is altered in these patients. NF-kB expression is down-regulated in PBMCs from septic patients [248] and some DAMPs, including HMGB1 and S100A9, are increased and correlate with sepsis mortality [249,250]. Molecules that inhibit TLR signalling are also found to be altered during sepsis. For instance, monocytes isolated from septic patients up-regulate IRAK-M when re-stimulated with LPS [251] and SIGIRR and MyD88s expression are both increased in septic monocytes [252].

During acute inflammation, it has been described important metabolism changes of leukocytes. In septic patients, metabolism pathways are decreased (immunometabolic paralysis) with a defective capacity to mount an immune response against new pathogens [253]. In addition, a shift to glycolysis is observed during hyperinflammation whereas it is reduced in immunotolerance [254]. Monocytes from septic patients also display a functional reprogramming with decreased inflammation and altered metabolic processes; interestingly, this reprogramming is mediated by a pathway dependent on HIF-1α [255].

Besides metabolic changes, there is also an epigenetic reprogramming during sepsis, that modifies gene expression by chromatin accessibility [256]. Numerous studies have shown that these epigenetic modifications are crucial for the acquisition of endotoxin tolerance. For instance, increased levels in repressive H3K9me2 mark in pro-inflammatory gene promoters are important during TNFα and IL-1β silencing in human monocytic cell lines [257,258]. In addition, HMGB1 and nucleosome linker histone H1 are necessary for endotoxin-mediated silencing of TNFα [259]. Specifically, the histone methyltransferase G9a, combined with HP1 and DNA methylation machinery, regulates TNFα expression during endotoxin tolerance [260]. Furthermore, IFN-γ inhibits endotoxin tolerance by facilitating TLR-induced chromatin remodelling to regulate the expression of inflammatory cytokines [261]. Another example has been described in lung macrophages from cecal ligation and puncture mice, an animal model of sepsis, where IRAK-M mediates TNFα silencing through histone acetylation and methylation [262]. IL-12 expression is also regulated by H3K4 and H3K27 methylation changes in dendritic cells from this mouse model [263]. In patients with sepsis, chromatin remodelling may occur similar to endotoxin tolerance to regulate immunosuppression. For instance, in a pilot

study, human monocytes from patients suffering sepsis have specific changes in H3K4me3, H3K27me3 and H3K9ac in relevant immune genes [264].

It is important to highlight that sepsis is very heterogeneous. The initial infection varies with respect to the pathogen, site of infection or diagnosis time. Furthermore, patients have different genetic backgrounds and comorbidities [265]. Due to the high rate of mortality during sepsis, it becomes necessary to find novel biomarkers and treatments that can improve this systemic condition.



OBJECTIVES

2. OBJECTIVES

When exposed to pathogens, immune cells mount an appropriate response that involves the activation of signalling cascades and downstream TFs as well as an interplay with epigenetic enzymes, which results in transcriptional changes that activate or repress specific genes. In recent years, it has been described the ability of innate immune cells to develop memory. Innate immune memory has been termed as trained immunity or endotoxin tolerance depending on whether there is an enhanced or diminished immune response respectively after a subsequent challenge. During the past years, different groups have made key contributions in relation to the participation of epigenetic mechanisms to innate memory. For instance, several publications have shown the occurrence of changes in the profile of histone modifications during trained immunity and endotoxin tolerance. Less studies have focused on DNA methylation, despite the relevance of this epigenetic modification, as well as the enzymes associated with its incorporation or removal, for the plasticity of monocytes. For this reason, we decided to focus on DNA methylation. In addition, there are several unsolved questions about the possible role of DNA methylation during the exposure to microbial agents and in sepsis. Sepsis is a major burden of the healthcare systems due to the associated high mortality rates and high medical costs. We were interested in studying in depth the mechanisms by which septic monocytes become tolerant, a major cause of reinfections after septic episodes. In this regard, this doctoral thesis aims at investigating DNA methylation changes in human monocytes associated with sepsis episodes and how the resulting epigenetic landscape may determine their tolerized phenotype. In addition, we aimed at unveiling the influence of the epigenetic mechanisms, specifically, DNA methylation, during the acquisition of innate memory.

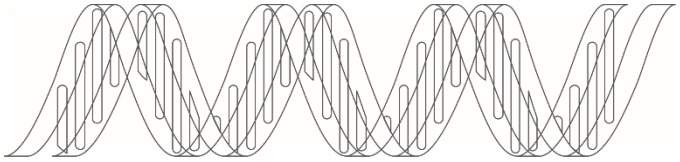
Therefore, we proposed the following specific objectives:

1) To obtain the DNA methylation patterns of monocytes isolated from patients with sepsis and study the potential role of this epigenetic mark in the acquisition of tolerance.

Obtaining the DNA methylation profile from patients with sepsis may allow to identify specific features related to basic and clinical aspects of this process: a) biomarkers associated with the clinical outcome in the context of systemic inflammation and organ damage; b) understanding the contribution of signalling pathways and TFs in the recruitment of DNA methylation/demethylation enzymes and their functional consequences.

2) To characterize epigenomic profiles, specifically, DNA methylation, during the acquisition of trained immunity and endotoxin tolerance by monocytes using *in vitro* models, as archetypical examples of innate immune memory.

DNA methylation alterations may participate in the acquisition of innate memory. In this context, it is relevant to define the mechanisms and dynamics involved in these alterations and explore the participation of TFs in targeting these methylation changes during innate memory. Furthermore, we will investigate specific signalling pathways involved in this process. Investigating the dynamics of DNA methylation, histone marks and gene expression may help to understand the role of epigenetic mechanisms in the acquisition of the phenotype.



MATERIALS AND METHODS

3. MATERIALS AND METHODS

This doctoral thesis comprises two related studies. In this section, the materials and experimental procedures are presented together, given that both studies share many protocols and technical approaches.

3.1 Patients and ethics statement

For studying the role of DNA methylation following sepsis, human blood samples were obtained from septic patients, non-infectious systemic inflammatory response syndrome (SIRS) individuals, and healthy subjects, that matched in age, sex and body mass index, from Hospital Universitario La Paz (Madrid) and Hospital Vall d'Hebron (Barcelona). The study was approved by the corresponding Hospital Ethics Committees, in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. Before providing blood samples, all individuals received oral and written information about the possibility that their blood would be used for research purposes and signed a consent form.

Septic patients were identified according to the diagnostic criteria for sepsis to the Society of Critical Care Medicine and the European Society of Intensive Care Medicine international conferences [231,266]. Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. Septic patients have to exhibit at least two of these next criteria for SIRS, in presence of infection:

- Body temperature > 38º or < 36º
- Heart rate higher than 90/min
- Hyperventilation (higher than 20 breaths/min) or $PaCO_2$ < 32 mmHg
- White blood cell counts higher than 12000 cells/µl or lower than 4000 cells/µl

The Sequential [Sepsis-related] Organ Failure Assessment (SOFA) score was also used. To compute this score, several clinical parameters were measured including PaO_2 (partial pressure of oxygen), platelets, creatinine and bilirubin levels and mean arterial pressure. All patients with sepsis have a SOFA score equal to or higher than 2 [231]. Blood samples were collected at the first 12 hours of sepsis diagnosis, before any therapy, and sepsis was confirmed using clinical and analytical data mentioned above. The clinical data of the samples included in the study are summarized in Table 1.

Finally, a group of non-infectious systemic inflammatory response syndrome (SIRS) was also included, formed by patients in the immediate (the first 24 hours) post-operative period of cardiac surgery.

Table 1. Clinical features of the patients included in this study.

							Basal cytokine levels (pg/ml)																								
Disease phenotype	Code	Sex	Age	Infection	Outcome	SOFA	TNFα	IL6	IL10	IL1B	Temperature	Heart rate	Respiratory rate	SatO2	FiO2	SaFi	Systolic blood pressure	Diastolic blood pressure	Creatinine	Bilirubin	Lactate	Procalcitonin	Hemoglobin	Hematocrit	GOT	GTP	INR	Leukocytes	Platelets	PCR	Focus of infection
Sepsis	SEP-1	Male	90		Survivor with relapse	3	18.21	1076.91	25.50	31.33	35.4	79	28	94	0.21	447	100	75	1.39	1.2	1.3	2.99	11.6	38	15	17	2.1	17200	174000	93.3	Respiratory
Sepsis	SEP-2	Female	93	Gram -	Survivor	3	5.15	6.70	11.34	4.55	38.1	57	22	92	0.21	438	157	85	0.82	0.2	1.4	0.14	13.7	43.1	43	22	1	16700	358000	117	Urinary tract
Sepsis	SEP-3	Male	94		Non survivor	3	0.00	1810.49	212.50	0.00	32	105	22	85	0.21	404	123	95	3.36	1.8	7.1	3.3	17.3	56	164	175	1.5	18200	224000	65.4	Respiratory
Sepsis	SEP-4	Female	85	Gram +	Survivor	3	0.00	39507.10	56.52	384.78	39.2	150	32	74	0.21	352	100	80	1.1	1.2	0.9	4.71	12	40.3	18	52	2.8	12300	168000	94.7	Others
Sepsis	SEP-5	Female	59	Gram -	Survivor	2	0.00	499.11	9.68	0.00	39	135	20	97	0.21	461	114	85	1.47	0.6	3	121.8	14.2	42.4	43	41	1	31200	221000	401	Urinary tract
Sepsis	SEP-6	Female	83	Gram -	Survivor	3	0.00	64.83	96.64	0.00	37	110	22	96	0.21	457	77	55	1.3	0.4	3	39.67	11.7	36.3	20	19	1.1	31000	138000	83.8	Urinary tract
Sepsis	SEP-7	Female	85	Gram +	Survivor	4	16.03	49.50	45.82	0.00	35	85	24	91	0.21	433	100	70	0.88	0.9	3	0.03	13.2	41	44	28	1	14900	157000	60.8	Respiratory
Sepsis	SEP-8	Female	68	Gram -	Survivor	3	7.89	92.61	16.60	9.86	36	78	18	99	0.21	471	88	65	1.03	0.9	2.7	18.99	11.9	36.3	104	142	1.3	12500	84000	124	Urinary tract
Sepsis	SEP-9	Male	75		Survivor with relapse	2	8.40	45.61	10.65	18.80	38	86	26	86	0.21	409	95	63	1.27	0.2	0.6	0.21	11.8	36.8	31	35	0.9	10100	181000	128	Respiratory
Sepsis	SEP-10	Female	56	Gram-	Survivor	4	4.35	13.54	3.76		36.5	75	24	97	0.35	237	95	60	0.91	4.29	4.9	8.61	11.6	32.2	133	44	1	63590	324000	7.97	Abdominal
Sepsis	SEP-11	Male	69	Gram+	Non survivor	7	1.04	14156.89	16.93		35.8	116	14	98	0.3	279	82	59	0.77	0.42	10	41.48	12.2	37.8	31	12	2.4	1040	127000	9.79	Abdominal
Sepsis	SEP-12	Male	61	Gram -	Survivor	2	0.00	40.36	19.04		35	72	23	93	0.3	313	79	66	1.65	0.6	7	55.96	9.2	28.8	122	39	1.11	860	181000	28.79	Urinary tract
Sepsis	SEP-13	Male	49	Gram+	Non survivor	7	7797.94	1417.48	223.24		37.4	96	32	77	0.4	153	71	49	1.63	1.16	8.9	46.91	15.6	48.9	52	27	1.76	10080	154000	3.69	Respiratory
Sepsis	SEP-14	Female	77	Gram-	Survivor	8	0.00	1045.88	30.42		36.9	121	20	97	0.5	194	107	51	2.3	0.38	3.3	46.55	8.2	26.2	49	23	1.26	6320	277000	38.94	Abdominal
SIRS-cardio	SIRS-1	Male	74		Survivor	7	0.00	71.57	117.08		37.2	75	20	98	0.6	163	124	59	1.96	1	1.9		10.8	33.2	53	7	1.1	17630	143000		
SIRS-cardio	SIRS-2	Male	60		Survivor	2	0.58	77.35	27.06		36.9	76	18	97	0.5	194	136	89	0.74	0.35	1		11.8	35.5	18	16	1.17	15890	166000		
SIRS-cardio	SIRS-3	Female	69		Survivor	3	6.90	275.75	110.17		36.1	45	17	99	0.5	198	139	68	0.83	0.6	1.5		11	33.7	50	16	1.2	12200	106000		
SIRS-cardio	SIRS-4	Male	68		Survivor	3	3.22	70.35	72.20		36.8	85	21	95	0.45	211	110	60	0.9	0.45	1.9		12.3	38.4	250	149	1.31	15120	151000		
Healthy control	CON-1	Female	59				0.00	0.00	0.00	14.27																					
Healthy control	CON-2	Male	47				0.00	1.29	2.56	4.21																					
Healthy control	CON-3	Male	61				0.00	4.88	0.00	0.00																					
Healthy control	CON-4	Female	27				0.00	0.00	7.67	0.00																					
Healthy control	CON-5	Male	48				4.13	30.38	0.00	12.73																					
Healthy control	CON-6	Male	35				3.50	9.47	10.12	10.56																					
Healthy control	CON-7	Male	49				17.24	32.31	13.74	21.37																					
Healthy control	CON-8	Male	51				57.80	27.73	11.86	39.05																					
Healthy control	CON-9	Male	57				8.75	20.99	9.06	13.90																					
Healthy control	CON-10	Male	67				5.00	3.06	6.78																						
Healthy control	CON-11	Female	60				2.33	1.67	2.24																						

3.2 Human sample preparation and purification of monocytes

For monocyte isolation, peripheral blood mononuclear cells (PBMCs) were obtained from blood samples by density gradient centrifugation (800 g for 30 min, in the absence of braking at 16-18º) using Lymphocytes Isolation Solution (Rafer). PBMCs, the white interface, were transferred to a new falcon and we proceeded to erythrocyte lysis using ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.125 mM EDTA pH 7.2). Following incubation with human FcR Blocking Reagent (Miltenyi Biotec), PBMCs were stained with CD14-PE (Miltenyi Biotec), CD66b-APC (STEMCELL™ Technologies) and Fixable Viability Dye eFluor™ 520 (Thermo Fisher Scientific) for 20 minutes on ice protected from light and, subsequently, cells were fixed with 2% formaldehyde methanol-free (Thermo Fisher Scientific). After that, cells were washed three times with MACS buffer (4% fetal bovine serum (FBS) and 2 mM EDTA in phosphatase-buffered saline (PBS)). Finally, pure monocytes were isolated as CD14+CD66b- cells using flow cytometry sorting (MoFlo Astrios), and the purified samples were pelleted and stored at -80ºC.

3.3 Cell culture and stimulation experiments

For *in vitro* experiments, buffy coats from anonymous blood donors were used. These were obtained through the Banc de Sang i Teixits (Barcelona), which follows the principles set out in the World Medical Association (WMA) Declaration of Helsinki. All donors received detailed oral and written information and signed a consent form prior to any donation.

As mentioned previously, PBMCs were isolated by density gradient centrifugation. Then, PMBCs were washed twice with PBS and incubated with magnetic CD14 MicroBeads (Miltenyi Biotec) for 15 minutes at 4ºC; after that, CD14+ monocytes were isolated using positive selection with a magnetic cell separator. Following cell separation, purified monocytes were resuspended in Roswell Park Memorial Institute (RPMI) Medium 1640 + GlutaMAXTM-1 (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS) or 10% human pooled serum (One Lambda, Thermo Fisher Scientific), 1% Penicillin/Streptomycin (Gibco) and were maintained in a humidified incubator at 37º and 5% CO_2 for the corresponding days. Purified CD14+ cells were used for diverse stimulation experiments (Figure 11).

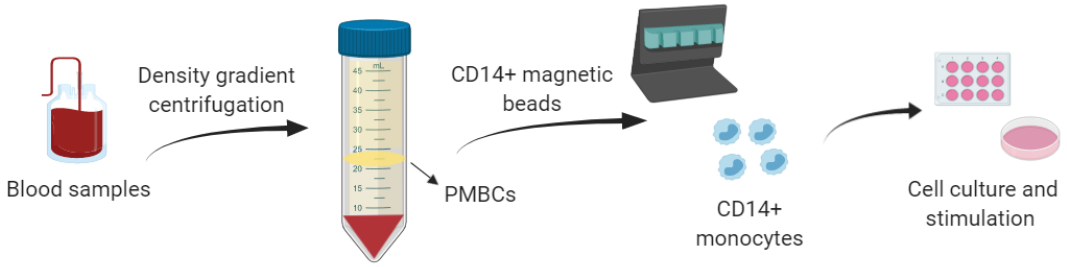


Figure 11. Schematic representation of the procedure to purify CD14+ monocytes from blood samples. After density gradient centrifugation, PBMCs are incubated with CD14+ MicroBeads and pure monocytes are isolated, which are then cultured and stimulated to the diverse experiments.

Two *in vitro* assays were set up for studying innate immune memory. Depending on the amount needed, $2x10^6$ or $4x10^6$ monocytes/well were grown in 12-well or 6-well plates, respectively. For studying endotoxin tolerance, monocytes were pre-incubated for 24 h with 10 ng/ml of lipopolysaccharide (LPS, from *E coli* O111:B4, Sigma-Aldrich) or 10 µg/ml of Pam3CSK4 (P3C, InvivoGen). As a model of trained immunity, monocytes were exposed to 10 µg/ml of β-glucan (InvivoGen), also for 24 h. In both cases, after the first stimulus, cells were washed and left to rest for 3 days with medium supplemented with 10% human pooled serum (One Lambda, Thermo Fisher). Then, the cells were again stimulated with LPS at the same concentration. After one day, cells and supernatants were collected by the following studies. For JAK2 inhibition experiments, cells were grown in presence of different concentrations of fedratinib, (formerly known as TG101348, Santa Cruz Biotechnology) in the same conditions mentioned above.

In experiments aiming at recapitulating human sepsis, PBMCs were either non-treated (control) or treated with 10 ng/ml of LPS and cultured in poly-2-hydroxyethyl methacrylate (poly-HEMA) (Santa Cruz Biotechnology) coated plates for 4 days. After that, monocytes were purified by cell sorting as CD14+CD66b- cells. Alternatively, CD14+ monocytes were isolated using positive selection with CD14 MicroBeads (Miltenyi Biotec) and cultured in the same conditions as PBMCs. Purified monocytes were pelleted and stored until DNA or RNA extraction. Supernatants were collected and stored at -80ºC until cytokine measurement.

3.4 Transfection of primary human monocytes

ON-TARGETplus siRNA (GE Healthcare Dharmacon) against TET2 was used to perform knockdown experiments in monocytes with ON-TARGETplus Non-targeting Control Pool used as a negative control. Monocytes were transfected with siRNAs using

Lipofectamine 3000 Reagent (Invitrogen). siRNAs were added 24h before the first stimulus and then, stimulations were performed as mentioned above. Finally, the levels of the target factors were examined by qRT-PCR at day 4.

3.5 DNA isolation, bisulfite modification and pyrosequencing

In the case of samples from patients for studying DNA methylation changes following sepsis, genomic DNA was extracted using ReliaPrep™ FFPE gDNA Miniprep System (Promega). This kit provides an efficient DNA extraction method to reverse the crosslinking between proteins and DNA generated after formaldehyde fixation. For samples corresponding to *in vitro* experiments, Maxwell® RSC Cultured Cells DNA Kit (Promega) was used for DNA purification following the manufacturer's protocols. In the case of isolating high numbers of cultured cells, genomic DNA was extracted by proteinase K protocol as follows. Cells were lysed in 750 µl of lysis buffer (50 mM Tris pH 8.8, 10 mM EDTA pH 8, 100 mM NaCl, 1% SDS) and incubated overnight at 55ºC with proteinase K at a final concentration of 50 µg/ml. The next day, 340 µl of 5 M NaCl were added at each sample, vortexed and incubated at RT for 10 minutes. Samples were centrifuged at 16000 g for 20 minutes and supernatants were transferred to new eppendorf tubes. Then, 450 µl of isopropanol were added and samples were incubated at RT for 10 minutes to allow DNA precipitation. We centrifuged at 16000 g 10 minutes and discarded supernatants. DNA pellets were washed with 70% ethanol for twice and were resuspended in *Milli-Q* water. DNA was quantified with Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific).

Bisulfite modification of 200-500 ng of genomic DNA isolated from cells was performed using EZ DNA Methylation-Gold™ Kit (Zymo Research), following the manufacturer's protocol. Bisulfite sequencing is commonly used for the detection of 5-methylcytosine (5mC) in the genomic DNA; bisulfite treatment converts unmethylated cytosines to uracil by deamination, that will be read as thymine during sequencing, whereas methylated positions remain as cytosines (Figure 12).

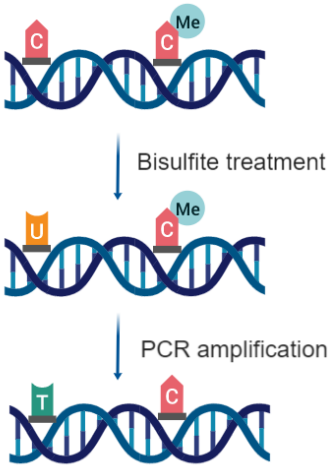


Figure 12. Scheme representing bisulfite treatment of genomic DNA. Treatment of DNA with sodium bisulfite converts unmethylated cytosines into uracil but not affects 5mC. After PCR amplification, unmethylated positions are read as thymine while methylated cytosines stay as cytosines.

After bisulfite modification, conventional PCR was performed with the IMMOLASE™ DNA polymerase PCR kit (Bioline). For each primer pair, we used the following mix: 17.3 µl of H_2O, 2.5 µl 10X buffer, 2.5 µl DTNPs, 1 µl $MgCl_2$, 0.5 µl forward (F) + reverse (R) primers, 0.2 µl immolase and 1 µl of modified DNA. PCR protocol was:

10 min at 95ºC
45 cycles
30 sec at 95ºC (denaturation)
30 sec at 56ºC-60ºC (annealing)
30 sec at 72ºC (extension)
7 min at 72ºC

The success of PCR amplification was assessed by 2% agarose gel electrophoresis. Primers for PCR amplification and sequencing were designed with the PyroMark Assay Design 2.0 software (QIAGEN). Primers are listed in Table 2. Finally, PCR products were pyro-sequenced with the PyromarkTM Q96 (or Q24) system (QIAGEN). Pyro Q-CpG software was used to obtain the percentage of methylation of each analysed CpG. Results from bisulfite pyrosequencing are represented as the mean methylation percentage in the graphs generated with GraphPad Prism 6.

Table 2. List of pyrosequencing primers used in this thesis.

[Btn]: Biotinylated primers

	CG	Forward Primer	Reverse Primer	Sequencing Primer
IL1R2	cg06528771	TGTTTTTTGGAAGTTTTGATTGTTTTTAGA	[Btn]TCCCAAAAATTTACTAATATCTACACTTAC	TTTTAGATTGTTATTTGTAGGGTA
IL15	cg10912126	[Btn]TGAGGTGAATTATTGGATTGTTTATTAGAA	CCCTCAAACAAACAAAAATAAAAACACATC	ACACATCATATCAAAACC
CCL20	cg08575688	GATGATATGATGGGGTTAGTTGATT	[Btn]ACAACAACCTAAAATAACCCTATTTATA	TGATTAATGGGGAAAATTTTAT
ETS1	cg00320216	AGTATTTGAAATAGAGTTGGTGTGTATAA	[Btn]TAACCTCAAAATCACACAAAAACACACTTC	AGAGTTGGTGTGTATAAT
IRAK2	cg14433987	[Btn]AAAGAAATGTTGTTGGAGATGT	CCTACATTCAAATCCCTCATTCTCTAATA	CCCAAAAAAAAACTACTAAC
HDAC9	cg16457652	[Btn]GTTGTTAGAGAAGGATATTTGTTAAGA	ACTAACAAAACTTCCCTCTCATTATAAAA	ACAAAAAATTCCCTTATAACTC
IL24	cg04008201	AATTTAGGTTTTATTTTATTTTAGGGGTTG	[Btn]AATCACCCATCACTTTAAAAACAATACA	TTTATTTTATTTTAGGGGTTGT
CD82	cg20498089	GGTGAGAGAGAGGAAAAATAATAGT	[Btn]CCAAACCTAAAAAAAAAAAAACATAACTTC	GGTATTGAAGATTTTTGGTT

3.6 DNA methylation profiling using universal bead arrays

Infinium MethylationEPIC BeadChip (Illumina) arrays were used to analyse DNA methylation. This platform allows > 850,000 methylation sites per sample to be interrogated at single-nucleotide resolution, covering 99% of reference sequence (RefSeq) genes. The samples were bisulfite-converted using EZ DNA Methylation-Gold™ Kit (Zymo Research) and were hybridized in the array following the manufacturer's instructions.

Each methylation data point was obtained from a combination of the Cy3 and Cy5 fluorescent intensities from the M (methylated) and U (unmethylated) alleles. For representation and further analysis, beta and M values were used. Beta value is the ratio of the methylated probe intensity to the overall intensity (the sum of the methylated and unmethylated probe intensities). The M value is calculated as the log2 ratio of the intensities of the methylated versus unmethylated probe. Beta values range from 0 to 1, in which 0 is no methylation and 1 is complete methylation and were used to derive heatmaps and to compare DNA methylation percentages from bisulfite pyrosequencing experiments. For statistical purposes, the use of M values is more appropriate.

3.7 Quality control, data normalization and detection of differentially methylated and variable CpGs

Methylation array data were processed in the statistical language R using methods from the Bioconductor libraries minfi and limma. Data quality was assessed using the standard pipeline from the minfi package [267]. The data were Noob-normalized (sepsis array) or Illumina-normalized (*in vitro* model) and after normalization, beta and M values were calculated. To exclude technical and biological biases, we develop pipeline with several filters as removing CpGs with sex-specific methylation or with SNPs overlapped. To minimize the potential confounding influence of age and gender, we used these parameters as covariates.

In patients' study, we considered a probe to be differentially methylated if it had a differential methylation of 15% ($\Delta\beta \geq 0.15$) and when the statistical test was significant [*p*-value < 0.01 and false discovery rate (FDR) < 0.05]. For *in vitro* experiments, a probe is considered to be differentially methylated if it has ≥ 20% difference in β-value and with *p*-value < 0.01 and FDR < 0.05. In addition, we use the iEVORA algorithm [268,269] to designate a probe as differentially variable (DVP). This algorithm detects the homogeneity of variances using the Bartlett's test (FDR < 0.001) and then selects those probes whose t-test is significant (*p*-value < 0.05 and FDR < 0.05) in order to regularize the variability test which is overly sensitive to single outliers.

Spearman's correlation was used to correlate methylation changes with cytokines concentration and SOFA score. Spearman's correlation coefficient is a nonparametric approach to measuring the strength of association of two variables being more reliable with non-linear data. CpG sites for which Spearman's correlation coefficient (rho) was higher than 0.5 and had a correlation *p*-value < 0.01 were selected for further analyses.

3.8 Gene ontology analysis

Gene ontology (GO) of DNA methylation data was carried out using the Genomic Regions Enrichment of Annotations Tool (GREAT, version 3.0.0). GREAT assigns biological meaning to a set of non-coding genomic regions by analysing the annotations of the nearby genes [270]. For gene identification, each gene was assigned a regulatory domain consisting of a basal domain that extends 5 kb upstream and 5 kb downstream from the differentially methylated CpG site. Enrichment is shown as $-\log_{10}$ raw binomial *p*-values.

3.9 Transcription factor enrichment analysis (HOMER)

Motif enrichment analyses were performed using HOMER motif discovery software to look for motifs that are over-represented in the target set relative to the background set (software v4.5) [271]. It was used to identify enrichment of TF binding motifs in the 500 bp-window upstream and downstream of the differentially methylated CpG sites. Annotated CpGs in the EPIC array were used as background.

3.10 Chromatin state discovery and characterization (ChromHMM)

Chromatin state discovery and characterization (ChromHMM algorithm) was used to analyse the enrichment of the different chromatin states for the corresponding CpGs sites [272,273]. The enrichment among chromatin states is defined using the 18-state ChromHMM model (Roadmap Epigenomics Integrative Analysis Hub, ChromHMM track of the UCSC Genome Browser) based on six chromatin marks (H3K4me3, H3K4me1, H3K27ac, H3K36me3, H3K27me3 and H3K9me3). A Fisher's exact test was used to assign odds ratio and *p*-value.

3.11 Analysis of DNase I hypersensitivity and ChIP-seq of histone modifications

DNase I hypersensitivity and ChIP-seq data of histone marks H3K27ac and H3K4me1 of monocytes and monocytes treated with LPS (MO LPS 24h) were downloaded from the BLUEPRINT portal. When more than one replicate was available, the peaks were consolidated, filtering the peaks with a q.value < 0.01 and a fold change >= 2 and using the program MSPC [274] with the parameters -w = 1E-4 and -s =1E-8. For the positional

enrichment graphs, we did overlaps between the genomic positions of the CpG sites of interest and the consolidated histone marks / DNase files, and Fisher's tests comparing with the EPIC background, shifting the CpG list in bins of 10bp, 2500bp upstream and downstream, and plotting the odds ratio of each bin.

3.12 Cytokine measurements

The endotoxin tolerance status of the septic and SIRS patients and healthy donors were evaluated by exposing whole blood to a stimulus of 5 ng/ml LPS (at 37 ºC), collecting supernatant after 3 hours (3000 rpm, 5 min). The cytokines levels from these samples were determined using the cytometric bead array (CBA) Flex Set (BD Biosciences), following the manufacturer's protocol. The collected data were analysed with FCAP Array Software v3.0 (BD Biosciences).

For *in vitro* experiments, the concentration of cytokines (TNFα and IL-10) was measured from the cell culture supernatants using an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (BioLegend).

In addition, the Pre-defined Human Inflammatory Panel LegendPlex™ (BioLegend) was used for the simultaneous analysis of 13 cytokines (CCL2, IFN-α2, IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33 and TNFα) related to inflammation in the same samples according to the manufacturer's instructions.

3.13 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Cells were homogenised in 200 µl of homogenization buffer and total RNA was isolated by Maxwell® RSC simplyRNA Cells Kit (Promega). The amount and purity of RNA was defined by measuring absorbance at 260nm and 280nm with Nanodrop 1000 spectrophotometer. Then, RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to manufacturer's instructions. cDNA synthesis was carried out by anchored oligo $(dT)_{18}$ primer, which binds at the beginning of the poly(A) tail of mRNAs.

Then, quantitative PCR (qPCR) was performed in triplicate using LightCycler® 480 SYBR Green Mix (Roche). qPCR was carried out using LightCycler® 480 II System (Roche) and results were analysed using the corresponding LightCycler® 480 Software (Roche). Standard double delta Ct method was used to determine the relative quantification of target genes and values were normalized against the expression of endogenous gene controls as *RPL38*. Primers sequences are listed in Table 3.

Table 3. List of qPCR primers.

	Forward Primer	Reverse primer
CCL20	TTTATTGTGGGCTTCACACG	GATTTGCGCACACAGACAAC
IL24	ACACAGGCGGTTTCTGCTAT	TCTGCATCCAGGTCAGAAGA
IL2RA	CATGGCCTACAAGGAAGGAA	TGGACTTTGCATTTCTGTGG
IL15	CTGCAGCCAGGACTCGAT	TTTCTGTGCTCCATGTGACG
IL1B	AGCTGATGGCCCTAAACAGA	GGAGATTCGTAGCTGGATGC
IL6	CTCAGCCCTGAGAAAGGAGA	TTTCAGCCATCTTTGGAAGG
RPL38	TGGGTGAGAAAGGTCCTGGTC	CGTCGGGCTGTGAGCAGGAA
IL36G	GGGCCGTCTATCAATCAATGTG	GGGTCACACTGTCACTTCGT
TM4SF19	TGGCACTCCTCCTTCCTAAC	GCAAATCAGGGCTCCAAGTA
RNF13	GGCCGGACTTCAAGGTGATT	AGCTGGACAGTCAAGATGGTG
CPM	TTCTTGTTGTGGGGCGGTTT	CCGCCCAACAGTCTCATCTC

3.14 Protein extraction and western blot

Nuclear and cytoplasmic protein fractions were obtained using hypotonic solution buffer (Buffer A; 10 mM Tris pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl supplemented with protease inhibitor cocktail (Complete, Roche) and phosphatase inhibitor cocktail (PhosSTOP, Roche)) to lyse the plasmatic membrane. Samples were homogenised in buffer A and incubated with rotation for 10 minutes at 4ºC. Then, we centrifuged 30'' at > 13000 rpm and collected the supernatant (cytoplasmic fraction). Finally, pellets were washed three times with 1 mL of Buffer A consisting of 5 minutes of incubation and centrifugation in order to discard cytoplasmic leftovers. Protein pellets were resuspended in loading buffer Laemmli 1X (Laemmli 2X: 0.125 M Tris-HCl pH 6.8, 4% SDS, 30% glycerol, 0.02% bromophenol blue). Proteins were sonicated for 2x10 seconds with the ultrasonic processor UP50H (Hielscher).

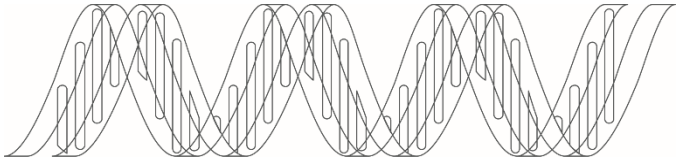
After that, proteins were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) using 10% acrylamide gels. Immunoblotting was performed onto polyvinylidene difluoride (PVDF) membranes according to standard procedures. Then, membranes were blocked with 5% of bovine serum albumin (BSA) and blotted with primary antibodies listed in Table 4. After overnight incubation, membranes were washed three times for 10 minutes with TBS-T (50mM Tris, 150mM NaCl, 0.1% Tween-20) and incubated 1 hour with HPR-conjugated mouse or rabbit secondary antibody solutions (Thermo Fisher) diluted in 5% milk (dilution 1/10000). At last, proteins were detected by chemiluminescence using WesternBright™ ECL (Advansta).

Table 4. List of antibodies used for western blot (WB).

Antibody	Company	Reference
Phospho-STAT3 (Tyr705) (D3A7) Rabbit mAb	Cell Signaling Technology	#9145
Phospho-STAT5b (Tyr694) (C11C5) Rabbit mAb	Cell Signaling Technology	#9359
Phospho-STAT1 (Tyr701) (58D6) Rabbit mAb	Cell Signaling Technology	#9167
Phospho-STAT6 (Tyr641) Antibody	Cell Signaling Technology	#9361
STAT3 (79D7) Rabbit mAb	Cell Signaling Technology	#4904
STAT5B	Millipore	#06-969
STAT1(42H3) Rabbit mAb	Cell Signaling Technology	#9175
LaminB1	Abcam	#ab65986

3.15 Statistical analysis

Data were analysed with Prism version 6.0 (GraphPad). Statistical analyses were performed using the non-parametric Mann-Whitney U test to determine differences between two non-paired group and the paired student *t*-test to compare the means of two matched groups, except as indicated. The levels of significance were as follows: **p*-value < 0.05, ***p*-value < 0.01, ****p*-value < 0.001.



RESULTS

4. RESULTS

The results of this doctoral thesis are presented in two separate sections that correspond to the two parallel studies carried out to investigate the relationship between the acquisition of endotoxin tolerance and DNA methylation changes, as well as their potential functional implications, directly related to the two main objectives, previously presented. Firstly, we studied the potential occurrence of DNA methylation changes following sepsis and how these changes might modulate innate responses against pathogens. Secondly, we investigated the potential DNA methylation alterations following *in vitro* PRR stimulation. With these two approaches, we intended to provide new knowledge on the mechanisms underlying these DNA methylation changes and their association with specific signalling pathways and gene expression programs.

4.1 DNA methylation profile of septic monocytes and their contribution to an endotoxin tolerance state

To explore the relationship between DNA methylation and endotoxin tolerance in the context of human pathological conditions, we decided to study patients with sepsis, which present a secondary hypo-responsive state similar to *in vitro* tolerized monocytes/macrophages. In this study, we also included patients with systemic inflammatory response syndrome (SIRS), which also present systemic inflammation, but without bacterial infection. SIRS patients were obtained from critical patients following the immediate postoperative period of cardiac surgery. The study of the possible role of DNA methylation in these pathologies is crucial because the mechanisms and specific signalling in relation to tolerance induction are poorly understood.

4.1.1 Monocytes from patients with sepsis display an aberrant DNA methylation profile

Firstly, peripheral blood monocytes were sorted as CD14+ CD66b- cells from septic patients, SIRS individuals following cardiac surgery and healthy controls (Figure 13A, Table 1). Using this cell sorting strategy, we ensured not to have contamination with other cell types, especially, with neutrophils. In pilot experiments we had noticed increased contamination with neutrophils due to enhanced CD14 expression in these cells after infection. Therefore, it became necessary to exclude that lineage by using CD66b, in addition to using positive selection with CD14.

To investigate the potential occurrence of DNA methylation alterations in these monocytes, we performed DNA methylation screening using EPIC arrays from Illumina. This strategy allows interrogating over 850,000 CpG sites of the genome (around 3% of

the total number of CpG sites in the human genome). Following hybridization, principal component analysis (PCA) showed samples from healthy controls and patients with sepsis separated along the axis of principal component 1 with SIRS patients between them (Figure 13B). In addition, a wider heterogeneity was observed in septic monocytes perhaps due to the diversity of infective bacteria. We identified 897 CpG sites that were differentially methylated (p-value < 0.01, FDR < 0.05, $\Delta\beta \geq 0.15$). Specifically, the analysis of the DNA methylation changes in septic monocytes with respect to those from healthy controls revealed the existence of two clusters: a group of 595 CpGs corresponding to sites that display gains of methylation (hypermethylation) and 302 changes that comprise CpGs that undergo loss of DNA methylation (hypomethylation) (Figure 13C).

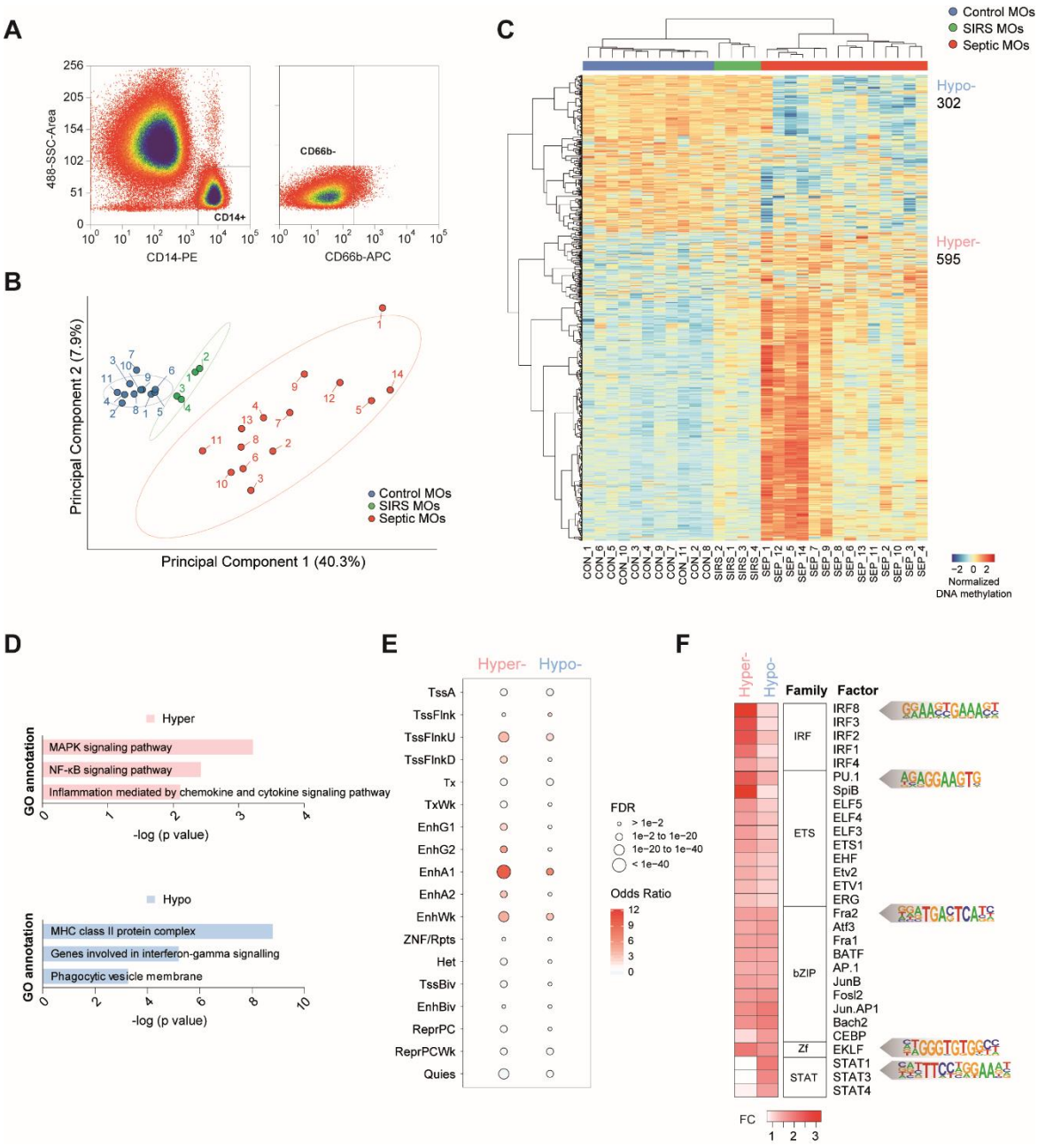
We then performed gene ontology (GO) analysis using the GREAT tool to identify the processes in which genes related to the found CpGs are involved. GO analysis of the hypermethylated CpGs revealed enrichment in MAPK signalling pathway, NF-kB signalling pathway and inflammation mediated by chemokine-cytokine signalling pathway. In parallel, for the hypomethylated cluster, MHC class II protein complex, genes involved in interferon-gamma signalling and phagocytic vesicle membrane were enriched (Figure 13D).

The analysis of the chromatin states of differentially methylated CpG sites revealed an enrichment in active and weak enhancers of both hyper- and hypo-methylated sets, characterized by H3K4me1 and H3K27ac marks (Figure 13E). These results are consistent with previous studies of our group and others [121,275,276], which have shown that changes in this lineage mainly take place far from promoters.

We also investigated the enrichment of TF binding motifs in 500 bp windows around the CpG sites of these two clusters displaying differential methylation. We found an overrepresentation of binding sites for IRF and ETS families in hypermethylated CpGs (Figure 13F). Previous reports have described that PU.1, a member of ETS family, can interact with DNMT3B [119] and could also be involved in endotoxin response [277]. In the case of the hypomethylated set, we observed an enrichment of binding motifs of the STAT family (Figure 13F). Previously, it has been described that STAT pathway has a crucial role in controlling cytokine responses during sepsis [278].

Finally, upon further inspection of the individual genes, we identified essential genes for the immune response against pathogens in those containing differentially methylated CpG sites (Table 5). For instance, we observed hypermethylation in the pro-inflammatory cytokine IL-1A, in the chemokine CCL22 and in CCR2 receptor, which are

associated with leukocyte chemotaxis and in gene encoding *STAT3*, crucial member for JAK/STAT signalling. Furthermore, hypomethylation was found in genes such as *HLA-A*, which is involved in antigen presentation, in the suppressor of cytokine signalling 1 (*SOCS1*), in the interleukin 1 receptor type 2 (*IL1R2)* or in the co-stimulatory molecule CD46, that suppress the immune response through IL-10 (Figure 13G). These examples emphasize the relationship between genes differentially methylated in sepsis and pathways connected to endotoxin tolerance and innate response.



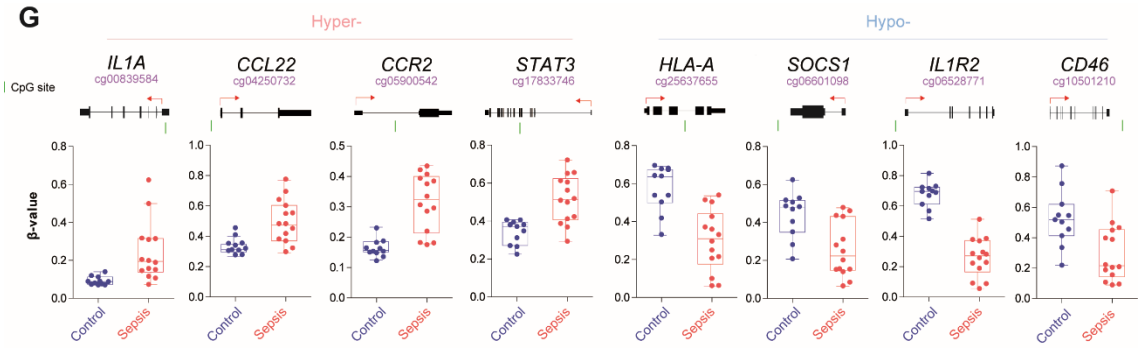


Figure 13. Global analysis of DNA methylation changes in septic monocytes. (A) Representative flow cytometry profiles indicating the sorting strategy and gates used in this study. Monocytes (CD14+ CD66b-) were sorted from healthy controls and patients (SIRS and sepsis). (B) Principal Component Analysis (PCA) of methylation heatmap data for control, SIRS and septic monocytes (in blue, green and red respectively). (C) DNA methylation heatmap showing differentially methylated CpGs between controls (CON, blue) and patients with sepsis (SEP, red). The heatmap includes all CpG-containing probes displaying significant methylation changes (15% of differential of β-values, *p*-value < 0.01 and false discovery rate (FDR) < 0.05). A scale is shown at the bottom right ranging from -2 (lower DNA methylation levels, blue) to +2 (higher methylation levels, red). (D) Gene ontology (GO) analysis of genes associated with differentially methylated CpGs sites showing the most relevant and significantly enriched categories resulting from the Genomic Regions Enrichment of Annotations Tool (GREAT). (E) Enrichment analysis of the different chromatin states for CpG sites corresponding to each methylation cluster (left to hypermethylation, right to hypomethylation). The relative enrichment of the different states is represented using the odds ratio. Dot size represents the FDR value. (F) TF binding motif analysis of differentially methylated CpGs between control and sepsis. The panel shows fold change (FC), transcription factor (TF) family and factor (selected TF with $p \leq 1e^{-05}$ for hypermethylated regions and $p \leq 1e^{-03}$ for hypomethylation). Motif logo is representative of the TF family. (G) Boxplots showing β-values obtained from the DNA methylation array. We observed hypermethylation and hypomethylation at important loci of immune system genes. CpG sites are marked with a green line in the gene scheme placed on top of each graph, where the TSS is marked with a red arrow.

Table 5. List of differentially methylated genes in septic monocytes.

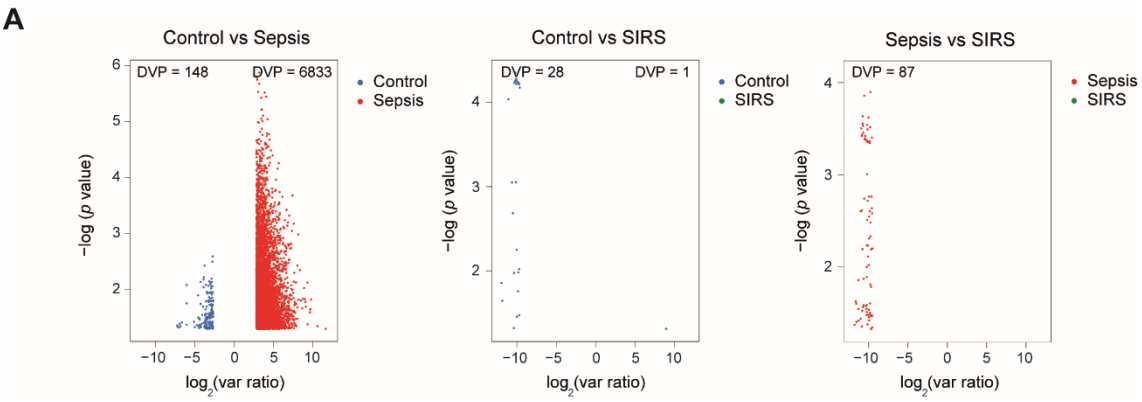
Gene	CpG ID	Position respect TSS	ΔBeta value	Relevance of gene in immune system
CLECL1	cg20894963	+331	0.21	C-type lectin-like protein that mediates immune cell-cell interactions.
TNFSF8	cg14107762	+2071	0.19	Pro-inflammatory cytokine that is a member of the tumor necrosis factor family and participates in defense response.
PIK3R5	cg19732273	+12206	0.20	Regulatory subunit of PI3K (phosphoinositide 3-kinase) complex. PI3Ks participate in Akt/mTOR signaling pathway that play important roles in differentiation, survival, cell growth or metabolism.
RNF216	cg07010622	+98729	0.26	E3 ubiquitin ligase that participates in protein modification; it also takes part in down-regulating TNF- and IL1- induced NFκB activation.
CCL22	cg04250732	-1029	0.16	C-C cytokine that display chemotactic activity for monocytes, dendritic cells, activated T-lymphocytes and natural killer cells to inflammatory sites.
LTA	cg00983060	-8641	0.18	Protein that belongs to the tumor necrosis factor family and could regulate inflammatory, defense and apoptotic processes.
RUNX3	cg27058497	-46	0.17	Runt-related transcription factor 3 is a member of the runt domain-containing transcription factor family that can activate or suppress the transcription of their targets through recognizing consensus DNA binding sequence 5'-PYGPYGGT-3'.
IL1A	cg00839584	+76	0.15	This cytokine is produced by monocytes and macrophages. It is a member of the interleukin 1 cytokine family which are mainly involved in inflammatory response.
IRF2	cg11802666	+50027	0.18	Encodes a member of Interferon regulatory transcription factor family. IRF2 represses IRF1-mediated transcriptional activation.
MAP3K1	cg25148456	-1861	0.17	Mitogen-activated protein kinase kinase kinase 1 acts as serine/threonine kinase activating ERK and JNK kinase cascades as well as NFκB pathway.
CD93	cg19010566	+4181	0.22	Membrane receptor that may play a role in phagocytosis in monocytes and macrophages.
CCR2	cg05900542	+2623	0.15	C-C chemokine receptor type 2 specifically mediates leukocyte chemotaxis leading to cell infiltration during inflammation processes.
CXC4R	cg07784959	+404	0.19	CXC chemokine receptor that mediates LPS-induced inflammatory responses.
STAT3	cg17833746	+50801	0.17	Signal transducer and transcription activator that mediates cellular responses to interleukins.
PRKCB	cg26562691	+3060	0.22	Serine-threonine protein kinase that mediates the activation of the canonical NFκB pathway.
IL1R2	cg06528771	-94321	0.41	Interleukin-1 receptor type II acts a decoy receptor preventing activity of IL1B or IL1A cytokines.
CD46	cg10501210	+71619	0.23	CD46 acts as a costimulatory factor for T lymphocytes promoting T-regulatory 1 cells which suppress immune response through IL-10.
HLA-A	cg25637655	+2506	0.27	HLA-A belongs to the Major Histocompatibility Complex (MHC) Class I and plays a central role presenting foreign antigens to the immune system.
ZAK	cg22093378	+75749	0.18	It is also known as mitogen-activated protein kinase kinase kinase 20 (MAP3K20). ZAK regulates JNK and p38 pathways through its kinase activity.
SOCS1	cg06601098	+30946	0.18	This gene encodes a member of suppressor of cytokine signaling (SOCS) family that is involved in negative regulation of JAK/STAT cytokine signaling.
HLA-C	cg13872627	+1827	0.16	It is a member of the MHC Class I molecules, responsible of antigen presentation during the immune response.
IL22	cg24596116	-17931	0.15	Interleukin 22 is a cytokine member of IL10 family involved in the inflammatory response through JAK/STAT and MAPKs pathways.
STAT4	cg04128669	-51040	0.20	Signal transducer and transcription activator that mediates cellular responses to interleukins.
S100A8	cg04681218	+6180	0.22	Member of the S100 protein family which has an important role in the regulation of inflammatory processes.

4.1.2 Septic monocytes exhibit increased DNA methylation variability

Given the intrinsic heterogeneity of septic cohort, we studied DNA methylation variability in our samples using a recently developed algorithm named iEVORA [268,269]. We observed a considerable and significant higher number of differentially variable CpG positions (DVPs) in septic patients (n=6833) compared to healthy controls (n=148) (Figure 14A).

Additionally, sepsis-associated DVPs mainly occurred at enhancers and TSS flanking regions (Figure 14B) and sequences surrounding these DVPs were enriched for binding motifs of ETS and IRF families, highlighting the importance of these TF families in the tolerized phenotype of septic monocytes (Figure 14C).

Then, GO analysis of these sepsis-associated DVPs revealed an enrichment in functional categories such as positive regulation of inflammatory response, leukocyte chemotaxis, defence response to bacterium and toll-like receptor binding (Figure 14D). For example, genes like *IL1A* and *TNF* display a wider methylation range in septic samples (Figure 14E). Overall, this increased DNA methylation variability in septic patients compared to healthy donors suggests that diverse factors, including the type of infection, the cytokine environment or the genetic background, could influence DNA methylation pattern of monocytes, which are highly plastic cells in response to a changing environment.



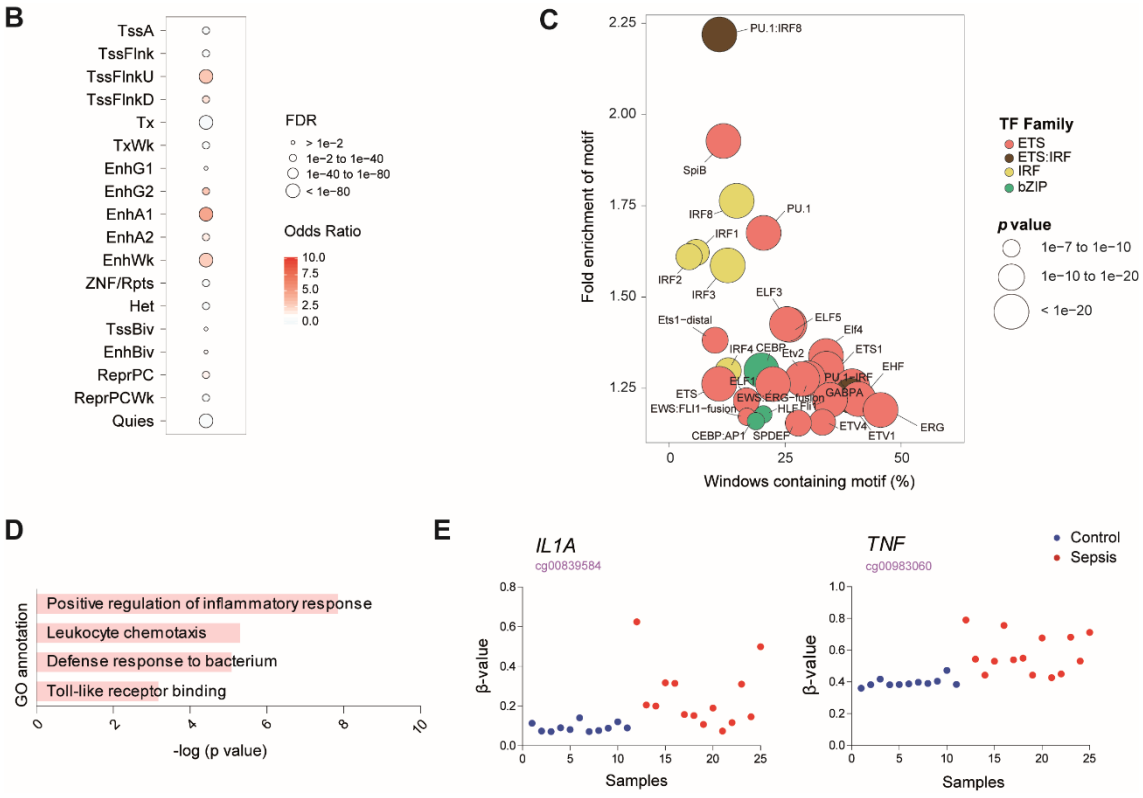


Figure 14. Analysis of differentially variable CpG positions (DVPs) in monocytes from patients with sepsis. (A) Volcano plot showing the *p*-value vs the variance ratio for healthy control, SIRS and sepsis-associated DVPs. DVPs were identified using iEVORA algorithm. (B) Enrichment of sepsis-associated DVPs at 18 distinct chromatin states using ChromHMM. (C) TF binding motif analysis for sepsis-associated DVPs. Bubbles are coloured according to TF family. The *p*-value is indicated by bubble size (selected TF with $p \leq 1e^{-07}$ for DVP regions). (D) GO categories resulted from GREAT analysis for sepsis-associated DVPs of section (A). (E) DNA methylation plot of representative examples displaying DNA methylation variability in sepsis.

4.1.3 DNA methylation changes in monocytes from septic patients correlate with increased IL-10 and IL-6 levels

Sepsis compromises host immune response, and, for that reason, some cytokines related to such dysregulated response were measured in whole blood of patients with sepsis and compared to healthy controls. We also examined the levels of cytokines following exposure to LPS (t = 3h) to study the acquisition of a tolerant state. These analyses showed a significant increase in IL-10 and IL-6 basal levels in septic patients and the acquisition of a tolerogenic phenotype in sepsis, with reduced TNFα, IL-6 and IL-1B levels and enhanced IL-10 cytokine following LPS stimulation (Figure 15A).

Given that septic patients display altered release of cytokines, we investigated the potential relationship between the observed DNA methylation changes and the levels of cytokines. To this end, we performed Spearman's correlation analysis between DNA methylation and IL-10 or IL-6 basal levels, which are increased in septic patients. In the

case of IL-10, we observed 855 hypermethylated CpGs and 389 CpGs that undergo hypomethylation changes in relation to increasing IL-10 levels (*p*-value < 0.01; r > 0.5, Δβ ≥ 0.15) (Figure 15B); and for these CpG sites, there was a significant difference of their median DNA methylation levels between septic and healthy controls (Figure 15C). Spearman's analysis for IL-6 cytokine revealed 2492 sites that display higher methylation levels and 909 CpGs that were hypomethylated in relation to IL-6 levels (*p*-value < 0.01; r > 0.5, Δβ ≥ 0.10) (Figure 16A).

Some of the CpGs correlating with IL-10 and IL-6 levels were located at or near genes with biological importance in relation to immune responses. GO analysis identified significant enrichment in categories related to fibroblast growth factor-activated receptor activity or chemokine receptor binding for hypermethylated IL-10 and IL-6 regions respectively and to MHC protein complex and interferon signalling for both hypomethylated CpG sets (Figure 15F and 16B).

Interestingly, our DNA methylation data related to these cytokines showed hypermethylation in many genes related to Wnt signalling pathway (*WNT3A*, *WNT6* and *AXIN2* among others), highlighting the importance of this route in sepsis. In fact, it has been recently described the role of Wnt signalling during inflammation and sepsis, in which Wnt family members can regulate the macrophage-mediated immune response [279,280]. Moreover, we found hypermethylation in the protocadherin (*PCDH*) gene cluster, which can regulate Wnt pathway [281] (Figure 15G). Regarding CpG sites hypomethylated in relation to IL-10 levels, we found an enrichment in genes involved in interferon signalling, which is essential for antimicrobial defence and restoring metabolic monocyte deactivation in patients with sepsis [282]. Finally, we identified a large genomic region within the HLA cluster, which is also related to IFN-γ and JAK/STAT pathway [283,284] (Figure 15G).

CpG sites displaying changes in DNA methylation in relation to IL-10 and IL-6 levels enriched for specific chromatin features. Specifically, we found an enrichment in enhancers for both hyper- and hypo- methylated regions with also TSS flanking upstream overrepresentation for hypermethylated IL-6 set (Figure 15D and 16C), suggesting that methylation-sensitive enhancers could be potentially regulating the gene expression.

Finally, the analysis of TF binding motifs revealed the enrichment of various sets of TFs in the IL-10-related CpGs. We observed a significant enrichment in basic leucine zipper (bZIP), IRF and ETS families for hypermethylated regions, which are all effectors downstream of cytokine stimulation. The hypomethylated set included CpGs that displayed enrichment in motifs of the ZF and STAT families, which also control immune

functions relevant to cytokine signalling (Figure 15E). Similar results were found in IL-6 related CpGs (Figure 16D). Altogether, these observations demonstrate changes in DNA methylation of monocytes related to the inflammatory environment in the blood of septic patients.

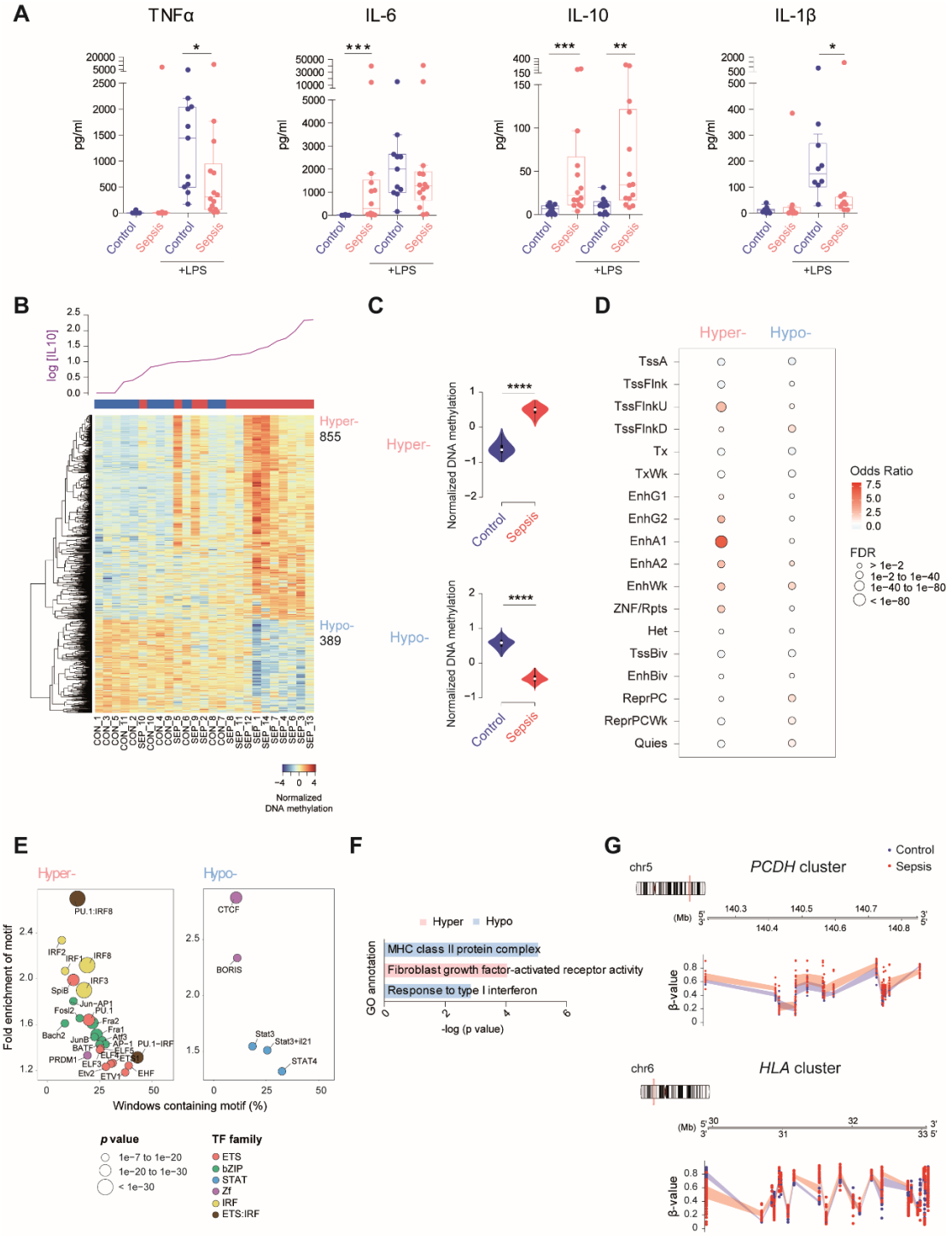
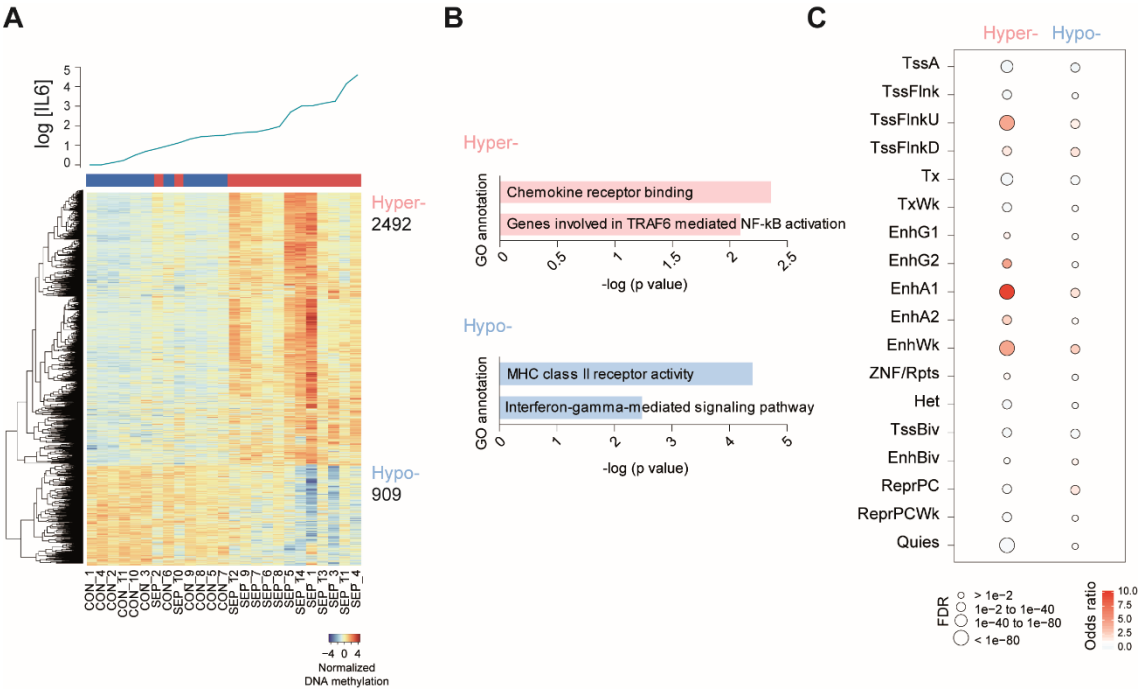


Figure 15. DNA methylation changes in septic monocytes parallel the increase of IL-10 levels. (A) Cytokine measurement using cytometric bead array (CBA) from control and septic

PBMCs before and after LPS stimulation (t=3h). Box and whiskers plots show median values. Mann-Whitney U test was used to determine significance (*p < 0.05, **p < 0.01, and ***p < 0.001). (B) DNA methylation heatmap of CpGs changes in relation to IL-10 basal concentration (represented on the top of the heatmap as log scale). Spearman's correlation was used with $p <$ 0.01, r > 0.5 and Δβ ≥ 0.15. A scale is shown at the bottom, wherein β-values range from −4 (lower DNA methylation levels, blue) to +4 (higher methylation levels, red). (C) Violin-plots corresponding to the 5mC-normalized data for control and sepsis presented in the heatmap in the previous section. The median and the interquartile range are represented. (D) Chromatin state characterization of differentially methylated sites for section (B). The relative enrichment of the different state assignments is representing using the odds ratio. FDR is represented by the size of the dots. (E) Bubble-scatterplot of TF enrichment for hypermethylated and hypomethylated CpGs. The x-axis shows the percentage of windows containing the motif and the y-axis shows the fold enrichment of the motif. Bubbles are coloured according to TF family. *p*-value is indicated by bubble size (selected TF with $p < 1e^{-07}$ for hypermethylated and hypomethylated regions). (F) GO categories resulted from GREAT analysis of differentially methylated CpGs related to IL-10 concentration. (G) Genomic tracks representing the clusters of differentially methylated CpGs for protocadherins (*PCDH*, upper scheme) and human leukocyte antigen (*HLA*, lower panel). Blue and red lines represent the confidence intervals for each average value.



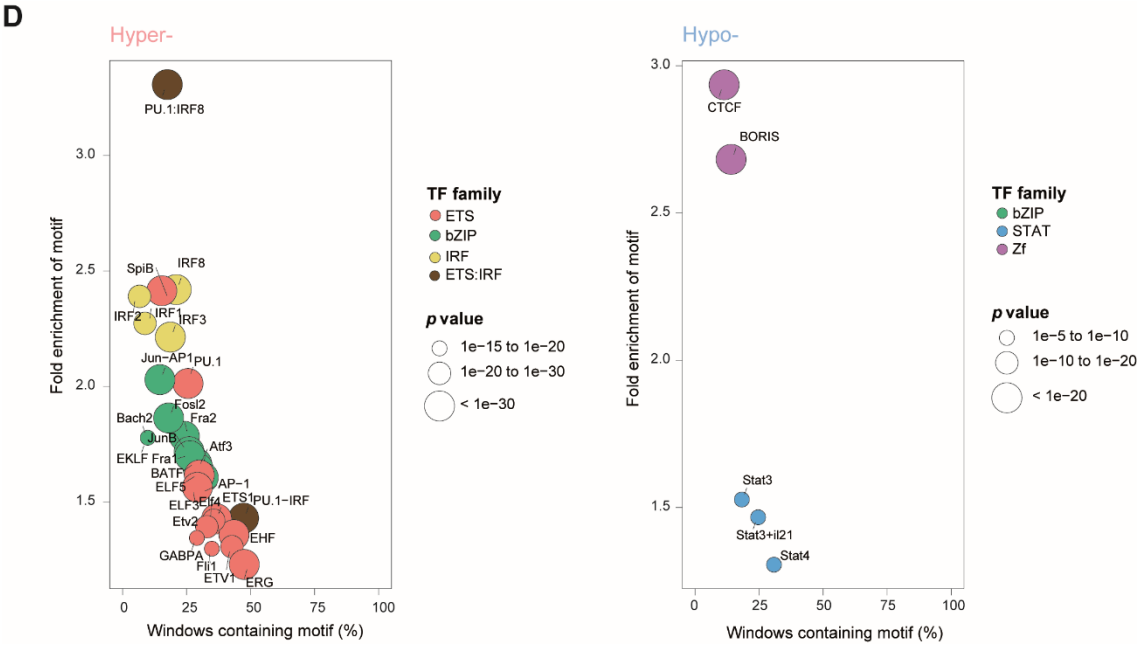


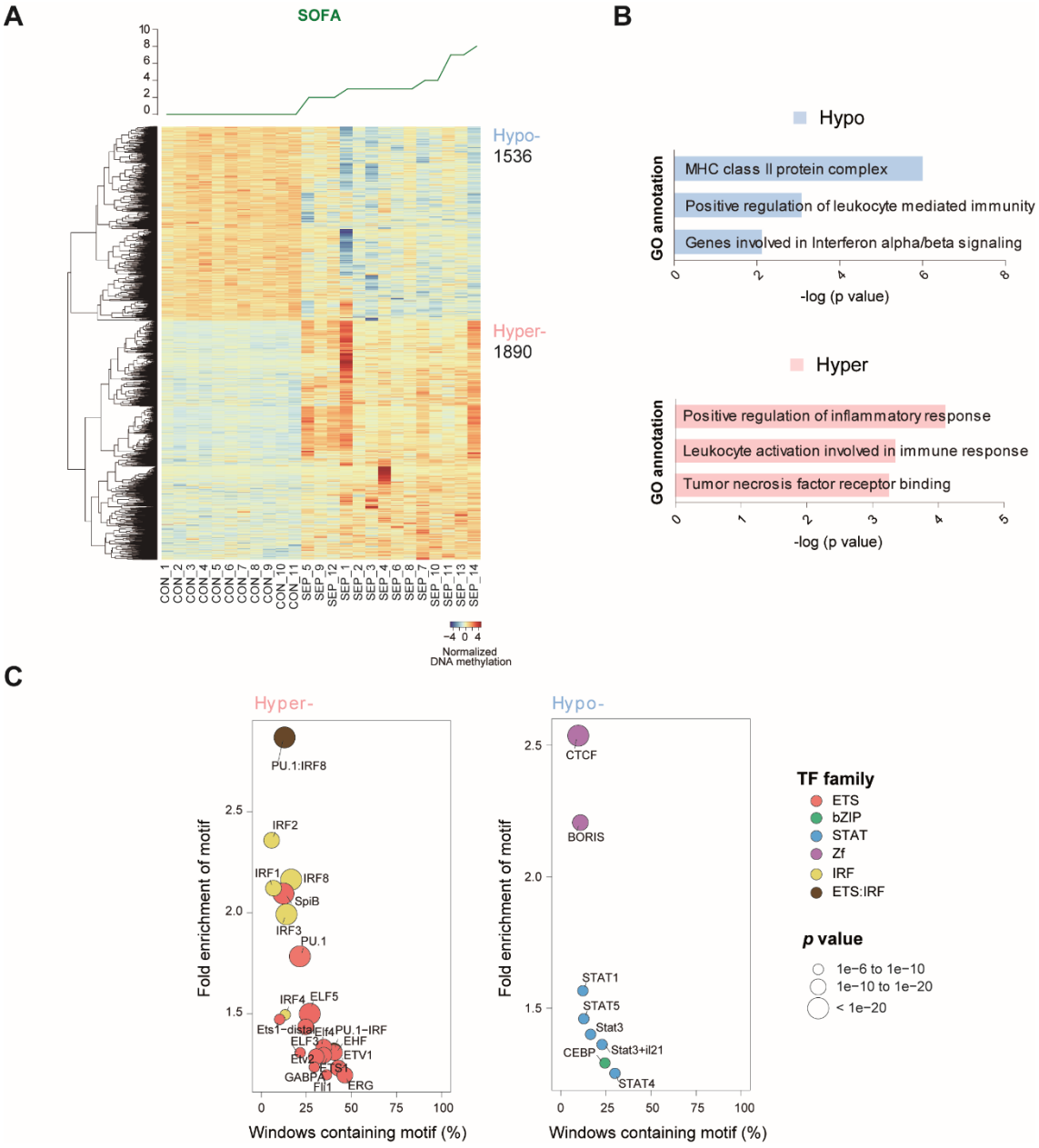
Figure 16. DNA methylation changes in septic monocytes parallel the increase of IL-6 levels. (A) DNA methylation heatmap of CpGs changes in relation to IL-6 basal concentration. Spearman's correlation was used with $p < 0.01$, $r > 0.5$ and differential β-value ≥ 0.1. A scale is shown at the bottom, wherein β-values range from −4 (lower DNA methylation levels, blue) to +4 (higher methylation levels, red). (B) GO categories for differentially methylated CpGs of section (A). (C) Enrichment of differentially hyper- and hypo-methylated CpGs among chromatin states, defined using the 18-state ChromHMM model. (D) HOMER motif analysis for methylation changes. The x-axis shows the percentage of windows containing the motif and the y-axis shows the fold enrichment of the motif. Bubbles are coloured according to TF family. *p*-value is indicated by bubble size (TF with $p < 1e^{-15}$ for hypermethylated regions and $p \leq 1e^{-5}$ for hypomethylation were represented).

4.1.4 Organ dysfunction associates with DNA methylation changes

Since 2016, clinicians have expanded the use of the Sequential [Sepsis-related] Organ Failure Assessment (SOFA) score for the diagnosis of sepsis. This score indicates the degree of organ dysfunction. Patients are nowadays diagnosed with sepsis when their SOFA score is 2 points or more, and the value increases for those patients having a worse prognostic. Therefore, we performed Spearman's correlation analysis to determine the occurrence of significant methylation changes in relation to the SOFA score. We found 1890 hypermethylated CpGs and 1536 hypomethylated CpG sites in relation to increasing levels of SOFA (*p*-value < 0.01; r > 0.6) (Figure 17A). We next performed GO analysis. For the hypermethylated set, we found an enrichment of functional categories involving regulation of inflammatory response and TNF receptor binding while MHC protein complex and genes involved in interferon signalling were overrepresented in the hypomethylated CpG set (Figure 17B).

When we investigated the enrichment of TF binding motifs for these sets of CpGs, we observed similar results to previously described, suggesting again that ETS, IRF or

STAT TF families could be participating in targeting epigenetic machinery to these differentially methylated regions (Figure 17C). Finally, we observed a correlation between the DNA methylation status of several relevant molecules such as HLA-A, IL-19, IL-15 or IL-27 with the organ dysfunction (Figure 17D). These data reflect that monocyte DNA methylome is related to organ damage, estimated by SOFA score.



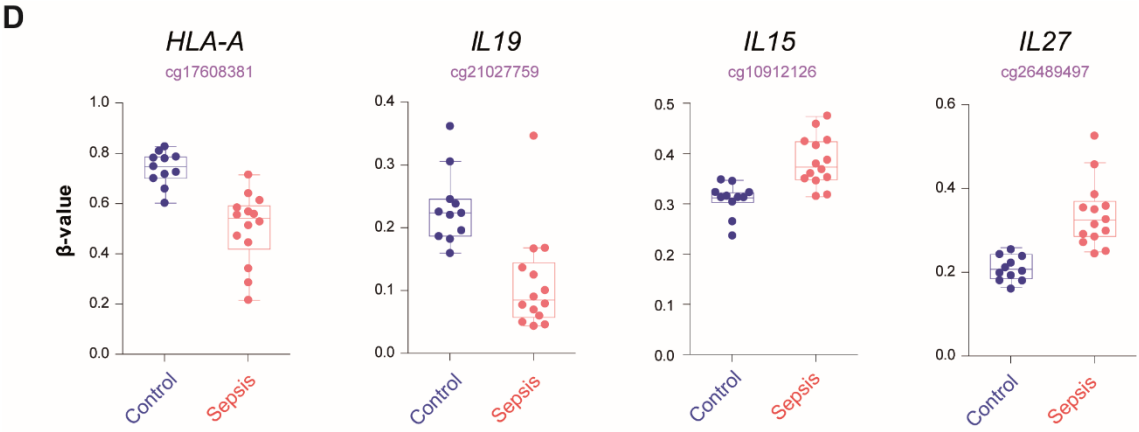


Figure 17. DNA methylation changes in septic monocytes parallel organic damage. (A) DNA methylation heatmap of CpGs changes in relation to the SOFA score (represented on the top of the heatmap). Spearman's correlation was used with $p < 0.01$, $r > 0.6$. A scale is shown at the bottom, wherein β-values range from −4 (lower DNA methylation levels, blue) to +4 (higher methylation levels, red). (B) Representation of selected GO categories resulted from GREAT analysis of differentially methylated CpGs related to SOFA. (C) Bubble-scatterplot of TF enrichment for hypermethylated and hypomethylated CpGs. The x-axis shows the percentage of windows containing the motif and the y-axis shows the fold enrichment of the motif. Bubbles are coloured according to TF family. *p*-value is indicated by bubble size (selected TF with $p \leq 1e^{-06}$ for hypermethylated and hypomethylation regions). (D) Boxplots showing β-values obtained of genes significantly correlating with the SOFA score.

4.1.5 Monocytes exposed to LPS partially recapitulate DNA methylation changes observed in sepsis

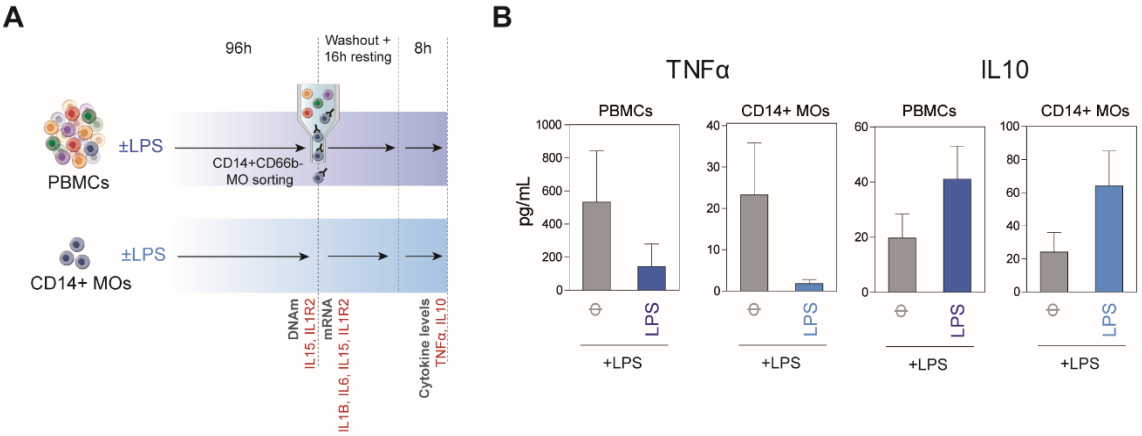
Our data confirmed the existence of DNA methylation changes in monocytes after bacterial infection. In addition, the specific DNA methylation profile is related to TLR stimulation and the resulting inflammatory environment. Thus, we explored the possibility of inducing *in vitro* methylation changes similar to those observed in septic monocytes.

We therefore exposed PBMCs isolated from healthy donors to LPS for 4 days and cultured in plates treated with poly-2-hydroxyethyl methacrylate (Poly-HEMA) to prevent monocyte attachment and minimize subsequent differentiation to macrophages. Previous results from our team and others had shown that differentiation from monocytes to macrophages results in drastic changes in DNA methylation [121,285], which we wanted to minimize for this experiment. Following incubation, monocytes were isolated by sorting CD14+CD66b- cells like in the isolation from patients with sepsis. In parallel, CD14+ monocytes were also exposed to LPS and compare it with cells without such stimulation (Figure 18A). ELISA of TNFα and IL-10 cytokines revealed in both cases the acquisition of tolerance after second LPS stimulation, with reduced levels of TNFα and higher IL-10 levels in those cells that had previous exposure to LPS (Figure 18B).

We next performed pyrosequencing of a selection of hyper- and hypo- methylated genes (*IL15* and *IL1R2* respectively) previously identified in monocytes from patients with sepsis. Here we found that monocytes isolated from PBMCs exposed to LPS underwent gain (*IL15*) or loss (*IL1R2*) of DNA methylation in the direction of those observed in patients with sepsis (Figure 18C). However, in the case of CD14+ monocytes directly exposed to LPS, these changes occur at a lower extent (Figure 18E). These findings suggested that perhaps signals from other blood cell types are necessary to induce in monocytes changes in DNA methylation following LPS treatment, similar to those observed in sepsis.

Interestingly, in these experiments, changes in DNA methylation occurred in association with changes in gene expression of these genes in the two sets of samples exposed to LPS, and not in controls. We inspected the mRNA expression levels of *IL15* and *IL1R2* through qRT-PCR analysis and we observed an enhanced expression in LPS samples. In addition, we also observed an increase in mRNA levels of pro-inflammatory cytokines (*IL-1B* and *IL-6*) in LPS-treated cells, as expected (Figure 18D and 18F).

Altogether, these experiments suggested that the gains and losses of DNA methylation and gene expression in monocytes isolated from patients with sepsis are the results of TLR stimulation and the generation of an inflammatory environment that parallels the acquisition of a tolerized state in these cells.



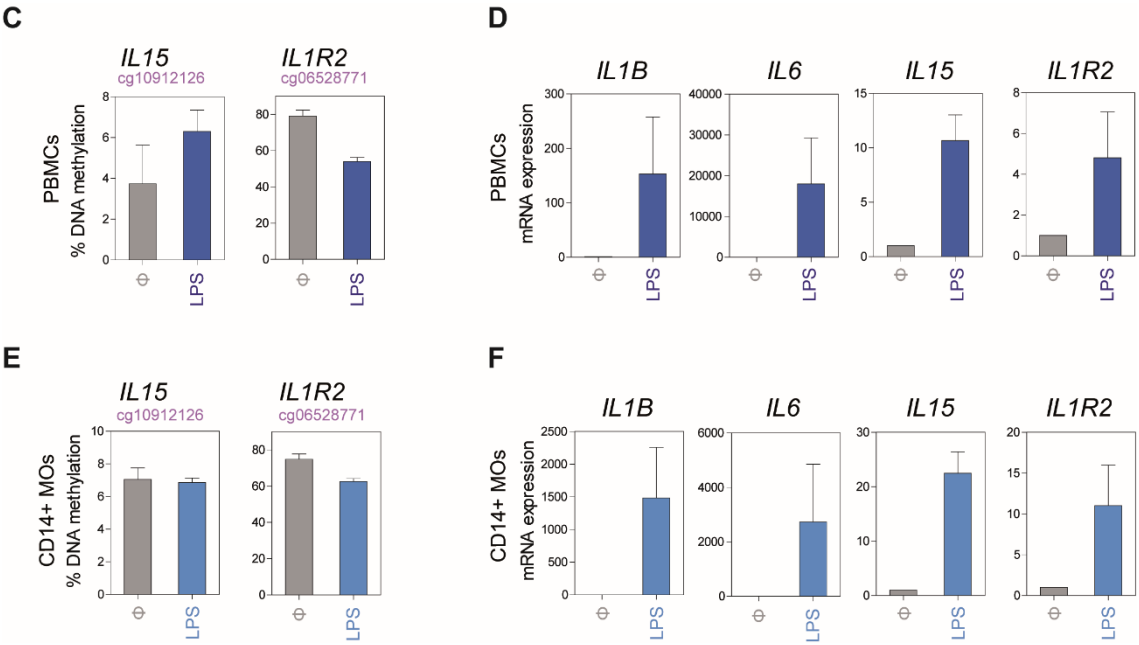


Figure 18. *In vitro* exposure to LPS induces the acquisition of tolerance and DNA methylation changes similar to those observed in sepsis. (A) Schematic diagram depicting our *in vitro* model for sepsis. PBMCs were cultured with or without LPS for 4 days and then, monocytes were sorted as CD14+CD66b- cells for subsequent analyses. In parallel, monocytes were isolated with magnetic CD14 antibody and cultured in the same conditions. (B) TNFα and IL-10 production, as determined by ELISA, from PBMCs (dark blue) or monocyte (light blue) supernatants, following washout and resting following the 4 initial days (with/without LPS) and a 8 h exposure to LPS, as indicated in the scheme in (A). (C) Bisulfite pyrosequencing of selected hypermethylated (*IL15*) and hypomethylated (*IL1R2*) genes in sorted CD14+CD66b- monocytes from PBMCs in the *in vitro* sepsis model. (D*) IL1B, IL6, IL15* and *IL1R2* mRNA levels were analysed by quantitative RT-PCR using *RPL38* as control in the same sorted monocytes. (E) Bisulfite pyrosequencing of *IL15* and *IL1R2* in CD14+ monocytes in the *in vitro* sepsis model. (F) *IL1B, IL6, IL15* and *IL1R2* mRNA levels analysed by qRT-PCR in CD14+ monocytes.

4.2 Role of DNA methylation during innate immune memory processes

The study of monocytes from patients with sepsis allowed the identification of widespread DNA methylation alterations in CpGs biologically relevant in the context of the acquisition of a tolerized state. The relationship between these DNA methylation changes with cytokine levels and organ dysfunction suggested that such changes are relevant to the acquirement of the phenotype. This notion is reinforced by the *in vitro* study included in that section. However, we decided to perform a new *in vitro* study, using a genomic approach, to explore the extent of the DNA methylation changes resulting from the stimulation of PRRs.

4.2.1 TLR stimulation results in specific demethylation during endotoxin tolerance

Several evidences have demonstrated the occurrence of vast changes in the profiles of some histone modifications during processes involving the generation of innate memory.

In parallel, some of these studies have studied the analyses of DNA methylation [286], which have a lower extent than histone modification changes [222]. Given the importance of DNA methylation changes, and its associated enzymatic machinery, in the context of monocyte/macrophage biology, it is relevant to study the downstream effects following the stimulation of PRRs. To investigate the potential role of this epigenetic modification during innate immune memory, we explored two *in vitro* approaches in human CD14+ monocytes isolated from healthy blood donors. On one hand, we pre-incubated monocytes for 24h with lipopolysaccharide (LPS) or Pam3Cys (P3C), which signal through TLR4 and TLR2 respectively, and are known to produce endotoxin tolerance. On the other, we exposed CD14+ cells to a 24h pulse of β-glucan, the main cell wall component of fungal pathogens and recognized by dectin-1 receptor. β-glucan is known to stimulate an enhanced secondary response, termed as trained immunity. In both cases, after the first stimulation, cells were washed and left to rest for 3 days in the presence of human pooled serum. Then, cells were again stimulated with LPS for another 24h (Figure 19A). Monocytes incubated with only RPMI medium were used as negative controls.

Firstly, the levels of TNFα, which is a pro-inflammatory cytokine, were measured by ELISA to interrogate the acquisition of memory in monocytes treated under the conditions mentioned above. We observed that cells previously exposed to LPS or P3C produced lower levels of TNFα (endotoxin tolerance) after second LPS stimulation, while for β-glucan cells, TNFα levels were enhanced (trained phenotype) (Figure 19B).

To obtain DNA methylation profiles of the aforementioned *in vitro* models, we hybridized bead arrays with bisulfite-treated biological triplicates of monocytes treated as indicated above after the 3-day resting period (with LPS, P3C or β-glucan and untreated) and compared with monocytes at day 0. Statistical analysis of the data revealed specific demethylation in LPS- and P3C- treated cells. To identify changes that are specific to TLR stimulation, we established a differential β-value (Δβ) over a threshold for changes that happen during the stimulation with LPS (MO-ML) or P3C (MO-MP) and not occurring in unstimulated cells (MO-MN) (Δβ MO-ML/MO-MP ≥ 0.2 and Δβ MO-MN ≤ 0.1; *p*-value < 0.01, FDR < 0.05). Under these conditions, we identified 260 CpG sites undergoing LPS-specific demethylation (Figure 19C, left panel) and 199 CpG sites displaying P3C-specific demethylation (Figure 20A, left panel). In parallel, we obtained the list of CpG sites that demethylate at a similar extent in both TLR-stimulated and non-stimulated monocytes, due to the differentiation to macrophages. We named those changes as LPS-non specific (n=273) (Figure 19C, right panel) and P3C-non specific (n=308) (Figure 20A, right panel), in comparison with those DNA methylation events that

are TLR-specific. These non-specific changes were set as those with differential β-value ≥ 20% between monocytes and 3-day rested cells (MN, ML, MP) and ≤ 10% differential methylation between 3-day rested cells with or without stimulus (Δβ MO-MN ≥ 0.2, Δβ MO-ML/MO-MP ≥ 0.2 and Δβ MN-ML/MN-MP ≤ 0.1) and *p*-value < 0.01, FDR < 0.05.

In contrast with our findings for TLR stimulation with LPS or P3C, for monocytes trained with β-glucan, we did not observe any significant methylation changes and for this reason, we did not continue studying that model.

Enrichment analysis of chromatin states (ChromHMM) revealed a significant enrichment of different enhancer categories (genic, weak and active enhancers) for specific demethylated sites (Figure 19D and Figure 20B). These results showed that a high proportion of methylation changes occur in regulatory regions and suggest that enhancers could be regulating gene expression associated with these methylation changes.

The analysis of the distribution of these DNA methylation changes to CpG islands revealed that the majority of them occur at open sea areas encompassing gene body and intergenic regions for LPS- and P3C- specific sites (Figure 20C).

We also analysed the enrichment at these LPS-specific demethylated CpG sites of various histone marks using public available monocyte ChIP-seq datasets in monocytes and LPS exposed monocytes from the Blueprint Consortium. We found a significant enrichment in H3K4me1 and H3K27ac (indicative of active enhancers marks) for these differentially methylated regions (Figure 19E). These observations suggest the participation of regulatory elements such as enhancers in this inflammatory response. Interestingly, the comparison between LPS-specific versus LPS-non specific changes in relation to chromatin accessibility data from Blueprint Consortium showed that non specific changes occur in chromatin sites more accessible in monocytes to those that occur in a LPS-specific manner (Figure 19 F). This also occurred to P3C-specific sites in comparison to P3C-non specific changes (Figure 20D). Altogether, these data suggest that TLR-specific demethylation (using LPS or P3C) require a pioneer factor that is able to access to closed chromatin following TLR stimulation to enable such specific demethylation.

Gene ontology (GO) analysis of CpG sites displaying LPS-specific or P3C-specific demethylation revealed the enrichment of functional categories associated with monocyte/macrophage cell biology including inflammatory response, cytokine activity, interleukin-1 receptor binding and response to bacterium for both conditions (Figure 21A, left panel and Figure 20E, top). In contrast, GO of LPS-non specific sites revealed

enrichment in categories like T cell costimulation, cortical cytoskeleton organization or negative regulation of cytokine production, perhaps more related to macrophage differentiation (Figure 21A, right panel and Figure 20E, bottom).

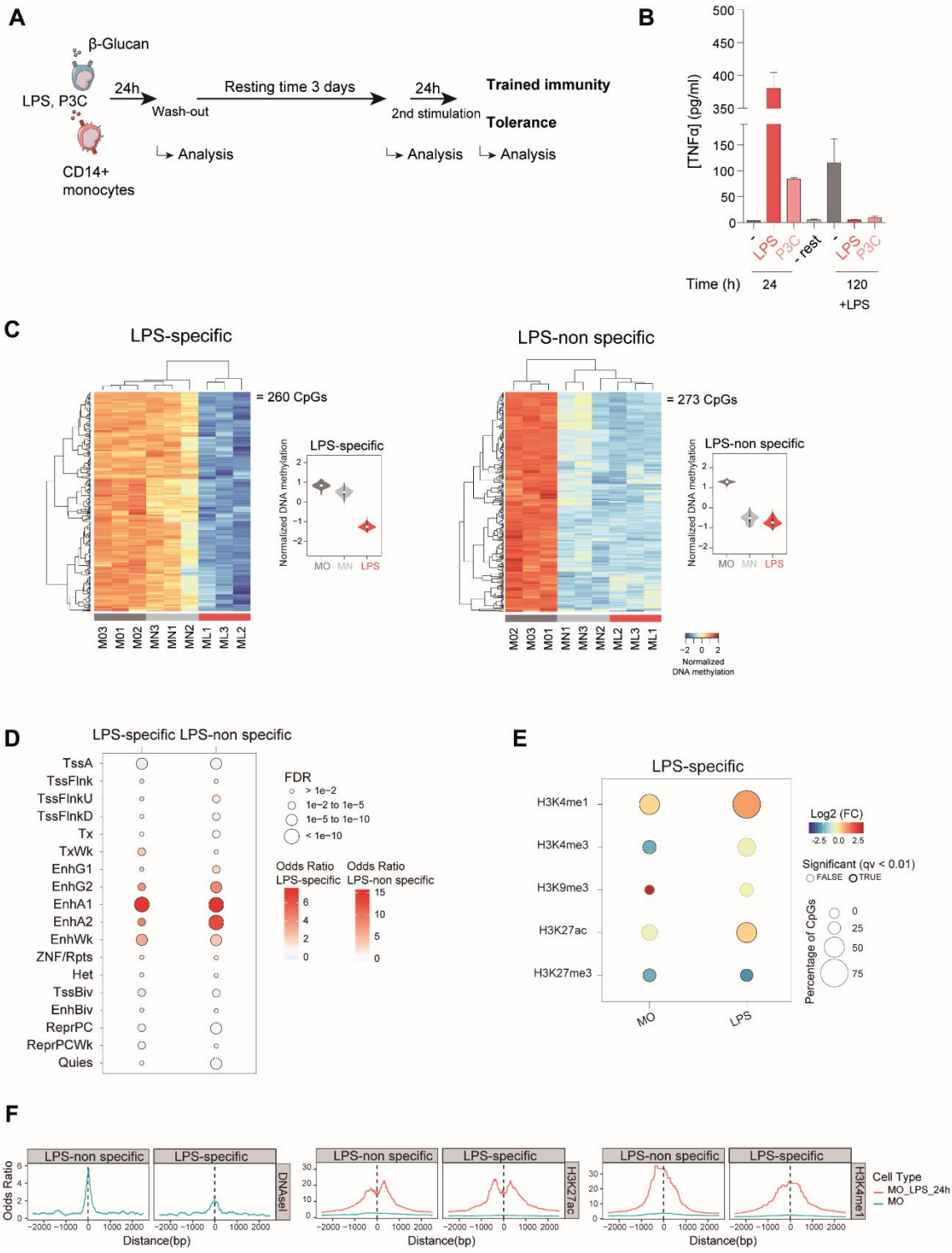


Figure 19. DNA methylation profile of LPS-primed human CD14+ monocytes. (A) Schematic diagram depicting *in vitro* experiments for innate memory models. (B) Release of TNFα from CD14+ monocytes isolated from healthy donor blood samples treated with LPS (10 ng/ml, 24h) or P3C (10 µg/ml, 24h), then washed and rested (3 days) and then treated again for 24h with LPS (10 ng/ml) all compared to not treated monocytes. (C) DNA methylation heatmap for LPS-specific

CpG sites (left heatmap) (Δβ-value ≥ 0.2, *p* -value < 0.01 and adjust *p*-value (FDR) < 0.05) and for non-specific sites (right heatmap). A scale is shown at the bottom right ranging from -2 (lower DNA methylation levels, blue) to +2 (higher methylation levels, red). Violin plots depicting normalized DNA methylation data of LPS-specific and LPS-non specific CpGs. (D) Chromatin states enrichment analysis for LPS-specific and LPS-non specific differentially methylated CpG sites based on ChromHMM monocyte published data. (E) Histone marks enrichment analysis of LPS-specific demethylated CpGs were carried out by crossing ChIP-seq data of H3K9me3, H3K4me3, H3K4me1, H3K27me3 and H3K27ac of monocytes and monocytes exposed to LPS downloaded from the Blueprint database. (F) DNase-seq and ChIP-seq data of H3K27ac and H3K4me1 of monocytes and monocytes treated with LPS were downloaded from the Blueprint database. Odds ratios were calculated for bins of 10 bp up to ±2500 bp centering around LPS-specific and LPS-non specific sites in which CpGs annotated in the EPIC 850K array were used as background. MO: monocytes (dark grey), MN: monocyte-macrophage cell (light grey), ML: LPS-treated monocyte (red). Graphs represent mean values of three healthy donors ± SEM.

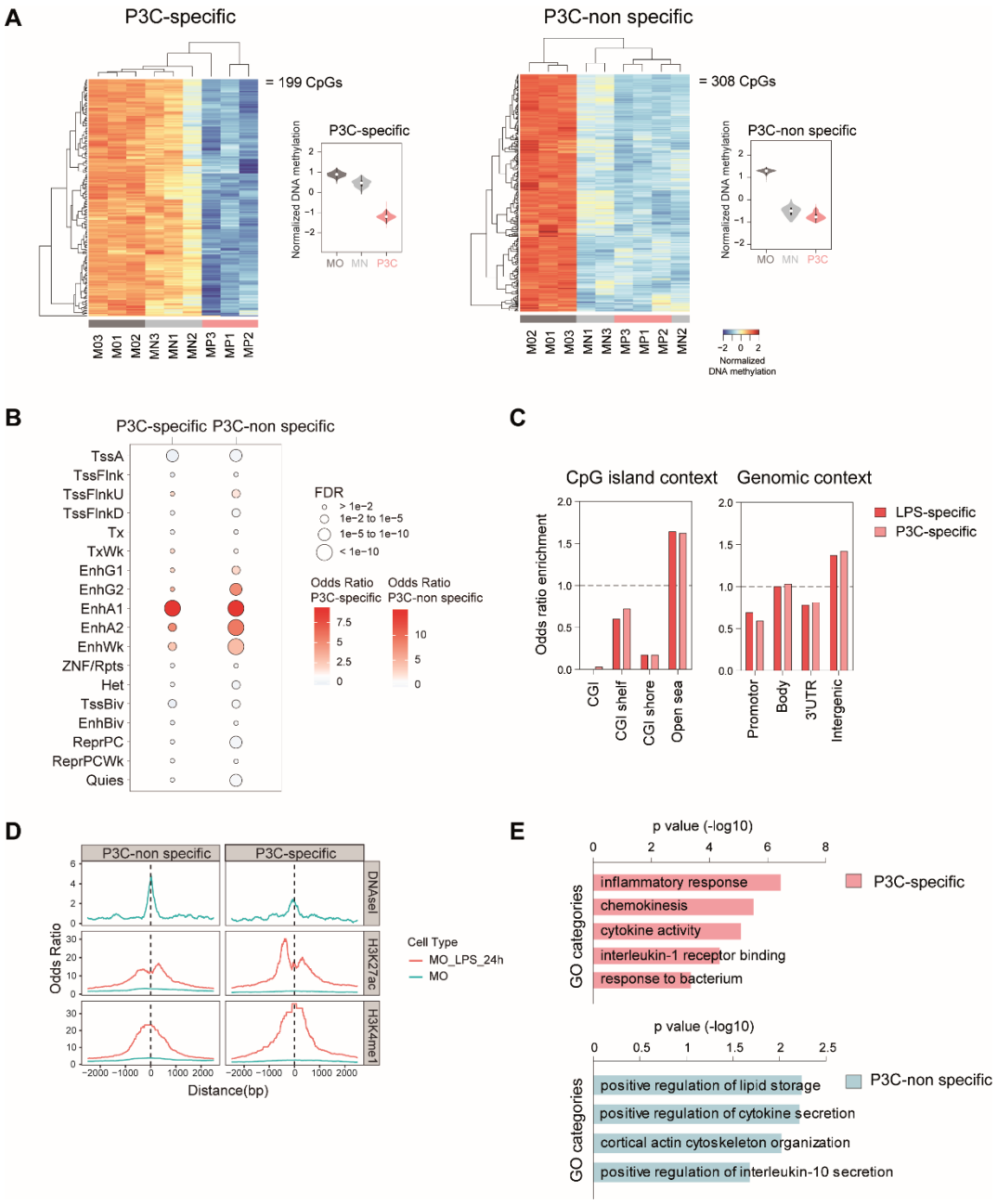
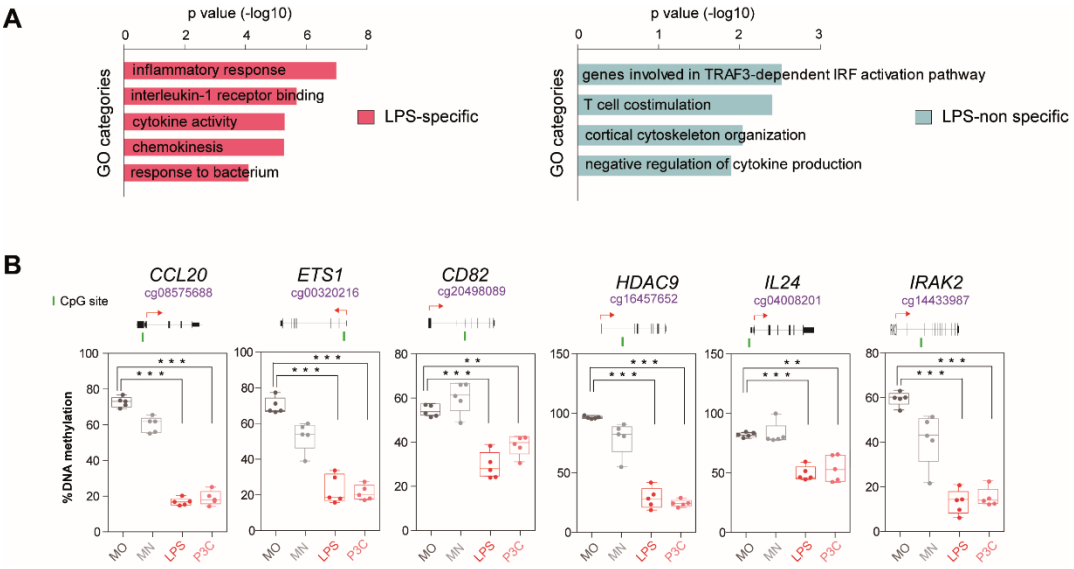


Figure 20. DNA methylation profile of P3C-primed human CD14+ monocytes. (A) DNA methylation heatmap for P3C-specific CpG sites (left heatmap) (Δβ-value ≥ 0.2, *p* -value < 0.01 and adjust *p*-value (FDR) < 0.05) and for non-specific sites (right heatmap). A scale is shown at

the bottom right ranging from -2 (lower DNA methylation levels, blue) to +2 (higher methylation levels, red). Violin plots depicting normalized DNA methylation data of P3C-specific and P3C-non specific CpGs. (B) Chromatin states enrichment analysis for P3C-specific and P3C-non specific differentially methylated CpG sites based on ChromHMM monocyte published data. (C) Distribution of differentially methylated CpGs in relation to CpG islands (CGI), including shores, shelves and open sea regions (left graph) and distribution of differentially methylated CpGs organised by genomic location (intergenic, promoter, gene body, and 3`UTR) (right graph) in LPS and P3C conditions. (D) DNase-seq and ChIP-seq data of H3K27ac and H3K4me1 were downloaded from the Blueprint database. Odds ratios were calculated for bins of 10 bp up to ±2500 bp centering around P3C-specific and P3C-non specific sites in which CpGs annotated in the EPIC 850K array were used as background. (E) Gene Ontology (GO) analysis of P3C-specific and P3C-non specific differentially CpG sites. MO: monocytes (dark grey), MN: monocyte-macrophage cell (light grey), MP: P3C-treated monocyte (pink).

Then, we selected some immune relevant genes, such as *CCL20*, *ETS1*, *CD82*, *HDAC9*, *IL24* and *IRAK2*, from our methylation screening. Bisulfite pyrosequencing of these representative CpGs confirmed their specific demethylation in LPS and P3C samples (Figure 21B). Indeed, we observed a significant correlation between DNA methylation changes and gene expression, where DNA demethylation generally associates with gene upregulation in those genes of which altered CpGs are located near to TSS (Figure 21C).

We then performed downregulation of *TET2* to assess the implication of this enzyme in the demethylation processes observed in our conditions. TET2 has also been implicated in catalysing demethylation in other monocyte-related differentiation processes [122,275]. Using specific siRNAs, we achieved around 50% of *TET2* downregulation after 3 days of treatment (Figure 21D). Under these conditions, demethylation of LPS-specific demethylated CpG sites was partially impaired (Figure 21E), demonstrating the implication of TET2 in this process.



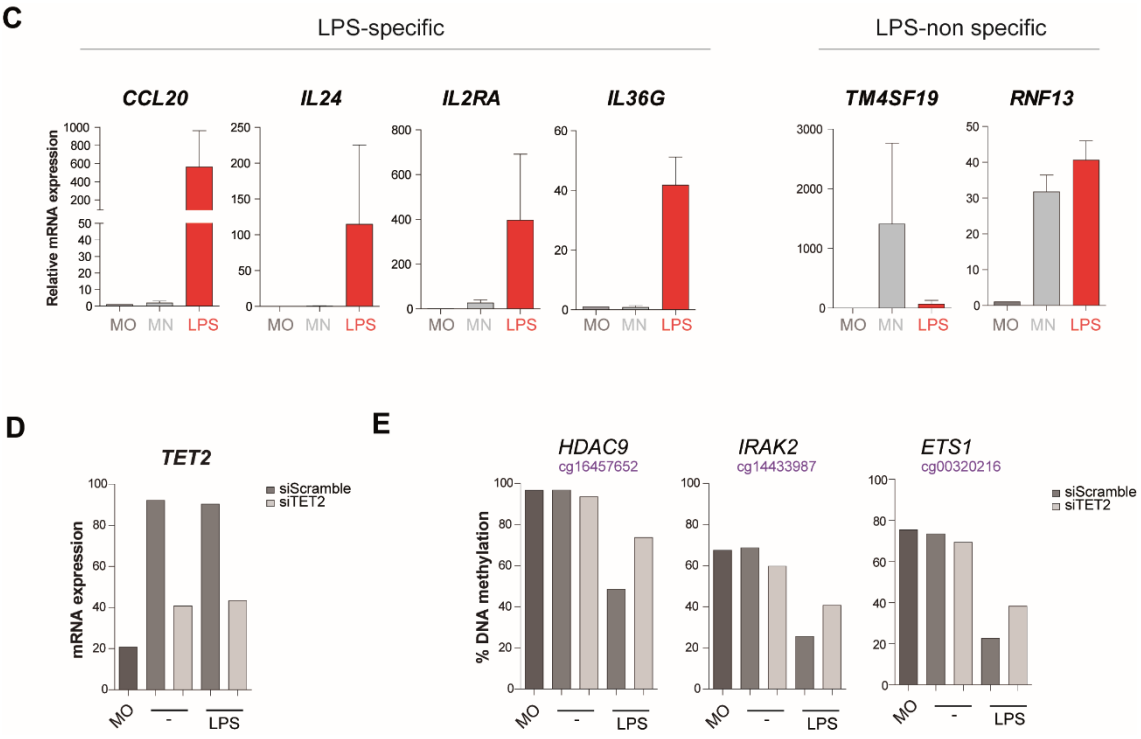


Figure 21. Methylation, gene expression and impact of TET2 downregulation on TLR-specific demethylation. (A) Gene Ontology (GO) analysis of LPS-specific and LPS-non specific differentially methylated CpGs. (B) Bisulfite pyrosequencing was performed to validate demethylation in the LPS and P3C samples. (C) mRNA expression levels of *CCL20*, *IL24*, *IL2RA* and *IL36G* that are CpGs associated with genes showing TLR-specific demethylation and *TM4SF19* and *RNF13* that are TRL-non specific. (D) qRT-PCR analysis to validate the downregulation of *TET2* by siRNA (siScramble used as control). Data are normalized against *RLP38*. (E) DNA methylation measured by bisulfite pyrosequencing in siRNA experiments. Statistical analyses were performed using paired student *t*-tests (* *p*-value < 0.05, ** *p*-value < 0.01 and *** *p*-value < 0.001). MO: monocytes (dark grey), MN: monocyte-macrophage cell (light grey), LPS: LPS-treated monocyte (red), P3C: P3C-treated monocyte (pink).

4.2.2 Involvement of JAK/STAT signalling in tolerance-specific DNA demethylation

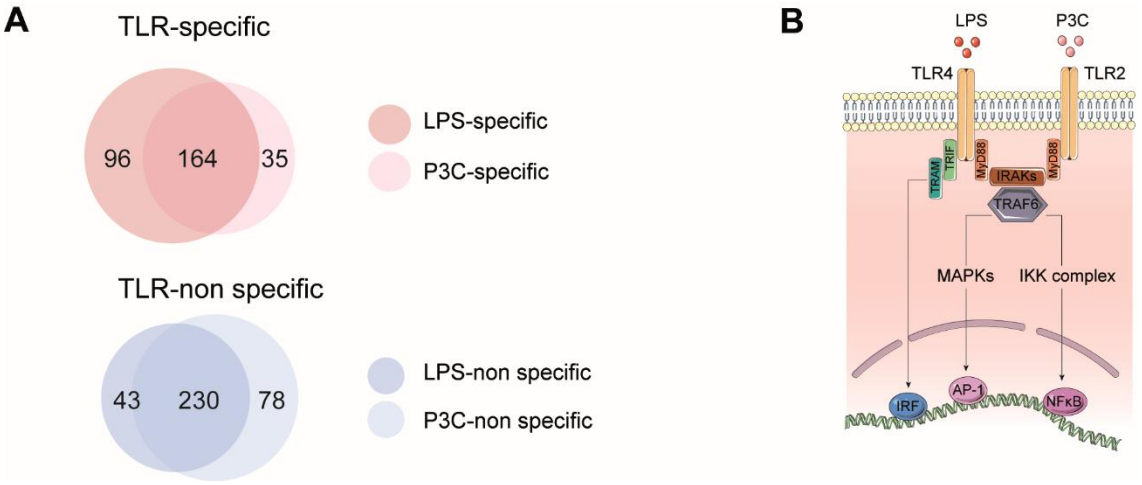
4.2.2.1 TLR-specific demethylation associates with STAT1, STAT3 and STAT5 protein phosphorylation

We then compared the demethylation events between LPS and P3C treated monocytes. The LPS- and P3C- specific DNA demethylation events displayed a high overlap (164 of common CpG sites) that we designated as TLR-specific (Figure 22A, top). The number of non specific demethylated sites between monocytes treated with LPS and P3C was also high (230 CpG sites) and were designated as TLR-non specific (Figure 22A, bottom). This similarity is relevant considering that LPS and P3C activate common TLR signalling pathways such as MAPKs and NF-kB. However, it is reported that LPS also stimulates the IFN route (Figure 22B). Given that DNA methylation changes occur downstream to the activation of signalling pathways and TFs, our results suggest that

the common pathways between TLR4 and TLR2 stimulation by LPS and P3C are the main responsible for the acquisition of the observed DNA demethylation.

Deposition or removal of epigenetic modifications is associated with TFs, which can be directly or indirectly involved in sequence-specific targeting. In our study, we performed separate HOMER analysis for the two groups of demethylated CpG containing-sequences previously identified (TLR-specific and TLR-non specific) in order to identify differential TF binding motifs between them. We found that both are enriched for common DNA binding motifs for TFs such as AP-1, Fos-related antigen (Fra) or NF-kB. Most importantly, for TLR-specific demethylated CpG sites, we identified enrichment for several STAT family members (Figure 22C). This finding suggested that STAT TFs might be important for LPS- and P3C-specific demethylation and perhaps relevant to TLR stimulation and the acquisition of endotoxin tolerance. Interestingly, for both LPS and P3C, we only identified binding motifs for STAT1, STAT3, STAT4 and STAT5.

We next analysed whether STAT activation is specific for LPS stimulation using WB. We observed an increase of the phosphorylated and activated forms of STAT1, STAT3 and STAT5 in monocytes treated with LPS compared to the non-treated controls (Figure 22D, left panel). By contrast, we could not detect phosphorylation of STAT6 either (Figure 22D, right panel). STAT1, STAT3, STAT4 and STAT5 are JAK2 targets, whereas STAT6 is a target for JAK3.



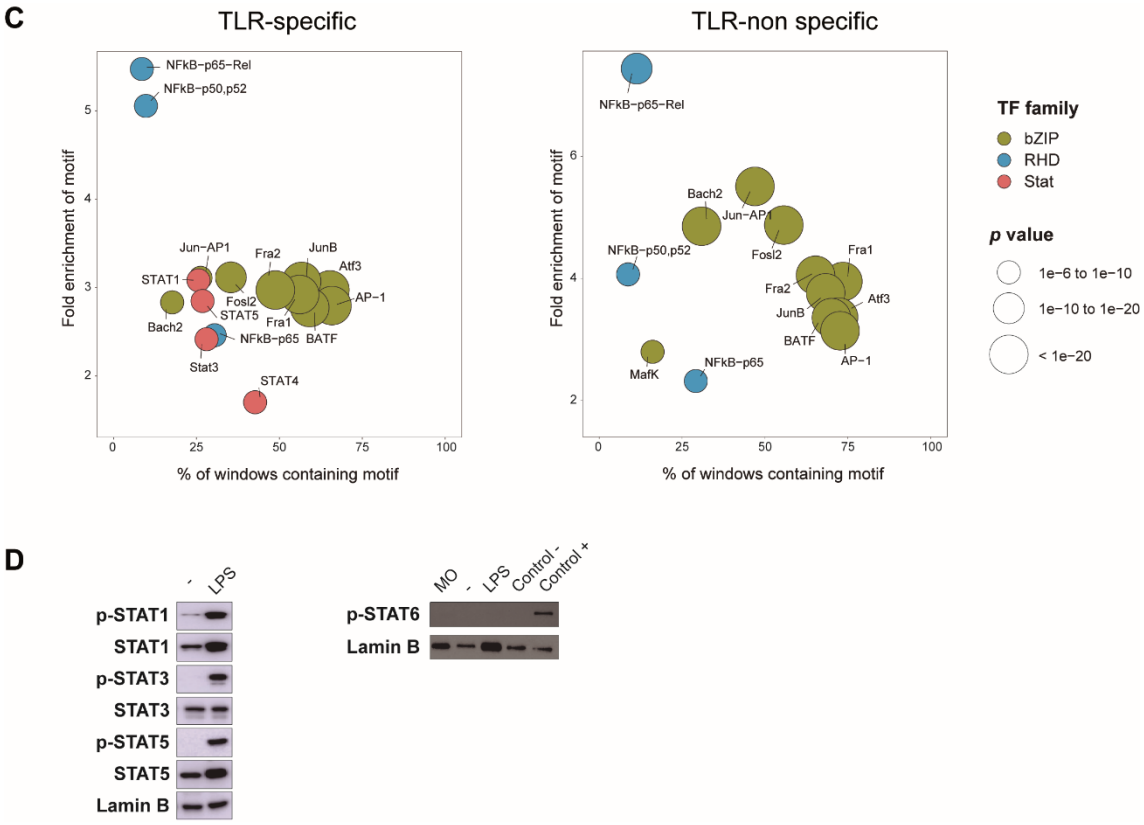


Figure 22. Involvement of JAK/STAT signalling pathway in tolerance-specific demethylation. (A) Venn diagrams representing the degree of overlap of TLR-specific (top) and TLR-non specific (bottom) demethylated CpGs between LPS and P3C stimuli. (B) Schematic diagram depicting TRL2 and TLR4 signalling pathways. (C) Bubble scatterplot of TF binding motif enrichment for TLR-specific and TLR-non specific differentially methylated CpG sites. The x-axis shows the percentage of windows containing the motif and the y-axis shows the fold enrichment of the motif. Bubbles are coloured according to TF family. *p*-value is indicated by bubble size (selected TF with $p \leq 1e^{-06}$). (D) Western blot from protein levels of STAT1, STAT3 and STAT5 (left panel) and STAT6 (right panel) in LPS stimulation compared to non-treated cells. LaminB is used as loading control.

4.2.2.2 Inhibition of JAK2 does not prevent demethylation but impairs activation of TLR-specific demethylated genes

To investigate the potential role of JAK2/STAT3/5 pathway in modulating tolerance-specific DNA methylation changes, we performed inhibition experiments using TG101348, a selective JAK2 inhibitor. We performed *in vitro* experiments as previously described. CD14+ cells were treated with LPS (10 ng/ml) during 24h in the presence or absence of increasing concentrations of TG101348 inhibitor (cell viability was assessed to select used concentrations). Then, monocytes were washed and left to rest for 3 days (with or without JAK2 inhibitor) and after that, cells were again stimulated with LPS (24h). Phosphorylated protein levels were investigated using WB in which we observed that STAT phosphorylation completely disappeared following JAK2 inhibition at 500nM (Figure 23A).

To examine the effects of JAK2 inhibition in innate immune response, we measured a panel of human inflammatory cytokines/chemokines using the LEGENDPlex™ Human Inflammation Panel (BioLegend), a bead-based multiplex assay that allows simultaneous quantification of 13 inflammatory molecules using a flow cytometer. Here, we observed a similar trend in the behaviour of cytokine release when JAK2 is inhibited and perhaps only JAK2 inhibition is not enough to observe some changes. Surprisingly, the JAK2 inhibitor appeared to accentuate the effects and TNFα levels were even more reduced and we detected higher levels of IL-10 after the second stimulation in those cells previously exposed to LPS in TG101348 treated cells compared to not inhibited cells (Figure 23B).

The identification of binding motif enrichment for STAT family members (STAT1, STAT3, STAT4 and STAT5) in specific LPS demethylated CpG sites suggested a direct implication of JAK2 in such demethylation events. We therefore expected impaired demethylation in these CpGs after JAK2 inhibition. DNA methylation levels of specific CpG sites were examined by bisulfite pyrosequencing, in genes like *CCL20*, *IRAK2*, *IL24* and *HDAC9*. Interestingly, we observed that JAK2 inhibition does not affect DNA methylation of those CpGs that are specifically demethylated following LPS treatment (Figure 23C), suggesting that JAK2 inhibition does not prevent their specific demethylation.

As aforementioned LPS-specific demethylation of these CpGs located at promoter regions occurred in association with up-regulated expression of these genes. Although JAK2 inhibition did not affect LPS-specific DNA methylation, contrastingly, we observed that this inhibition impaired gene expression. By using qRT-PCR, we found reduced gene levels in TG101348 treated cells (Figure 23D). These findings suggested us that additional mechanisms, probably histone modifications, associate with the JAK2/STAT3/5 pathway and that inhibition of this pathway impairs the activation of these genes, whereas DNA demethylation still takes place. It also suggested that DNA demethylation of these genes is a downstream consequence, and that perhaps their demethylation is not required for TF binding and gene activation, as recently described in the context of human dendritic cells after infection [276].

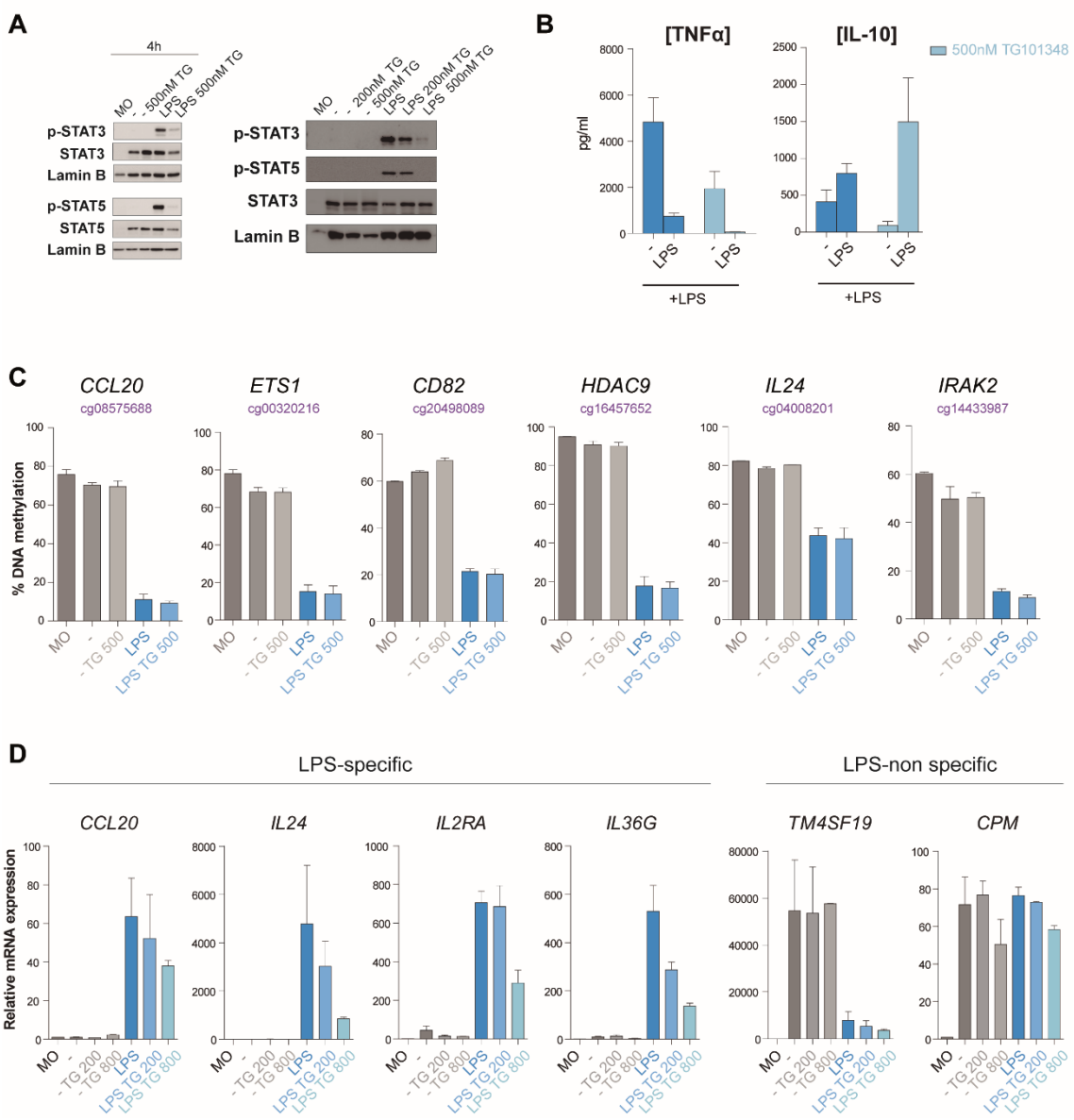
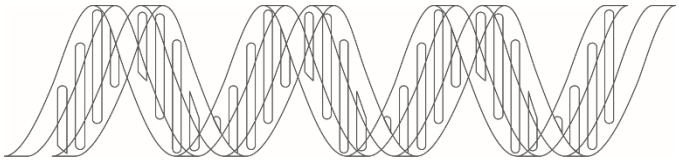


Figure 23. JAK2 inhibition effects on DNA methylation and gene expression. (A) Western blot from protein levels of STAT family in LPS stimulation and JAK2 inhibition compared to non-treated cells after 4 hours (left) or 96h (right). LaminB is used as loading control. (B) TNFα and IL-10 levels measured by ELISA to assess the tolerance state after JAK2 inhibition. (C) DNA methylation levels by bisulfite pyrosequencing in TG101348 (500nM) treated cells vs non treated cells. *CCL20, ETS1, CD82, IRAK2, IL24* and *HDAC9* are representative examples of CpGs that are LPS-specifically demethylated. (D) Relative mRNA levels of selected LPS-specific genes that have CpG site near to TSS and of *TM4SF19* and *CPM* that are LPS-non specific genes. Expression values were normalized against the expression of the endogenous gene *RPL38*. TG: JAK2 inhibitor (TG101348). Blots are representative of more than 2 independent experiments.

To explain these results, we can speculate and propose the following model. After LPS stimulation, monocytes trigger the recruitment of signal-regulated TFs, which activate an early gene expression program. Later on, TET2 is activated, maybe by interaction with some pioneer TFs such as PU.1 [119]. Consequently, loss of methylation might allow the binding of methyl-sensitive TFs, which in turn contributes to the later

transcriptional response. In this way, DNA methylation might play a role in modulating or stabilising the innate response by facilitating the binding of these methyl-sensitive TFs. Moreover, DNA methylation is associated with the gain of histone activation marks (H3K4me1 and H3K27ac) which may also participate in the regulation of gene expression programs of TLR-specific genes.



DISCUSSION

5. DISCUSSION

The studies presented in this doctoral thesis represent two alternative strategies to explore the participation of epigenetic mechanisms, and more specifically DNA methylation, in the context of bacterial infection and systemic inflammation. We have investigated: **1)** the occurrence and biological implications of DNA methylation alterations in monocytes from patients with sepsis in relation to those from healthy individuals, and **2)** the occurrence of DNA methylation changes in *in vitro* TLR-stimulated monocytes. Using both experimental approaches, we have identified DNA methylation alterations in monocytes associated with the acquisition of endotoxin tolerance. In addition, we have shown that these changes are associated with TFs and pathways downstream to TLR and inflammatory cytokines signalling. In this section, the results obtained in these two parallel studies are discussed.

5.1 DNA methylation alterations following sepsis

Our study demonstrates for the first time the existence of DNA methylation alterations in human monocytes from individuals following a sepsis episode in relation to the acquisition of a tolerized phenotype. Most notably, changes occur in genes relevant to the function of these cells including the interferon-gamma-mediated pathway and MHC class II proteins. On one hand, the observed methylation changes in patients with sepsis suggest their participation among the mechanisms leading to the generation of an aberrant phenotype of these cells. On the other, correlation analyses of the DNA methylation profiles in relation to IL-10 and IL-6 levels, which are increased in patients with sepsis, suggest a potential mechanism downstream to these cytokines participating in the defective generation of DNA methylation alterations. Furthermore, *in vitro* analysis of the influence of bacterial LPS and inflammatory context in determining the acquisition of DNA methylation alterations in monocytes also shows how these changes associate with aberrant transcriptional levels of dysregulated genes. Finally, our analysis shows increasing changes in DNA methylation in relation to organ dysfunction.

Monocytes play a pivotal role in the innate immune system being critical for many infectious and chronic inflammatory diseases with high medical implications and associated economic costs. In the case of sepsis, the huge variability in patients to treatments and recovery highlights the influence of other modifying elements, such as epigenetic mechanisms, in their progression. Our study opens up the possibility that DNA methylation changes could be used as a biomarker for patients with sepsis.

We have shown that DNA methylation changes occur at CpG sites near or at genes relevant for the immune response against pathogens such as MHC class protein

complex, NF-kB signalling pathway or interferon-gamma-mediated signalling pathway. Furthermore, many of these differentially methylated genes are connected between specific signalling pathways that are important during pathogen infection. For instance, we have observed methylation changes in TLR- and IL-1 routes, including genes such as *IL1A*, *IL1R2*, *IL1R1*, *TAB2*, *TAB1, MAP2K1* and *MAP3K1*. Another route involved is JAK/STAT signalling with *IL23A*, *IL19*, *IL22, IL27, TYK2, JAK1, STAT3, STAT4, SOCS5* or *SOCS3* undergoing specific methylation changes in monocytes from patients with sepsis (Figure 24). DNA methylation changes at these sites could regulate the activation or repression of related gene transcription.

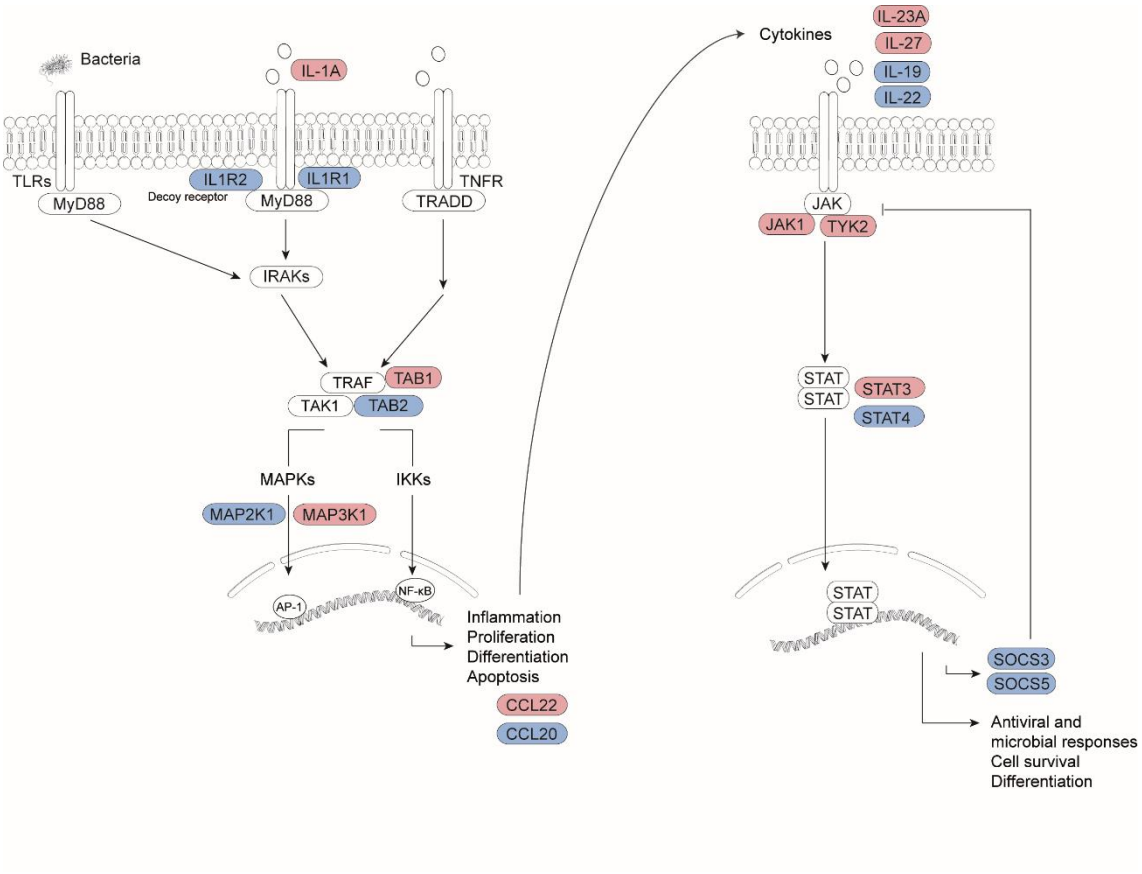


Figure 24. Scheme depicting molecules displaying DNA methylation changes in important signalling pathways related to immunity and sepsis. Molecules whose encoding genes displayed DNA methylation alterations in this study are shown in red and blue for hypermethylation and hypomethylation respectively.

The observed methylation changes suggest their role in the acquisition of the aberrant phenotype. In particular, there was enrichment in specific TFs relevant to innate responses; for instance, for the hypermethylated set, there was enrichment in IRF and ETS families while STAT proteins were enriched in hypomethylated CpG sites. These results suggest the participation of these TF families in targeting DNMTs or TET proteins to these genomic regions, although the specific participation would require further analysis. Previous studies have shown the ability of TFs, such as PU.1 and C/EBPα, to

recruit DNMT3B and TET2 [119,287]. Alternatively, binding or release of these TFs to those genomic sites may alter the accessibility of DNMTs and TET enzymes.

Most importantly, we observed that DNA methylation is related to the inflammatory cytokine environment of septic patients, specifically, with IL-10 and IL-6 basal cytokine levels, which are significantly increased in patients. In these analyses, we also found enrichment in genes involved in the interferon-gamma signalling category. It has been described that IFN-γ treatment partially restores metabolic and immunological programs in tolerized cells [288] and that IFN-γ recovers monocyte function in septic patients [282]. In addition, IFN-γ enhances host defence and innate immune responses by activating inflammatory M1 macrophages via the JAK/STAT1 signalling pathway [30] and disrupts the anti-inflammatory effects of IL-10 cytokine by suppressing IL-10-STAT3 signalling [289].

Our findings also showed a relationship between DNA methylation changes and the SOFA score, which is a measurement of organ damage. We have found that SOFA correlates with the methylome of septic monocytes. Specifically, these changes are involved in antigen processing and presentation via MHC and regulation of inflammatory response. Additionally, DNA methylation changes in relation to SOFA occurs in sites that are enriched for ETS, bZIP or STAT TF motifs. We can only speculate on how organ damage can be associated with DNA methylation changes. The most direct explanation is that organ damage is the result of systemic inflammation. In this sense, we have proven that inflammatory cytokines are able to shape the DNA methylome in monocytes. However, SIRS patients included in our study showed more moderate methylation changes in comparison to patients with sepsis, suggesting that bacterial infection is also an important driver of DNA methylation changes by other mechanisms [290].

We finally proved that stimulation of TLR, specifically with LPS, in monocytes, induces similar changes in DNA methylation as those observed in monocytes from patients with sepsis. Here we also showed that methylation changes associate with aberrant transcriptional levels of dysregulated genes. In this line, genome-wide high-throughput analysis of healthy PBMCs and monocytes stimulated with LPS for 4 days would be useful to test the observed changes in monocytes from patients with sepsis.

Several potential limitations should be taken into account when interpreting the above results due to the size and features of the cohort, including genetic background [291,292]. It would be necessary to use vaster cohorts, including more patients with Gram-positive or -negative bacterial infection and in different stages following sepsis episodes. Our data do not allow to draw conclusions about the timing of which those DNA

methylation alterations are produced. In other words, we cannot conclude that methylation changes are responsible for disease onset and progress. Furthermore, the study was focused on monocytes, although sepsis-induced immunosuppression is described by defects in both innate and adaptive cells.

It would also have been interesting to obtain blood samples from patients surviving a sepsis episode 3 or 6 months after their admission in the emergency room, which would allow determining the traces of epigenetic memory in their new peripheral blood circulating monocytes, which would contribute to obtaining new clinical perspectives. Moreover, we could have investigated the long-term contribution of DNA methylation to the tolerized state of monocytes.

When an infection occurs, monocytes act very fast to remove the invading microorganisms. Innate immune cells have the ability to broadly respond to pathogens and internal damage by a tightly regulated transcriptional and epigenetic programs. It is of critical importance to better understand the epigenetic modifications in these host innate responses. These epigenetic marks are stable, transmissible to progeny and dynamically regulated. In this way, it has been described that the epigenetic drug iBET151, which is a small molecular histone mimic bromodomain and extra terminal domain (BET) inhibitor, reverses the tolerized phenotype of immune cells, with increased cytokine release upon LPS re-stimulation [223]. In addition, HDAC inhibitors have resulted in feasible options to treat sepsis in numerous animal models [293]. Therefore, the use of epigenetic drugs could represent an interesting therapeutic target during infection.

The present study has led us to identify a specific DNA methylation profile associated with sepsis. Therefore, DNA methylation could contribute to stabilizing the state of tolerance of septic monocytes. A better understanding of the role of DNA methylation in these patients by exploiting the aforementioned aspects may open up their use as a potential epigenetic biomarker.

5.2 Epigenetic regulation of innate immune memory: specific DNA demethylation during endotoxin tolerance

In the second part of the present doctoral thesis, we have studied DNA methylation alterations during monocyte activation in an inflammatory context and their contributions to the acquisition of innate immune memory. Our results indicate that the acquisition of endotoxin tolerance in monocytes is accompanied by TET2-dependent specific demethylation associated with TLR stimulation. Such specific changes appear to be related to the activation of the JAK2/STAT3/5 pathway. Interestingly, inhibition of the

JAK2 pathway in our *in vitro* model for endotoxin tolerance is able to impair the upregulation of genes activated in a TLR-specific manner.

In addition to be a source of macrophages and dendritic cells, monocytes also play a critical role themselves in host defence. Monocytes, which are able to detect PAMPs and DAMPs, response by secreting several pro-inflammatory cytokines and chemokines that activate immune defence. Once innate immune cells sense microbial and damaged molecules, they induce sophisticated transcriptional programs involving gene expression changes. Signal-regulated TFs, such as IRF, NF-kB or AP-1, bind to the promoters and enhancers of their target genes after stimulation and regulate immune-related gene expression [160]. Epigenetic modifications can also regulate these gene programs by modulating chromatin accessibility to TFs; these alterations are hereditable and important for long-term maintenance of transcriptional programs.

Furthermore, monocytes can adapt their immune response following subsequent encounters with pathogens, in a process known as innate immune memory. This immunological memory shows enhanced or reduced immune response after secondary challenges, known as trained immunity and endotoxin tolerance respectively, as mentioned in the introduction.

The central feature of innate memory cells is the ability to mount a specific transcriptional response upon stimulation. Gene transcription is tightly regulated through epigenetic modifications by changing DNA accessibility to TFs and transcriptional machinery, as mentioned above. The engagement of TLRs with microbial pathogens activates downstream signalling pathways that result in the induction of specific transcriptional and epigenetic programs [294].

Despite innate immune memory has been associated with profound histone modifications that modify gene expression [222], much less is known about the role of DNA methylation. DNA methylation changes are more stable and longer than histone modifications [295] and could be associated with the establishment and maintenance of these adapted states.

For all these reasons, we decided to study the possible role of DNA methylation in endotoxin tolerance and trained immunity. For that, we performed *in vitro* stimulations of CD14+ monocytes with LPS and P3C, on the one hand, and with β-glucan, on the other, to provide tolerant and trained cells respectively. We observed specific demethylation patterns in LPS- and P3C- treated cells, whereas the methylation profile of β-glucan-treated cells resembled to naïve cells. These results are consistent with a

study published in 2016 by Novakovic *et al.* [223], in which they did not observe a role for DNA methylation in training macrophages.

We have observed several genes that become demethylated after TLR stimulation and these differentially methylated regions have relevance in monocyte biology and function. Specifically, we found enrichment in inflammatory response, cytokine activity or response to bacterium gene ontology categories.

After that, several genes important for immune responses were validated by pyrosequencing. Some of these examples were the chemokine *CCL20*, which has antimicrobial activity [296,297], the costimulatory molecule *CD82* or the inflammatory cytokine *IL-24* [298]. Other genes also demethylated were the TF *ETS1* and the molecule *IRAK2*, an essential regulator for IL-1R and TLR signalling [47]. Finally, we also included *HDAC9*, which codes for histone deacetylase 9 and regulates some transcriptional changes.

We found enrichment in enhancers for this TLR-specific demethylation. Furthermore, by using public ChIP-seq datasets, we examined active and repressive histone marks in LPS-specific regions. We identified that H3K4me1 and H3K27ac active histone marks were overrepresented in CpG sites that become demethylated after LPS stimulation. Finally, in those CpGs located near to TSS, we observed that loss of DNA methylation correlated with enhanced gene levels, indicating a possible regulation of gene expression by DNA methylation. These results are consistent with a study in human dendritic cells during MTB infection, where they observed a loss of methylation at enhancers and that demethylation strongly associated with gene expression changes of nearby genes [299].

DNA methylation can regulate transcription by multiple manners; TF binding or release can alter the accessibility of DNA methylation enzymes and impact on gene expression. Alternatively, DNMTs can interact with specific TFs, mediate gains of DNA methylation and modify gene expression. TET enzymes also bind to TFs in target DNA and mediate demethylation [98,300].

HOMER analysis for motif discovery was also performed in which we observed an enrichment of TFs belonging to the RHD and bZIF families, crucial TFs for immune cells and immune responses. More interestingly, we found an enrichment of STAT protein family in TLR-specific CpG sites, suggesting a role of this pathway in tolerance-specific demethylation.

We mainly focused on changes after LPS stimulation. LPS is the major component of the outer membrane of gram-negative bacteria; monocytes activated with LPS are able to induce MAPK, NF-κB and IRF signalling pathways. We had observed that LPS-specific demethylated regions display motif enrichment for STAT family, and for this reason, we decided to inhibit JAK/STAT signalling, in order to explore the effects of this inhibition. In addition, it has been described that STAT proteins interact with HAT and HDACs [301], thereby influence gene transcription, and could also mediate DNA methylation changes, through association with DNMTs. It would also be interesting to study the interaction of different STAT, such as STAT3 and STAT5, with the epigenetic machinery involved in differential gene expression, although co-immunoprecipitation experiments are required to detect these potential interactions.

Increasing our knowledge of how STAT proteins alter DNA methylation and gene transcription is important for understanding the role of these TFs after TLR-stimulation. Therefore, as aforementioned, we decided to inhibit JAK2 tyrosine kinase, by using the selective inhibitor fedratinib, also known as TG101348.

Firstly, following JAK2 inhibition, we analysed some cytokines and chemokines involved in inflammatory responses [302]. It has been described that STAT3 is necessary for anti-inflammatory IL-10 effects; therefore, JAK2 inhibition, associated with STAT3, could have pro-inflammatory effects following TLR stimulation [303]. However, we observed a similar behaviour of cytokine release in cells treated or not with TG101348 inhibitor.

Secondly, we studied the effects of the JAK2 inhibition on specific DNA demethylation and gene expression. Surprisingly, this inhibition does not affect DNA demethylation, whereas it is able to impair enhanced gene expression specific to LPS stimulation. These results suggest that alternative mechanisms to DNA methylation could be involved. For instance, histone modifications could be relevant in the differential expression of genes that become demethylated. We could hypothesize that some TFs, such as the STAT family members, bind their genomic targets after stimulation and are responsible to later convert it in accessible sites to other TFs or to epigenetic machinery such as DNMTs and TET enzymes.

To study the causal-effect relationship between two factors, firstly, it is necessary to demonstrate the association among them. In this case, we have observed that demethylation is linked with changes in gene expression in specific LPS genes during endotoxin tolerance. Secondly, we are going to determine the time order of the variables, by time-course experiments. Finally, we could suggest that an alternative variable, such

as histone modifications, is also affecting the relationship, being a complex regulatory network.

The connection between DNA methylation and gene expression changes is controversial and maybe it depends on the cell context. For instance, in a previous study from our laboratory, Vento-Tormo and colleagues described that, during dendritic cell differentiation, certain inflammatory genes became demethylated, however, these genes only become upregulated once LPS activates these cells [121]. In other examples from our lab, focusing on monocyte-to-osteoclast differentiation, some genes were shown to become hypermethylated only after they become silenced [119]. More recently, a study by Barreiro's group demonstrated that, in human dendritic cells, DNA demethylation in response to infection is a downstream consequence of TF binding [276].

We are planning to investigate the dynamics of our model to determine the role of DNA methylation in gene expression of LPS-specific sites. We will describe if the loss of DNA methylation anticipates or not the overexpression of these genes. In this line, we are exploring if DNA demethylation is the direct cause of altered gene expression or if DNA methylation is a downstream consequence of gene expression changes. The study of additional epigenetic mechanisms such as histone modifications and their mutual interplay is also necessary to define the possible cause and effect relationship between gene expression and epigenetic marks. Repressive (H3K27me3 and H3K9me3) or active (H3K4me3 and H3K27ac) histone marks could influence this complex network facilitating or preventing gene induction.

There are some open questions raised by our study. To what extent does DNA methylation be responsible for long-term effects in innate memory cells? What will be the role of this epigenetic mark in regulating gene expression programs? Is DNA demethylation a downstream consequence of transcriptional activation programs induced after TLR stimulation?

Future experiments will also include RNA-sequencing in order to better characterised the impact of JAK2 inhibition and to study the potential association between DNA methylation and gene expression during innate immune tolerance. In this context, JAK inhibitors may provide novel opportunities in the treatment of pathogenic infections; for instance, it has been described that JAK2 inhibition with AG490 rescued mice from polymicrobial sepsis [304].

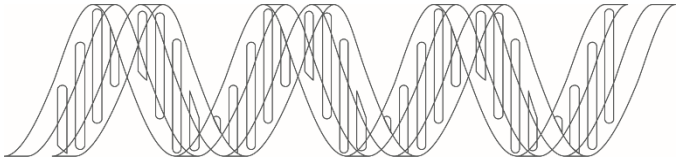
We also performed experiments to describe TET2 implication as the enzyme responsible for these methylation changes. It is expected that active demethylation is

involved in our model due to the absence of cell proliferation. In this way, by using specific siRNAs, we observed that TET2 downregulation impairs LPS-specific demethylation. So, after LPS stimulation, we found demethylation that is TET2-dependent.

Given that monocytes have a short half-life, recent studies have focused on interrogating whether epigenetic modifications, that it is known to occur on innate cells, are also participating in myeloid progenitors' cells in the bone marrow [305]. If it exists a reprogramming in hematopoietic cells, this could explain the long-term effects observed in these memory processes. In this way, we can speculate that epigenetic modifications are retained in progenitor cells and their epigenetic signatures can be inherited by daughter cells; however, it is only a hypothesis. More experiments using next-generation sequencing techniques for epigenetic analysis in progenitor cells are needed.

Furthermore, it is important to note that working with buffy coats from diverse blood donors is sometimes quite complicated. There are uncontrolled parameters as sex, age, health condition, genetic background, etc, that strongly influence immune response. Therefore, when we analysed the data, we usually found great variations and it is necessary to perform many replicates to obtain significant statistical results.

In summary, the *in vitro* approach in this thesis demonstrated the complex network between cell signalling, gene transcription and epigenetic machinery when LPS binds to TLR4 receptor. During this part of the doctoral thesis, we have speculated on whether DNA methylation plays a role in fine-tuning functionality of monocytes during innate responses. Further investigation of cell and molecular mechanisms during tolerance acquisition will allow developing novel therapeutic targets to improve the resolution of associated inflammation.



CONCLUSIONS

6. CONCLUSIONS

The conclusions obtained during this doctoral thesis can be summarized as follows:

1. Monocytes from patients with sepsis display widespread DNA methylation alterations. Sepsis-specific changes occur at genes associated with pathways relevant to host defence against pathogens.

2. DNA methylation changes correlate with IL-10 and IL-6 levels, which are increased in patients with sepsis. These differentially methylated genes associate with transcription factors and cell signalling important for innate immune responses.

3. DNA methylation changes in relevant genes are also associated with organ dysfunction, estimated by SOFA score.

4. *In vitro* stimulation of monocytes with LPS partially recapitulates the DNA methylation alterations observed in patients with sepsis.

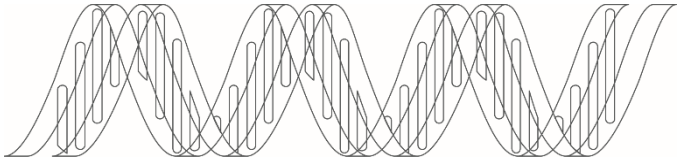
5. TLR stimulation of human monocytes, and the acquisition of endotoxin tolerance, is accompanied by the specific loss of DNA methylation. 260 and 199 CpG sites undergo TLR-mediated DNA demethylation following LPS and P3C stimulations, respectively.

6. CpG demethylation and transcriptional activation associated with the acquisition of endotoxin tolerance occur at genes involved in monocyte/macrophage cell biology and function, including *CCL20*, *IL24* or *IRAK2*.

7. The observed loss of DNA methylation is associated with an active mechanism of demethylation mediated by TET2. When TET2 is silenced, there is an impairment of DNA demethylation.

8. JAK2 inhibition impairs the enhanced expression of genes related to TLR stimulation.

9. Specific DNA demethylation, after TLR stimulation, might have a role in fine-tuning or conferring stability to endotoxin tolerance by its association with active histone marks and methyl-CpG sensitive factors.



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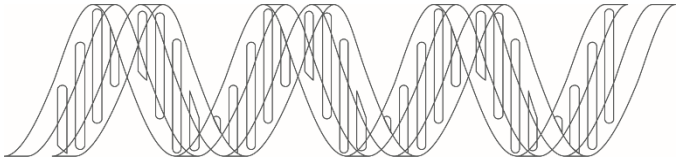

SUPPLEMENTARY INFORMATION

8. SUPPLEMENTARY INFORMATION

I. Lorente-Sorolla, Clara (et al.). Epigenetics in Autoinflammation. Chapter 3, Textbook of Autoinflammation. Springer (2019).

II. Lorente-Sorolla, Clara (et al.). Inflammatory cytokines and organ dysfunction associate with the aberrant DNA methylome of monocytes in sepsis. *Genome Medicine*. 2019, 11:66. doi: 10.1186/s13073-019-0674-2.

Epigenetics in Autoinflammation

3

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Abstract

The molecular mechanisms of inflammation involve a series of processes that start as extracellular signals that interact with membrane-bound receptors, cell signaling cascades, nuclear factors, and epigenetic enzymes that activate a specific gene expression program. Environmental factors and/or genetic defects can result in constitutive activation of this program. Recent studies highlight the relevance of epigenetic (dys) regulation in these processes and suggest several implications of these mechanisms and alterations in the clinical management of patients with autoinflammatory diseases. In this chapter, we provide an overview of the latest findings related to the epigenetic control in the function of myeloid cells as main effectors of inflammation, as well as the latest findings in the field of autoinflammatory diseases.

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Keywords

Autoinflammation · Epigenetics · DNA methylation · Myeloid cells

Abbreviations

5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
AID	Activation-induced cytidine deaminase
AIM2	Absent in melanoma 2
AP-1	Activator protein
ASC	Apoptosis-associated speck-like protein
C/EBPα	CCAAT/enhancer binding protein
CAPS	Cryopyrin-associated periodic syndromes
CD	Crohn disease
CNO	Chronic non-bacterial osteomyelitis
CREB	cAMP response element-binding protein
DAMPs	Danger-associated molecular patterns
DNMTs	DNA methyltransferases
EBF1	Early B cell factor 1
ETS	E26 transformation-specific
FCAS	Familial cold autoinflammatory syndrome
FMF	Familial Mediterranean fever
HATs	Histone acetyltransferases

HDACs	Histone deacetylases
HIDS	Hyperimmunoglobulinemia D syndrome
HMTs	Histone methyltransferases
HSCs	Hematopoietic stem cells
IKKs	IκB kinases
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinases
IRF	Interferon-regulatory factors
I-SRE	Intronic enhancer element
JmjC	Jumonji domain-containing proteins
JNK	c-Jun N-terminal kinases
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
MKD	Mevalonate kinase deficiency
MWS	Muckle-Wells syndrome
NLR	NOD-like receptor
NOMID	Neonatal-onset multisystem inflammatory disease
PAMPs	Pathogen-associated molecular patterns
PAX5	Paired box protein 5
PGE2	Prostaglandin E2
PRRs	Pattern-recognition receptors
STAT	Signal transducer and activator of transcription
TET	Ten-eleven translocation
TNF	Tumor necrosis factor
TRAF	Tumor necrosis factor receptor-associated factor

Key Points

- **Inflammation involves a series of linked processes that range from extracellular stimulation to transcription factor-mediated and epigenetic control**
- **Epigenetic alterations have been associated with both monogenic and genetically complex autoinflammatory diseases**

3.1 Introduction

Inflammation is an adaptive response triggered by infection or tissue damage. It is induced as host defense against invading pathogens, as tissue-repair response, or as homeostatic state restoration [1]. The inflammatory response is very complex and controlled by different regulatory networks which are responsible for modulation of inflammation and its resolution. However, inflammation is sometimes dysregulated and becomes detrimental; there are many diseases such as autoimmune and autoinflammatory disorders, sepsis, atherosclerosis, type II diabetes, or cancer that lead to inflammation [2].

The initial phase of typical inflammatory response is induced by pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). PAMPs are molecular structures or molecules that are shared by most pathogenic bacteria and some viruses, whereas DAMPs are molecules that are actively excreted or passively released by stressed or dying cells and further enhance inflammatory or cell-death signaling. PAMPs and DAMPs act through germ-line encoded pattern-recognition receptors (PRRs) which are important in microbial recognition and in regulation of inflammatory response [1]. These receptors, which act as key components in this initial process, are mainly expressed by myeloid cells including monocytes, macrophages, neutrophils and dendritic cells (see Chap. 4). Cellular activation drives the release of inflammatory cytokines (tumor necrosis factor-TNF, interleukin- IL-1β, IL-6), chemokines (such as chemokine (C-C motif) ligand 2, CCL2, and chemokine (C-X-C motif) ligand 8, CXCL8) as well as prostaglandins (like prostaglandin E2, PGE2). Cytokines and chemokines not only activate these innate immune cells but also can exert induction of acute-phase proteins in the liver, fever and fatigue by acting on the hypothalamus, platelet activation, and a multitude of cellular processes [3] (see Chap. 6).

In particular, engagement of extracellular or intracellular PRRs triggers cell signaling pathways that lead to the recruitment of signaling proteins including members of the tumor necrosis factor receptor-associated factor (TRAF) family and various protein kinases such as IL-1 receptor-associated kinases 1 and 4 (IRAK1 and IRAK4). These molecules activate several effector molecules and transcription factors. The most important activated signal routes are mitogen-activated protein kinases (MAPKs) and IκB kinases (IKKs) (Fig. 3.1a). MAPKs include, among others, c-Jun N-terminal kinases (JNKs) and p38 which

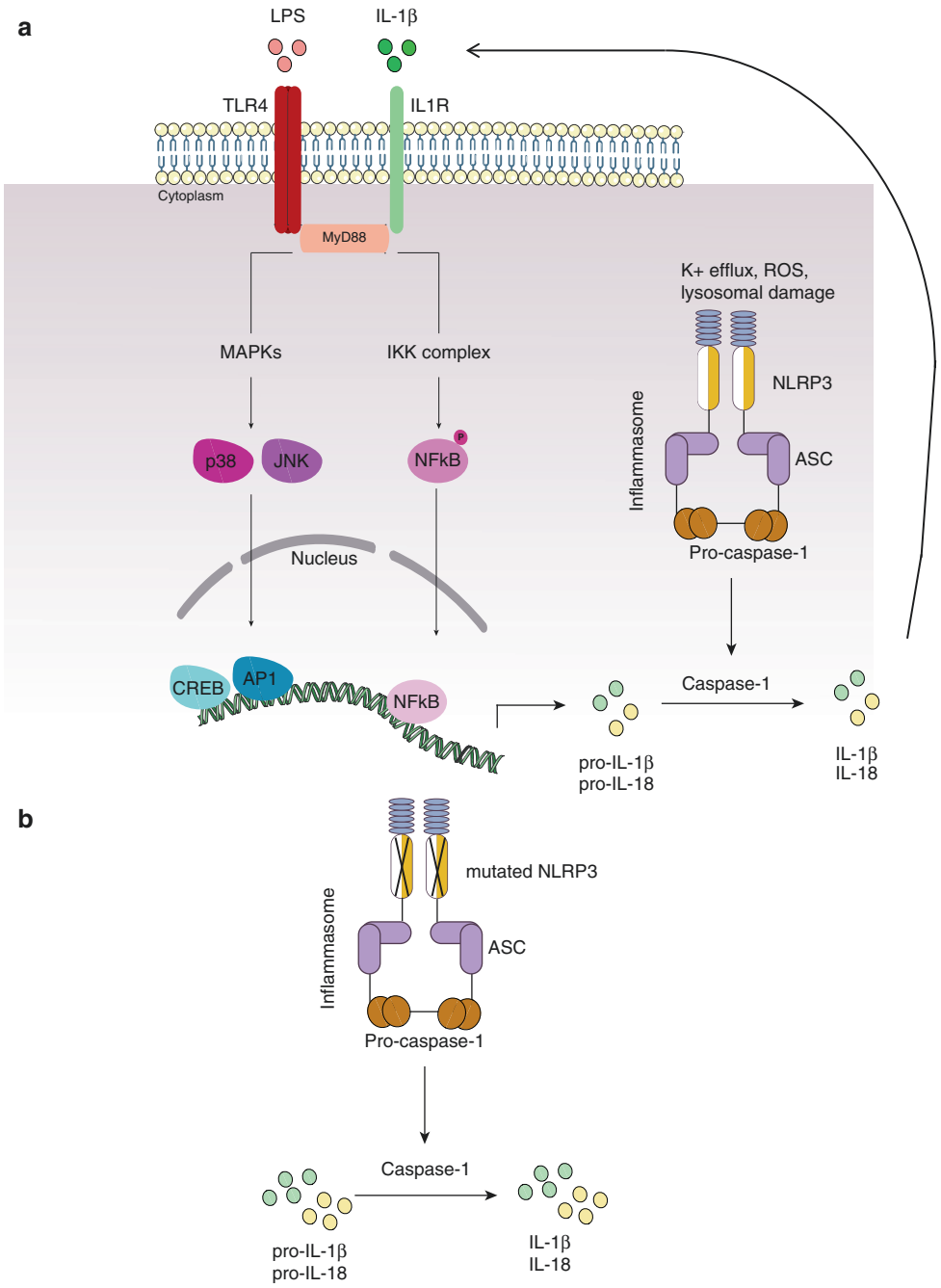

Fig. 3.1 Mechanisms of inflammation and links with epigenetic changes (**a**) Scheme depicting cellular pathways in monocytes/macrophages related to inflammation. Toll-like receptor 4 (TLR4) is a pattern recognition receptor (PRR) that recognizes bacterial antigens, such as lipopolysaccharide (LPS). Interleukin-1 receptor (IL-1R) is a cytokine receptor which binds interleukin 1 (IL-1). Activation of TLR4 and IL-1R result in activation of myeloid differentiation primary response 88 (MYD88), which subsequently activates transcription factor NF-kB and mitogen-activated protein kinase (MAPKs). Both have an effect on the activation of specific genes either by direct binding to chromatin or through the activation of downstream transcription factors, such as cAMP response element-binding protein (CREB) or activator protein (AP1). Transcription factors influence the acquisition of epigenetic changes. Increased transcription of the *IL1B* gene leads to an amplification loop involving the activity of the inflammasome (**b**) Mutations in the inflammasome subunit NLRP3 lead to an increase of IL1-β production and therefore impacts the nuclear effects of inflammation

phosphorylate and activate several transcription factors such as activator protein (AP-1) and cAMP response element-binding protein (CREB). Secondly, IKKs participate in the activation of the NF-κB transcription factor and subsequently all their gene targets [4]. Altogether, inflammation is able to activate several crucial signaling pathways to immune cells.

A key signaling pathway that controls the innate immune response by regulation of inflammation and tissue repair is mediated by the inflammasome. Inflammasomes are multimeric complexes that assemble following the detection of microbial pathogens and DAMPs (potassium efflux, reactive oxygen species, monosodium urate crystals, cathepsin) [5]. Inflammasomes consist of a sensor molecule [including members of the NOD-like receptor (NLR) or absent in melanoma 2 (AIM2)], an adaptor molecule (apoptosis-associated speck-like protein, ASC) and the effector molecule pro-caspase-1. Once inflammasomes are assembled, they activate caspase-1 which processes inactive proinflammatory cytokine precursors of IL-1β and IL-18 into their mature forms. In addition, inflammasome activation leads to an inflammatory cell death pathway known as pyroptosis [5, 6] (see Chap. 5). The inflammatory response activates various signaling pathways that regulate expression of numerous mediators. As mentioned above, reversal of this response is crucial to return to homeostasis; if inflammasome activation persists over time and there is a lack of inhibition, inflammation-related disorders occur. Therefore, several mechanisms inhibit or attenuate inflammation including anti-inflammatory cytokines (IL-10, IL-37, etc), receptor antagonists (IL-1R, TNFR), complement inhibitors, negative regulators of Toll-like-receptor signaling, prostaglandins and lipid mediators [2].

During the past 25 years, a group of disorders characterized by a dysregulated inflammatory response has been established under the term of autoinflammatory diseases. Autoinflammatory diseases, often linked to genetic defects, are characterized mainly by systemic or organ specific inflammation and recurrent fever in the relative absence of autoreactive T cells, high autoantibody titers or any detectable pathogen [7]. Thereby, the term autoinflammation is connected with dysregulation in the innate immune system [8]. Episodic fever, rash, swelling of joints and other tissues and overproduction of IL-1β are common findings associated with these diseases [8, 9].

New insights into the pathogenesis of autoinflammatory diseases have recently provided increasing evidence that epigenetic modifications are involved. For this reason, the identification of these epigenetic changes is crucial for patient diagnosis and new therapies.

3.2 Epigenetic Control in Immune Cells

Key Points

- **Epigenetic mechanisms involve the establishment of transcriptional activity states through the chemical and reversible modification of DNA and histones**
- **Epigenetic control participates both in the acquisition of cell identity and activation of inflammatory cells**

Epigenetics has been defined as the set of mechanisms that register, signal or perpetuate altered activity states without changing the DNA sequence. Epigenetic modifications play an important role in the regulation of gene expression [10]. In general, epigenetic mechanisms mainly involve modification of amino acid residues in the histone N-terminal ends, and DNA methylation (Fig. 3.2). Some also include non-coding RNAs-mediated processes as epigenetic mechanisms [11].

3.2.1 DNA Methylation and Histone Modifications

In mammals, DNA methylation generally refers to the addition of a methyl group to cytosine (5mC) and it takes place in CpG dinucleotides, although methylation has recently been demonstrated to occur in other nucleotides, in very low

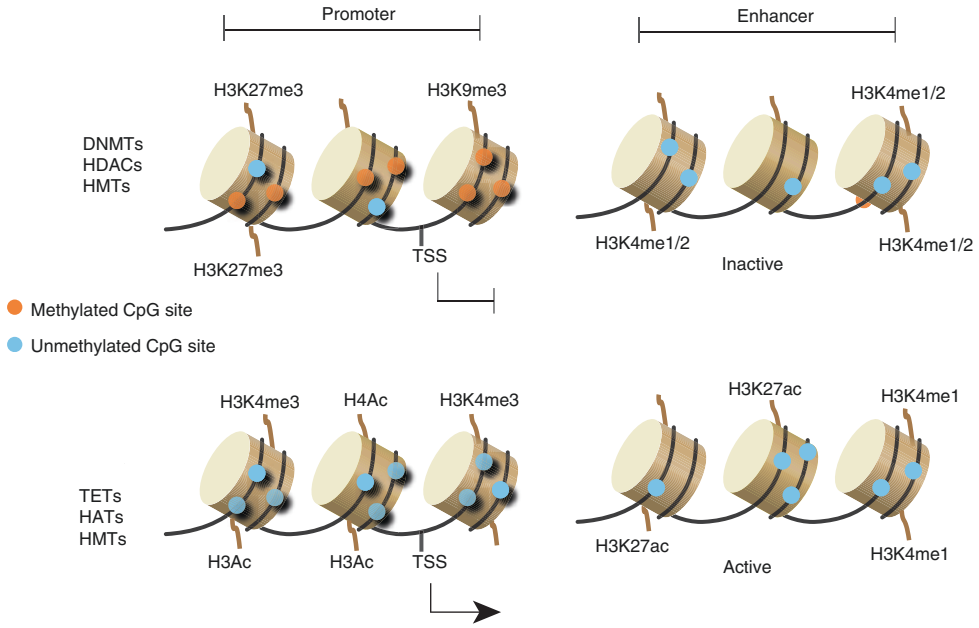

Fig. 3.2 A summary of epigenetic modifications and their relationship with transcriptional activity. Two groups of modifications are presented: methylation of cytosines, that occurs at CpG dinucleotides and post-translational modifications of histones that mainly occur at the N-terminal tails. Although these modifications occur along the entire genomic DNA sequence, in this figure two specific genomic regions have been chosen: gene promoters (left) and enhancers (right). Inactive promoters (top) are enriched in histone modifications such as trymethylation of lysine 27 of histone 3, H3K27me3 (and sometimes H3K9me3), are devoid of acetylated histones and can be heavily methylated for some CpG island containing promoters. Active promoters are enriched in trymethylation of lysine 4 of histone 3, H3K4me3, hyperacetylated histones (H3Ac and H4Ac) and low levels of 5mC. Enhancers regulate transcription at a long distance and are marked by H3K4me1 (in both active and inactive enhancers) and with H3K27Ac only in active ones. Enzymes implicated in these processes are DNA methyltransferases (DNMTs), histone acetyltranferases (HATs) and deacetylases (HDACs), ten eleven translocation (TET) and histone methyltransferases (HMTs)

proportion. The incorporation of a methyl group to cytosines is catalyzed by a family of enzymes known as DNA methyltransferases (DNMTs). DNMT1 is responsible for the maintenance of DNA methylation during replication whereas DNMT3A and DNMT3B are involved in the establishment of *de novo* methylation [12]. The reversion or loss of methyl groups, known as DNA demethylation, can be passive, caused by the inefficient maintenance of methylation during DNA replication, or active. Active DNA demethylation involves the activity of ten-eleven translocation (TET) enzymes that catalyze the conversion of 5mC to 5-hydroxymethylcytosine (5hmC), and further oxidize it into other intermediate forms that are then excised by thymine DNA glycosylase (TDG). The activity of the DNA repair machinery later restores the incorporation of an unmethylated cytosine [12, 13]. DNA methylation can influence gene expression through different mechanisms including the interference of transcription factor binding or the recruitment of histone modifiers or chromatin remodeling complexes containing methyl-CpG binding domain proteins. These effectors can alter chromatin accessibility or competence and modulate gene transcription. DNA methylation gains in promoters are generally associated with transcriptional repression. However, the effects of DNA methylation changes on gene transcription can be different depending on the genomic location.

Histone proteins interact closely with DNA and are responsible for packing DNA, by wrapping it around the histone octamer. Amino acid residues in histones are subjected to covalent post-translational modifications that include methylation, phosphorylation, acetylation and others [14]. Histone acetylation is the addition of an acetyl group carried out by histone acetyltransferases (HATs); acetylation plays important roles in chromatin dynamics, gene silencing, DNA repair, etc. Histone deacetylases (HDACs) catalyze the removal of the acetyl group to histone residues providing an equilibrium reaction. Another important modification is the methylation of histone lysine or arginine residues by different families of histone methyltransferases (HMTs). Methylation also affects gene transcription, promoting activation or repression, depending on the modified residue. Histone demethylation is the removal of methyl groups in modified histones via histone demethylases as Jumonji domain-containing proteins (JmjC). These modifications participate in regulating gene expression in very different ways depending on various factors, such as genomic location. For instance, acetylation of histones H3 and H4 and trimethylation of each K4, K36 and K79 of histone H3 are associated with a more open chromatin structure and correlate with ve transcription. By contrast, histone deacetylation and methylation of K9 and K27 of histone H3 mark silenced regions [15].

3.2.2 Epigenetic Control of Differentiation of Hematopoietic Stem Cells

Epigenetic control is crucial to determine cell fate decisions. Extracellular signals that are internalized through receptors and signaling cascades establish a crosstalk with lineage-specific transcription factors, which interact with epigenetic complexes. Immune cells are a very good example of this interplay between extracellular signals, signaling pathways, transcription factors and epigenetic enzymes. Immune cell differentiation involves the differentiation from hematopoietic stem cells (HSCs) to a large number of cell types, which are mainly grouped in two branches, lymphoid and myeloid cells. In the past few years a number of epigenomic studies have delineated the range of both DNA methylation and histone modification changes that occur during the differentiation of HSCs [16]. In human hematopoiesis, distinct DNA methylation changes are pivotal to promoting the commitment to lymphoid or myeloid differentiation. A genome-wide methylation analysis during hematopoietic cell differentiation revealed an increase in DNA methylation levels during lymphoid cell differentiation whereas a loss of methylation is associated with myeloid cell differentiation [17]. In addition, DNMT3A and DNMT3B are needed to *de novo* methylate and repress genes encoding transcription factors involved in the self-renewal capacity of hematopoietic stem cells, subsequently allowing cell differentiation [16]. Analysis of the epigenome of HSCs has shown that important transcription factors for hematopoiesis, including CCAAT/enhancer binding protein (C/EBPα), early B cell factor 1 (EBF1) and paired box protein 5 (PAX5) are demethylated and are also enriched in both activating H3K4me3 and repressive H3K27me3 (bivalent) histone marks [18]. One of the conclusions of the aforementioned studies is that myeloid and lymphoid cells are very different in relation to the participation of the epigenetic machinery. For instance, TET2 is a key enzyme in the acquisition of myeloid cell identity since the discovery that C/EBPα activates TET2 during C/EBPα-mediated B cell to macrophage reprogramming of pre-B cells. Furthermore, mutated TET2 has been described to be related with several myeloid malignancies [19]. Moreover, activation-induced cytidine deaminase (AID), comprehensively studied for its role in class-switch recombination and somatic hypermutation in B lymphocytes, has also been reported to participate in promoting DNA demethylation changes during B cell differentiation [20], although this role remains controversial. Histone modifiers are also important in myeloid- or lymphoid-specific cell development and identity. For example, the histone demethyltransferase KDM2B acts as key

regulator during lymphoid differentiation since ectopic expression of KDM2B favors lymphoid commitment [21]. It is also necessary to take into account that mutations in genes involved in epigenetic regulation are very common in leukemia. Finally, class II histone deacetylase HDAC7 has a transcriptional repression role of myeloid specific genes and its downregulation is crucial during C/EBPα-mediated reprogramming of B cells into macrophages [22].

3.3 Epigenetic Control in Inflammation

Although inflammation encompasses the activity of both innate and adaptive immune cells, myeloid cells are the main effectors of the inflammatory process; monocytes, macrophages, neutrophils or dendritic cells are very plastic and can display epigenetic modifications. Therefore, it is crucial to understand epigenetic changes in these cells that can contribute to chronic inflammation and disease.

Many myeloid transcription factors, including signal transducer and activator of transcription (STAT) family members, interferon-regulatory factors (IRFs), NF-κB family and members of the ETS (E26 transformation-specific or E-twenty-six) family such as PU.1, can recruit or associate DNMTs and histone modifying enzymes. This implicates a role of epigenetic mechanisms in the differentiation into inflammatory cell types, as well as in immune-related gene transcription [23, 24]. In this regard, chromatin structure is crucial to control NF-κB-regulated genes such as proinflammatory cytokines. As an example, following lipopolysaccharide (LPS) stimulation of macrophages, TLR-induced genes have been categorized into two classes: tolerized genes, which include inflammatory genes, show repressed expression whereas non-tolerized genes, which include antimicrobial mediators, increase their levels of expression. These changes in expression are related to the fact that histone acetylation and H3K4 methylation are only maintained on the promoters of the non-tolerized genes [4].

Activation of dendritic cells and macrophages is also regulated by epigenetic modifications. For instance, dendritic cell development and maturation is accompanied by significant DNA demethylation [25]. During macrophage polarization, lower expression of DNMT3B promotes a shift towards the M2 (anti-inflammatory) macrophage phenotype [26]. Furthermore, chromatin remodeling is also important in the acquisition of the M2 phenotype; demethylation of H3K27 by Jmjd3 and the absence of HDAC3 lead to M2 polarization [16]. Also, in the case of monocyte to macrophage differentiation, epigenetic reprogramming associated with the acquisition of specific epigenetic signatures has been observed. For example, priming of monocytes or macrophages by an initial stimulus (such as LPS or β-glucan) renders a tolerized or trained phenotype, respectively, both associated with epigenetic alterations in H3K4me3, H3K27Ac and H3K4me1. In this context, initial engagement of PRRs leads to a global acetylation and H3K4me3 mark in proinflammatory genes. Subsequent challenges produce loss of H3K4me3 and acetylation in the case of tolerized genes and leads to silent gene expression. By contrast, trained immunity retains initial histone marks in promoters of important genes (such as TNF and IL-6) facilitating gene transcription [23, 27].

3.4 Perspectives on Autoinflammatory Diseases

Key Points

- **Untreated CAPS patients show exacerbated DNA demethylation of several inflammasome-related genes whereas this demethylation is reverted in CAPS treated with anti-IL-1 drugs**
- **Upregulation of miR-4520a has been observed in FMF patients**
- **In CNO, a complex autoinflammatory disorder, the expression of IL-10 and IL-19 is decreased through impaired chromatin remodeling**

Autoinflammatory disorders comprise a wide range of pathologies characterized by hyperinflammation and recurrent attacks of fever as well as activation of innate immune cells [8]. Advances in genomic techniques (such as next-generation sequencing) have resulted in the inclusion as autoinflammatory syndromes both hereditary monogenic disorders such as cryopyrin-associated periodic syndromes (CAPS) (see Chap. 19) and familial Mediterranean fever (FMF) (see Chap. 16), as well as multifactorial and complex diseases such as Behçet disease (see Chap. 35), chronic non-bacterial osteomyelitis (CNO) (see Chap. 31) and Crohn disease (CD) among others [7]. The implication of epigenetic factors in monogenic and complex inflammatory diseases might be very different.

Several studies have reported that epigenetic modifications may participate in the development and pathogenesis of autoinflammatory diseases. Altered DNA methylation, covalent histone modification and miRNAs dysregulation have been linked as additional factors in these pathologies (Table 3.1).

3.4.1 Cryopyrin-Associated Periodic Syndromes (CAPS)

Despite the fact that monogenic autoinflammatory syndromes are caused by mutations in specific inflammatory-related genes, heterogeneous patient phenotypes and diverse drug response within the same disorder may suggest contribution of epigenetic factors [28]. For instance, cryopyrin-associated periodic syndromes (CAPS) are described as a spectrum of heterogeneous phenotypes with different degrees of severity; this suggest that additional factors as epigenetic modifications may contribute to the disease [29]. CAPS are monogenic autoinflammatory diseases which, in increasing order of severity, include: familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and neonatal-onset multisystem inflammatory disease (NOMID). Gain-of-function mutations of *NLRP3* gene (Fig. 3.1b), a well-known member of the inflammasome family, cause this rare disease, resulting in markedly increased IL-1β production and secretion [30]. Furthermore, *NLRP3* mosaicism (in the myeloid compartment) has been described in some CAPS patients with diverse disease severity, including adult-onset CAPS [28]. A recent study showed that DNA demethylation of several inflammasome-related genes is more efficient in monocytes from untreated CAPS patients than those of healthy counterparts (Fig. 3.3). Interestingly, monocytes from patients with CAPS treated with IL-1 inhibitors display methylation levels similar to those seen in control subjects, suggesting the effectiveness of the drug in preventing the exacerbated demethylation of inflammasome genes [30]. Another study found that NOMID patients present significantly down-regulated expression of miR-29c and miR 103-2

Table 3.1 Evidence of epigenetic contributions to autoinflammatory disease

Disease	Gene/protein	Epigenetic alterations	References
FMF	*MEFV*/ pyrin	Gains of DNA methylation of *MEFV* gene/Upregulation of miR-4520a	[32, 33]
CAPS	*NLRP3*/ NLRP3	DNA demethylation of inflammasome-related genes in untreated CAPS patients/miRNAs regulation in NOMID patients	[30, 31]
MKD	*MVK*/MVK	Trained immunity phenotype of monocytes	[34]
BD	Complex	Different DNA methylation patterns in monocytes and CD4+ cells	[35]
CNO	Complex	Failure of H3 phosphorylation at serine residue 10 (H3S10p) at the *IL-10* proximal promoter	[36]
CD	Complex	*DNMT3A* as a susceptibility gene/Differential methylation in several immune-related genes	[37, 38]

FMF Familial Mediterranean fever, *CAPS* Cryopyrin-associated periodic syndromes, *MKD* Mevalonate kinase deficiency, *BD* Behçet disease, *CNO* Chronic non-bacterial osteomyelitis, *CD* Crohn disease

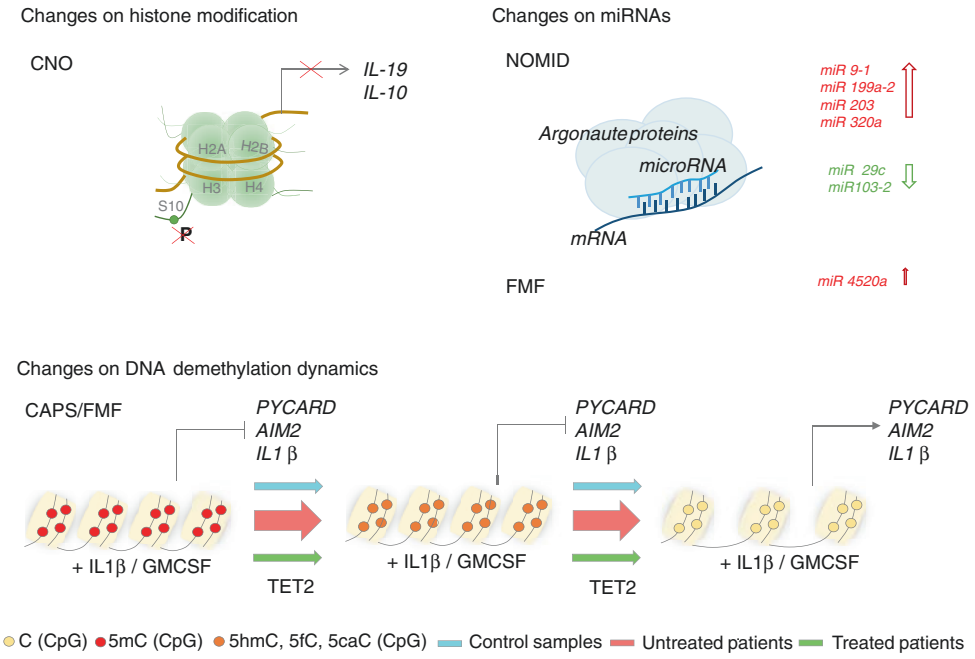

Fig. 3.3 Epigenetics in autoinflammatory diseases. Several autoinflammatory diseases have been associated with epigenetic changes. In chronic non-bacterial osteomyelitis (CNO), IL-19 and IL-10 expression is impaired due to a failure of histone H3 phosphorylation at serine residue 10 (H3S10p) in the promoter region. Changes in miRNA levels have been associated with neonatal-onset multisystem inflammatory disease (NOMID) (increase of miR 9-1, miR 199a-2, miR 203 and miR 320a, and a decrease of miR 29c and miR103-2 in their skin) and with familial Mediterranean fever (FMF) (increase in miR-4520a). In cryopyrin- associated periodic syndromes (CAPS) and FMF, changes in DNA demethylation dynamics have been reported. DNA demethylation (in light circles) is associated with increased gene expression of some inflammasome-related genes (*PYCARD, AIM2* and *IL1B*). Figure from Álvarez-Errico D, Vento-Tormo R, Ballestar E (2017) Genetic and epigenetic determinants in autoinflammatory diseases. Front Immunol. https://doi.org/10.3389/fimmu.2017.00318 [7]

but an increase of miR 9-1, miR 199a-2, miR 203 and miR 320a in skin lesions, suggesting the involvement of miRNA-mediated dysregulation in this disease (Fig. 3.3). Furthermore, several genes encoding histone modifiers were found to display aberrant expression levels in lesional skin compared to normal skin [31].

3.4.2 Familial Mediterranean Fever (FMF)

Another example of the role of epigenetics in monogenic autoinflammatory diseases involves familial Mediterranean Fever (FMF), an autosomal recessive disorder characterized by recurrent attacks of fever, caused by mutations in the *MEFV* gene, which encodes the pyrin protein [29]. Pyrin, which is expressed mainly in myeloid cells, is implicated in inflammation by the activation of caspase-1, which is responsible for the maturation of IL-1β and IL-18. Changes in DNA methylation dynamics have been described in FMF (Fig. 3.3). A slightly increased methylation of the second exon of *MEFV* in peripheral leukocytes from FMF patients is associated with reduced *MEFV* expression level [32]. In addition, upregulation of miR-4520a expression levels has recently been reported in patients with FMF (Fig. 3.3) [33].

3.4.3 Mevalonate Kinase Deficiency (MKD)

Mevalonate kinase deficiency (MKD), also known as hyperimmunoglobulinemia D syndrome (HIDS), is caused by loss-of-function mutations in *MVK,* which lead to accumulation of mevalonate [29] (see Chap. 17). Monocytes from patients with MKD have a trained immunity phenotype; analysis of H3K27ac histone mark by ChIP-sequencing also shows different peaks in patients compared to healthy controls [34].

3.4.4 Behçet Disease

There are also studies implicating epigenetic dysregulation on the disease course of multifactorial and complex autoinflammatory diseases. It has been suggested that changes in global DNA methylation may be responsible for the pathology of Behçet disease. Behçet disease is a chronic multi-systemic inflammatory disorder characterized by complex and numerous symptoms (recurrent oral and genital ulcers, skin lesions, uveitis, among others) [8]. Genome-wide DNA methylation analysis in monocytes and CD4+ cells of patients with Behçet disease shows different methylation levels in comparison to healthy controls; specifically, 383 differentially methylated CpGs were identified in BD monocytes, and CD4+ lymphocytes displayed 125 differential CpGs sites. This aberrant methylation is associated with important genes for structural and functional cytoskeletal proteins in monocytes and for antigen processing and presentation in CD4+ cells. Importantly, patients with Behçet disease in remission following treatment show a partial restoration of the DNA methylation pattern, similar to controls [35].

3.4.5 Chronic Non-bacterial Osteomyelitis (CNO)

There is evidence of epigenetic contribution in chronic non-bacterial osteomyelitis (CNO). CNO is an autoinflammatory disorder that mainly affects bones and it is occasionally associated with inflammatory bowel disease. CNO presents an imbalance of pro- and anti-inflammatory cytokines and regulatory signals; in particular, decreased IL-10 and IL-19 expression has been reported in these patients. This repression is suggested to be caused by chromatin remodeling; an altered histone H3 phosphorylation at serine residue 10 (H3S10p) in the promoter region impairs cytokines expression (Fig. 3.3). Moreover, a differential DNA methylation in IL-10 intronic enhancer element (I-SRE) has also been observed, giving strong support to the hypothesis of epigenetic contribution to pathophysiology in CNO [36].

3.4.6 Crohn Disease

Epigenetic modifications have also been observed in Crohn disease (CD). CD, which is one of the main types of inflammatory bowel disease, is a polygenic disease that presents a dysregulated response to intestinal microbiota in genetically susceptible individuals. In 2010, *DNMT3A* was identified by genome-wide association studies (GWAS) as a susceptibility gene for CD, suggesting a possible relationship between altered DNA methylation and the disease [37]. Further genome-wide methylation analysis in CD patients shows a specific methylation pattern in peripheral blood of patients compared to controls, with methylation changes in several important immune response genes including *MAPK13*, *FASLG*, *PRF1*, *S100A13*, *RIPK3*, and *IL-21R* [38]. Moreover, miRNA expression profiles of CD have also been studied in tissue and peripheral blood, although it is still necessary to further study the implications of their dysregulation to understand the possible role of miRNAs in CD diagnosis or therapy [7].

3.5 Conclusions

Based on the observations we described, it is becoming clear that epigenetic mechanisms likely contribute to the pathophysiology of

autoinflammatory diseases (Table 3.1 and Fig. 3.3). An increasing number of studies has addressed the participation of different epigenetic mechanisms involved in differentiation and function of myeloid cells, including their role in inflammation. Future efforts in the knowledge of autoinflammation could establish epigenetic modifications as crucial factors in related diseases, allowing the identification of attractive targets for novel therapeutic interventions. Although we are still far from understanding the complete extent of epigenetic alterations in autoinflammatory syndromes, a better knowledge of epigenetic deregulation in these patients will help to open new therapeutic approaches in these diseases.

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Inflammatory cytokines and organ dysfunction associate with the aberrant DNA methylome of monocytes in sepsis

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Abstract

Background: Sepsis, a life-threatening organ dysfunction caused by a dysregulated systemic immune response to infection, associates with reduced responsiveness to subsequent infections. How such tolerance is acquired is not well understood but is known to involve epigenetic and transcriptional dysregulation.

Methods: Bead arrays were used to compare global DNA methylation changes in patients with sepsis, non-infectious systemic inflammatory response syndrome, and healthy controls. Bioinformatic analyses were performed to dissect functional reprogramming and signaling pathways related to the acquisition of these specific DNA methylation alterations. Finally, in vitro experiments using human monocytes were performed to test the induction of similar DNA methylation reprogramming.

Results: Here, we focused on DNA methylation changes associated with sepsis, given their potential role in stabilizing altered phenotypes. Tolerized monocytes from patients with sepsis display changes in their DNA methylomes with respect to those from healthy controls, affecting critical monocyte-related genes. DNA methylation profiles correlate with IL-10 and IL-6 levels, significantly increased in monocytes in sepsis, as well as with the Sequential Organ Failure Assessment score; the observed changes associate with TFs and pathways downstream to toll-like receptors and inflammatory cytokines. In fact, in vitro stimulation of toll-like receptors in monocytes results in similar gains and losses of methylation together with the acquisition of tolerance.

Conclusion: We have identified a DNA methylation signature associated with sepsis that is downstream to the response of monocytes to inflammatory signals associated with the acquisition of a tolerized phenotype and organic dysfunction.

Keywords: Sepsis, DNA methylation, Cytokines, Endotoxin tolerance, Monocytes

Background

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. Sepsis can induce acute kidney injury and multiple organ failures and represents the most common cause of death in intensive care units [2, 3]. The immune response during sepsis is complex and varies over time, with the concomitant occurrence of both pro-inflammatory and anti-inflammatory mechanisms [3]. Despite intense study, the cellular and molecular basis of human sepsis remains still unclear and effective therapies are lacking.

In many cases, sepsis survivors continue to succumb to secondary challenges, latent infections, or malignancies several years after the initial septic episode [4]. It has been recognized that sepsis leads to the acquisition of tolerance, a state of reduced responsiveness to

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subsequent stimulation after a primary bacterial insult that results in reduced cytokine production by monocytes and macrophages [5]. As a result, most patients with sepsis rapidly display signs of profound immunosuppression, associated with an increase in hypoxia inducible factor-1α expression that drives functional reprogramming [6]. Such immune reprogramming is in part due to disruption of homeostasis and defective cellular energy metabolism which underlies the inability to respond to secondary or further stimulation [7]. A number of mechanisms are involved in the homeostasis of the immune system, where monocytes are pivotal. These cells recognize pathogen patterns or intercept and phagocytose antigens, critical steps in eliminating bacterial infections, halting the increase in viral load, and eradicating certain neoplastic growths. Several monocyte subtypes are also crucial to the de-escalation of inflammation and in wound healing [8]. In vitro experiments mimicking septic conditions have shown that upon endotoxin re-challenge with Gram-negative bacteria or only lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, tolerized monocytes/macrophages show a drastic downregulation of inflammatory cytokines (e.g., tumor necrosis factor [TNF] α, interleukin [IL]-6, IL-1β, IL-12) in parallel with the upregulation of anti-inflammatory cytokines like IL-10, transforming growth factor (TGF) β, and IL-1RA as compared to non-tolerized cells challenged with the same stimuli. These tolerant monocytes/macrophages also show an impaired antigen presenting capacity correlated with decreased expression of human leukocyte antigen (HLA)-DR and some costimulatory molecules [9] and upregulation of the immune checkpoint ligand PD-L1 [10].

The acquisition of endotoxin tolerance is accompanied by a remodeling of the epigenomic profiles [11–13]. Most studies have focused on histone modification changes. Following LPS stimulation of macrophages, toll-like receptor (TLR)-induced genes are categorized into two classes: tolerized and non-tolerized genes. Tolerized genes, which include inflammatory genes, show repressed expression whereas non-tolerized genes increase their levels. Transcriptional activation of non-tolerized genes is associated with high levels of histone acetylation and H3K4me3 at their promoters [11]. In addition, monocytes exposed to LPS showed changes in H3K27ac, H3K4me1, and H3K4me3 [12]. It has also been shown that during endotoxin tolerance, leukocytes display increased levels of repressive H3K9me2 mark at the promoter regions of the *IL1B* and *TNF* genes [14, 15]. Specifically, the H3K9 histone methyltransferase, G9a, is essential for silencing the *TNFA* gene [16].

In this context, DNA methylation changes have received less attention than histone modifications for several reasons. It has been mainly because DNA methylation has a more limited range of effects than histone modifications [17]. Nevertheless, DNA methylation changes are generally highly relevant for the biology of myeloid cells [18]. On the one hand, various studies have demonstrated the relevance of DNA (cytosine-5)-methyltransferase 3A (DNMT3A) and ten-eleven translocation methylcytosine dioxygenase 2 (TET2), both enzymes respectively essential for the de novo incorporation and oxidation/removal of methyl groups to cytosines, to the function of monocytes, dendritic cells, and macrophages [19, 20]. On the other hand, DNA methylation is generally associated with the stabilization of a transcriptional and functional state; thus, it is appealing to hypothesize that sepsis results in prolonged acquisition of DNA methylation changes of the monocytes well beyond the acute phase of sepsis, and perhaps contributing to stabilize the state of tolerance of monocytes. Most importantly, TET2 has a role in resolution of inflammation by recruiting HDAC2 to repress inflammatory genes [21] and to promote sepsis-induced emergency myelopoiesis [22].

In this study, we investigated DNA methylation changes in monocytes from individuals who have experienced an episode of sepsis. We performed DNA methylation profiling where we compared sorted monocytes from patients with sepsis and healthy controls. The analysis revealed the existence of significant DNA methylation differences between the two groups in CpG sites mapping at genes relevant for monocyte-related immune responses. Most importantly, we identified a significant relationship between DNA methylation data and IL-10 and IL-6 cytokine levels, which are significantly increased in patients with sepsis, as well as with organ dysfunction. We have determined that changes in DNA methylation are determined by TLR stimulation and the altered levels of inflammatory cytokines. Our findings also highlight the implication of TLR stimulation and cytokines under sepsis in establishing and perpetuating the dysregulated epigenetic signature and phenotype of monocytes.

Methods

Human samples

We selected and diagnosed patients with sepsis based on the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) [1]. For each patient, we calculated the Sequential [Sepsis-related] Organ Failure Assessment (SOFA) score. The study included 14 patients with bacterial infections with SOFA ranging from 2 to 8. Patients were obtained from La Paz University Hospital and Vall d'Hebron University Hospital. Blood samples were collected at the first 12 h of sepsis diagnosis, which was confirmed using clinical and analytical data. The

clinical data of the patients included in the study are summarized in Table 1 and Additional file 1: Table S1. We also studied a group of individuals with non-infectious systemic inflammatory response syndrome (SIRS), formed by 4 patients in the immediate postoperative period of cardiac surgery. In this group, the blood samples were obtained in the first 24 h of the postoperative period. Finally, we also included blood samples from 11 healthy controls collected from the blood donor service of La Paz University Hospital and Vall d'Hebron University Hospital. The Committee for Human Subjects of La Paz University Hospital (PIE2392) and Vall d'Hebron University Hospital (PR (ATR)122/2019) approved the study, which was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. All samples were in compliance with the guidelines approved by the local ethics committee, and all patients (sepsis, SIRS, and healthy controls) received oral and written information about the possibility that their blood would be used for research purposes and signed informed consent.

Purification of human monocytes from patients with sepsis, SIRS, and healthy control samples

Peripheral blood mononuclear cells (PBMCs) were obtained from blood by density gradient centrifugation using lymphocyte isolation solution (Rafer, Zaragoza, Spain). PBMCs were stained with CD14-PE (Miltenyi Biotec, Bergisch Gladbach, Germany), CD66b-APC (STEMCELL™ Technologies, Vancouver, Canada), and Fixable Viability Dye eFluor™ 520 (ThermoFisher Scientific, MA, USA), and then, cells were fixed with 2% formaldehyde methanol-free (ThermoFisher Scientific). Pure monocytes were isolated as CD14+CD66b− cells using flow cytometry sorting (MoFlo Astrios EQ, Beckman Coulter Spain, L'Hospitalet de Llobregat, Barcelona, Spain). Purified samples were pelleted and stored at − 80 °C.

For in vitro experiments, we obtained buffy coats from anonymous donors through the Catalan Blood and Tissue Bank (CBTB). The CBTB follows the principles of the World Medical Association (WMA) Declaration of Helsinki. Before providing blood sample, all donors received detailed oral and written information and signed a consent form at the CBTB. PBMCs were isolated by density gradient centrifugation. Then, PBMCs were resuspended in Roswell Park Memorial Institute (RPMI) Medium 1640 + GlutaMAXTM-1 (Gibco, Life Technologies, CA, USA) containing 10% human pooled serum (One Lambda, ThermoFisher Scientific Brand, Canoga Park, CA, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin, and cells were non-treated (control) or treated with 10 ng/ml LPS from *Escherichia coli* (O111:B4, Sigma-Aldrich, Darmstadt, Germany), and cultured in poly-HEMA (Santa Cruz Biotechnology, Dallas, TX, USA)-coated plates for 4 days. After that, monocytes were purified by cell sorting using the same strategy as mentioned above. Alternatively, CD14+ monocytes were isolated using positive selection with CD14 magnetic Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in the same conditions as PBMCs. Purified monocytes were pelleted and stored until DNA or RNA extraction. Supernatants were collected and stored at − 80 °C until cytokine measurement.

The endotoxin tolerance status of the septic patients and healthy donors was evaluated by exposing whole blood to a stimulus of 5 ng/ml LPS, collecting supernatant after 3 h to determine the state of innate immune system (IIS) during initial infection.

Cytokine measurements

The cytokine levels in whole blood were determined using the cytometric bead array (CBA) Flex Set (BD Biosciences, San Jose, CA, USA), following the manufacturer's protocol. The collected data were analyzed with Flow Cytometric Analysis Program (FCAP) Array Software v3.0 (BD

Table 1 Summary of the patient cohorts in the study

	Healthy controls	SIRS-cardio patients	Septic patients	*p* value (sepsis vs control)	*p* value (sepsis vs SIRS)	*p* value (SIRS vs control)
N	11	4	14			
Age (mean ± SD)	51 ± 11.8	67.8 ± 5.8	74.6 ± 14.5	0.0010***	0.3662	0.0130**
Sex (% female)	27.3	25	57.1	0.1353	0.2568	0.9299
SOFA	0	3.8 ± 2.2	3.9 ± 2.0	N/A	0.8226	N/A
Basal cytokine levels						
TNFα (pg/ml) (mean ± SD)†	9.0 ± 17.0	2.7 ± 3.1	561.4 ± 2082.8	0.9774	0.7845	0.7396
IL6 (pg/ml) (mean ± SD)†	11.9 ± 13.1	123.8 ± 101.4	4273.4 ± 10,789.5	0.0002***	0.7906	0.0050***
IL10 (pg/ml) (mean ± SD)†	5.8 ± 5.1	81.6 ± 41.4	55.6 ± 72.9	0.0005***	0.1236	0.0050***

p values of continuous data were calculated using Mann-Whitney *U* test and categorical data were calculated using chi-squared test. Statistically significant tests are represented as $^{**}p < 0.01$ and $^{***}p < 0.005$

†These correspond to the basal levels of the cytokines measured in the serum

Biosciences). For in vitro experiments, the concentration of cytokines was measured from the cell culture supernatants using an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (BioLegend, San Diego, CA, USA).

DNA methylation profiling using universal bead arrays, bisulfite sequencing, and pyrosequencing

Infinium MethylationEPIC BeadChip (Illumina, Inc., San Diego, CA, USA) array were used to analyze DNA methylation. This platform allows > 850,000 methylation sites per sample to be interrogated at single-nucleotide resolution, covering 99% of reference sequence (RefSeq) genes. The samples were bisulfite-converted using EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA) and were hybridized in the array following the manufacturer's instructions.

Each methylation data point was obtained from a combination of the Cy3 and Cy5 fluorescent intensities from the M (methylated) and U (unmethylated) alleles. For representation and further analysis, we used beta and *M* values. Beta value is the ratio of the methylated probe intensity to the overall intensity (the sum of the methylated and unmethylated probe intensities). The *M* value is calculated as the log2 ratio of the intensities of the methylated versus unmethylated probe. Beta values range from 0 to 1, in which 0 is no methylation and 1 is complete methylation, and were used to derive heatmaps and to compare DNA methylation percentages from bisulfite pyrosequencing experiments. For statistical purposes, the use of *M* values is more appropriate.

Bisulfite pyrosequencing was used to validate CpG methylation changes. DNA was isolated using ReliaPrep™ FFPE gDNA Miniprep System (Promega, Madison, WI, USA) for methylation array samples and with Maxwell® RSC Cultured Cells DNA Kit (Promega, Madison, WI, USA) for in vitro model. Bisulfite modification of genomic DNA isolated from monocytes was performed using EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocol. Primers for PCR amplification and sequencing were designed with the PyroMark® Assay Design 2.0 software (QIAGEN, Hilden, Germany). See list of primers in Additional file 2: Table S2. PCRs were performed with the IMMOLASE™ DNA polymerase PCR kit (Bioline Reagents Limited, London, UK), and the success of amplification was assessed by agarose gel electrophoresis. PCR products were pyrosequenced with the PyromarkTM Q24 system (QIAGEN, Hilden, Germany).

Quality control, data normalization, and detection of differentially methylated and variable CpGs

Methylation array data were processed in the statistical language R using methods from the Bioconductor libraries minfi, lumi, and limma. Data quality was assessed using the standard pipeline from the minfi package. The data were Snoob-normalized and, after normalization, beta and *M* values were calculated. To exclude technical and biological biases, we developed a pipeline with several filters as removing CpGs with SNPs overlapped. To minimize the potential confounding influence of age and gender, we used these parameters as covariates.

In this study, we considered a probe to be differentially methylated if it had a methylation differential of 15% ($\Delta\beta \geq 0.15$) and when the statistical test was significant [$p < 0.01$ and false discovery rate (FDR) < 0.05]. In addition, we used the iEVORA algorithm [23] to designate a probe as differentially variable. This algorithm detects the homogeneity of variances using Bartlett's test (FDR < 0.001) and then selects those probes whose *t* test is significant ($p < 0.05$ and FDR < 0.05) in order to regularize the variability test which is overly sensitive to single outliers.

Spearman's correlation was used to correlate methylation changes with cytokine concentration. Spearman's correlation coefficient is a nonparametric approach to measuring the strength of association of two variables being more reliable with non-linear data. We used the parameters specified in each section for Spearman's analysis.

Gene ontology analysis, transcription factor (TF) enrichment analysis, and chromatin state discovery and characterization (ChromHMM)

Gene ontology (GO) was analyzed using the Genomic Regions Enrichment of Annotations Tool (GREAT, version 3.0.0) (http://great.stanford.edu/public/html/). GREAT assigns biological meaning to a set of non-coding genomic regions by analyzing the annotations of the nearby genes [24]. For gene identification, we assigned a window that extends 5 kb upstream and 5 kb downstream from the differentially methylated CpG site. This window allows the analysis of CpGs located in regulatory regions distant to a TSS. Enrichment is showed as $-\log_{10}$ raw binomial *p* values.

We used the findMotifsGenome.pl program of the HOMER suite to look for motifs that are overrepresented in the target set relative to the background set (software v4.5). It was used to identify enrichment of TF binding motifs in the 500-bp window upstream and downstream of the differentially methylated CpG sites [25]. Annotated CpGs in the EPIC array were used as background.

Chromatin state discovery and characterization (ChromHMM algorithm) was used to analyze enrichment of the different chromatin states for the corresponding CpG sites [26]. The enrichment among chromatin states is defined using the 18-state

ChromHMM model (Roadmap Epigenomics Integrative Analysis Hub, ChromHMM track of the UCSC Genome Browser) (http://www.roadmapepigenomics.org/) based on six chromatin marks (H3K4me3, H3K4me1, H3K27ac, H3K36me3, H3K27me3, and H3K9me3). A Fisher's exact test was used to assign odds ratio and *p* value.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA was isolated by Maxwell® RSC simplyRNA kit (Promega, Madison, WI, USA) and reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. qRT-PCR was performed in triplicate using LightCycler® 480 SYBR Green Mix (Roche, Basel, Switzerland). Expression values were normalized against the expression of the endogenous gene controls as *RPL38*. See list of primers in Additional file 2: Table S2.

Statistical analysis

Data were analyzed with Prism version 6.0 (GraphPad). Statistical analyses were performed using the Mann-Whitney test, except as indicated. The levels of significance were as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Results

Monocytes from individuals who have undergone sepsis display an aberrant methylation signature

We first performed DNA methylation screening on monocytes, sorted from peripheral blood as CD14+CD66b− cells (Fig. 1a and Additional file 3: Figure S1), from a cohort of 14 septic patients (Table 1 and Additional file 1: Table S1) and compared it with those sorted from a cohort of 11 healthy controls. We also included 4 patients with systemic inflammatory response syndrome (SIRS) following cardiac surgery. For the analysis, we used bead arrays to interrogate the DNA methylation status of > 850, 000 CpG sites across the entire genome covering 99% of RefSeq genes. In the analysis, to minimize the potential confounding influence of age and gender (shown in Table 1), we used these parameters as covariates.

Principal component analysis (PCA) showed the two groups of monocytes from patients with sepsis and controls separated along the first principal component (Fig. 1b), with the SIRS in between. Overall, we observed a wider heterogeneity in septic monocytes than in control and SIRS monocytes (Fig. 1b), perhaps due to the diversity of infective bacteria (Additional file 1: Table S1). Monocytes from sepsis patients display 595 CpG sites with significantly higher methylation levels (hypermethylated) and 302 CpG sites with significantly lower methylation levels (hypomethylated) than control monocytes (Fig. 1c and Additional file 4: Table S3).

We performed gene ontology (GO) analysis to determine whether the differentially methylated genes were associated with relevant biological processes in sepsis. In the hypermethylated set, there was enrichment of important GO categories such as MAPK signaling pathway, NF-κB signaling pathway, and inflammation mediated by chemokine-cytokine signaling pathway. In the hypomethylated group, the functional categories were also relevant in the context of immune cell biology, including the MHC class II protein complex, genes involved in interferon-gamma (IFN-γ) signaling, and phagocytic vesicle membrane (Fig. 1d). The analysis of the chromatin states of differentially methylated CpG sites revealed the enrichment in active and weak enhancers (characterized by H3K4me1 and H3K27ac) for both the hyper- and hypomethylated sets and also for transcription start site (TSS) flanking regions in the case of the hypermethylated set (additionally marked by H3K4me3) (Fig. 1e).

We then inspected the enrichment of TF binding motifs among the two sets of differentially methylated CpG sites. We observed a significant overrepresentation of binding sites of the interferon regulatory factor (IRF) and ETS TF families in hypermethylated regions in septic monocytes (Fig. 1f). Previous reports have shown that ETS factors such as PU.1 can recruit DNA methyltransferases [27]. Hypermethylation could also antagonize the function of these TFs in endotoxin response [28]. Sequences around hypomethylated CpG sites were enriched for binding motifs of the signal transducer and activator of transcription (STAT) family (Fig. 1f). According to previous studies, the Janus kinase (JAK)/STAT pathway plays a critical role in protective immunity during sepsis via controlling cytokine responses (reviewed in [29]).

Inspection of individual genes among those containing differentially methylated CpG sites made it possible to identify some with essential functions in monocyte/macrophage biology and function. These included genes such as *IL1A*, *CCL22*, *CCR2*, and *STAT3* in the hypermethylated set and *HLA-A*, *SOCS1*, *IL1R2*, and *CD46* in the hypomethylated set (Fig. 1g and Table 2). IL1A is a pro-inflammatory cytokine. Different pro-inflammatory cytokines induce JAK activation, as well as the phosphorylation and activation of transcription activators STAT3, STAT5, and STAT6. CCL22 and CCR2 associate with leukocyte chemotaxis. HLA-A belongs to the MHC class I and is involved in antigen presentation. *SOCS1* encodes a member of the suppressor of cytokine signaling (SOCS) family that is involved in negative regulation of JAK/STAT cytokine signaling. Another example is *CD46*, encoding a costimulatory factor for T

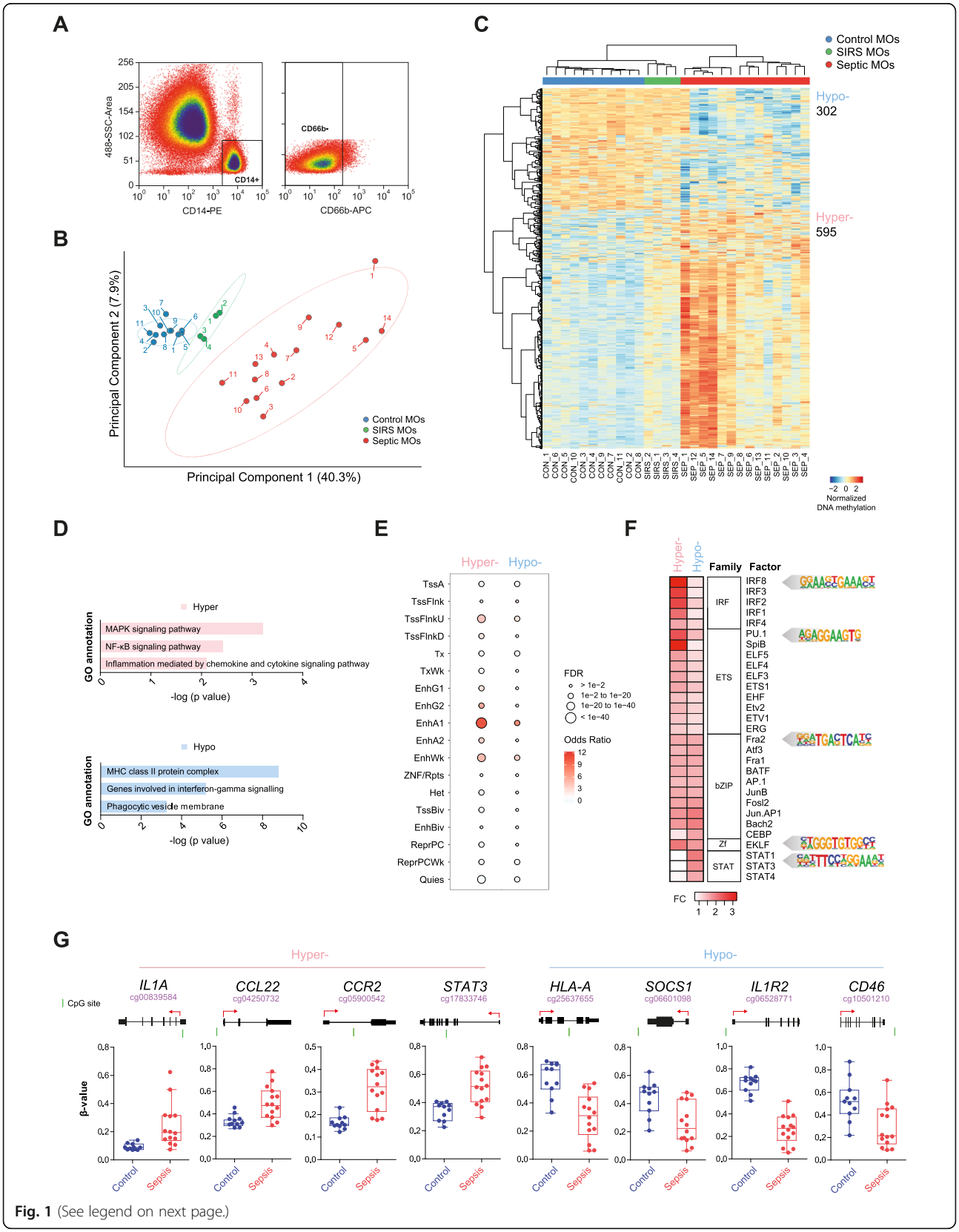

Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 Global analysis of DNA methylation changes in septic monocytes. **a** Representative flow cytometry profiles indicating the sorting strategy and gates used in this study. Monocytes (MOs) (CD14+ CD66b−) were sorted from healthy controls and patients (SIRS and sepsis). **b** Principal component analysis (PCA) of methylation heatmap data for control, SIRS, and septic monocytes (in blue, green, and red respectively). **c** DNA methylation heatmap showing differentially methylated CpGs between controls (CON, blue) and patients with sepsis (SEP, red). The heatmap includes all CpG-containing probes displaying significant methylation changes (15% of differential of beta values, $p < 0.01$ and false discovery rate (FDR) < 0.05). A scale is shown at the bottom left ranging from − 2 (lower DNA methylation levels, blue) to + 2 (higher methylation levels, red). **d** Gene ontology (GO) analysis of genes associated with differentially methylated CpG sites showing the most relevant and significantly enriched categories resulting from the Genomic Regions Enrichment of Annotations Tool (GREAT). **e** Enrichment analysis of the different chromatin states for CpG sites corresponding to each methylation cluster (left to hypermethylation, right to hypomethylation). The relative enrichment of the different states is represented using the odds ratio. Dot size represents the FDR value. Tss, transcription start site; Enh, enhancer; Repr, repressed region; PC, Polycomb. **f** TF binding motif analysis of differentially methylated CpGs between control and sepsis. The panel shows fold change (FC), TF family and factor (selected TF with $p \leq 1e^{-05}$ for hypermethylated regions and $p \leq 1e^{-03}$ for hypomethylation). Motif logo is representative of the TF family. **g** Box plots showing β-values obtained from the DNA methylation array. We observed hypermethylation and hypomethylation in important immune system genes. The CpG sites are marked with a green line in the gene scheme placed on top of each graph, where the TSS is marked with a red arrow

lymphocytes promoting T-regulatory 1 cells that suppress immune response through IL-10. All these examples highlight the relationship between the genes undergoing changes in DNA methylation and pathways related to the acquisition of endotoxin tolerance.

Septic monocytes also display increased DNA methylation variability

As indicated above, our PCA analysis indicated a higher heterogeneity in the DNA methylation profiles of monocytes from patients with sepsis. We used a recently developed algorithm, named iEVORA [23], to determine significant differentially variable CpG positions (DVPs) at an FDR < 0.05 between monocytes from patients with sepsis and healthy individuals. By using this method, we not only could confirm the occurrence of a higher number of DVPs in monocytes from patients with sepsis ($n = 6833$) versus healthy controls ($n = 148$) (Fig. 2a) but also determine that many of these sites occur at genes that also display significant DNA methylation changes (Additional file 5: Table S4).

Similarly to our previous analysis of differentially methylated sites, sepsis-associated DVPs mainly occur at enhancers and also TSS flanking regions (Fig. 2b). Sequences surrounding these DVPs were highly enriched in binding motifs of ETS and IRF families (Fig. 2c), again highlighting the role of these TFs in the acquisition of the tolerogenic phenotype of septic monocytes.

GO analysis revealed an enrichment of sepsis-associated DVPs in positive regulation of inflammatory response, leukocyte chemotaxis, and defense response to bacterium and toll-like receptor binding (Fig. 2d). We found sepsis-associated DVPs in genes important for immune response against infection and hyperinflammation such as *IL1A* and *TNF* (Fig. 2e and Additional file 5: Table S4).

All this suggests that a variety of factors encompassing bacterial infection and sepsis are driving DNA methylation and phenotypic changes in these cells in a similar manner, regardless of the infecting bacteria or individual-specific clinical outcome of the individual.

DNA methylation changes in monocytes from patients with sepsis correlate with increased IL-10 and IL-6 levels

Many of the characteristics of endotoxin-tolerized monocytes/macrophages resemble that of anti-inflammatory M2 macrophages [30, 31]. M2 macrophages show downregulated inflammatory cytokines (e.g., IL-12, TNFα) but upregulated anti-inflammatory cytokines (e.g., IL-10), scavenger receptor expression, and efficient phagocytosis. We wondered whether the DNA methylation patterns observed in monocytes from patients with sepsis might be associated with the generation of an immunosuppressive or tolerogenic environment in peripheral blood.

To address this question, we first tested the levels of a panel of cytokines associated with acute sepsis in cultured PBMCs from patients with sepsis and compared it to those in control individuals. We identified significantly increased levels of IL-10 and IL-6 in patients with sepsis (Fig. 3a). We also examined the levels of cytokines following the exposure to LPS. This analysis showed that the majority of the septic patients had acquired tolerance, with decreased levels of pro-inflammatory TNFα, IL-1β, and IL-6, following exposure to LPS, and increased secreted levels of anti-inflammatory IL-10, indicating a higher degree of tolerogenic properties on septic monocytes (Fig. 3a), as previously reported [32].

Given the greater heterogeneity of the methylation profiles of septic samples with respect to those from healthy controls, we investigated whether this could also be related to the wider range of cytokine levels, such as those observed for IL-10 or IL-6. To this end, we first performed Spearman's correlation between the DNA methylation data and the secreted IL-10 levels, for all patients with sepsis and control individuals. This analysis revealed that there are 855 CpG sites that become hypermethylated and 389 CpG sites hypomethylated in relation to increasing levels of IL-10 ($r > 0.5$; $\Delta\beta \geq 0.15$) (Fig. 3b and Additional file 6: Table S5). This analysis also showed that, for CpG sites associated with IL-10 levels, there is a significant difference of their median

Table 2 List of differentially methylated genes in septic monocytes

Gene	CpG ID	Position respect TSS	ΔBeta value	Relevance of gene in immune system
CLECL1	cg20894963	+331	0.21	C-type lectin-like protein that mediates immune cell-cell interactions.
TNFSF8	cg14107762	+2071	0.19	Pro-inflammatory cytokine that is a member of the tumor necrosis factor family and participates in defense response.
PIK3R5	cg19732273	+12206	0.20	Regulatory subunit of PI3K (phosphoinositide 3-kinase) complex. PI3Ks participate in Akt/mTOR signaling pathway that play important roles in differentiation, survival, cell growth or metabolism.
RNF216	cg07010622	+98729	0.26	E3 ubiquitin ligase that participates in protein modification; it also takes part in down-regulating TNF- and IL1- induced NFκB activation.
CCL22	cg04250732	-1029	0.16	C-C cytokine that display chemotactic activity for monocytes, dendritic cells, activated T-lymphocytes and natural killer cells to inflammatory sites.
LTA	cg00983060	-8641	0.18	Protein that belongs to the tumor necrosis factor family and could regulate inflammatory, defense and apoptotic processes.
RUNX3	cg27058497	-46	0.17	Runt-related transcription factor 3 is a member of the runt domain-containing transcription factor family that can activate or suppress the transcription of their targets through recognizing consensus DNA binding sequence 5'-PYGPYGGT-3'.
IL1A	cg00839584	+76	0.15	This cytokine is produced by monocytes and macrophages. It is a member of the interleukin 1 cytokine family which are mainly involved in inflammatory response.
IRF2	cg11802666	+50027	0.18	Encodes a member of Interferon regulatory transcription factor family. IRF2 represses IRF1-mediated transcriptional activation.
TLE1	cg20926353	+862	0.18	Transcriptional corepressor that inhibits NFκB related gene expression.
MAP3K1	cg25148456	-1861	0.17	Mitogen-activated protein kinase kinase kinase 1 acts as serine/threonine kinase activating ERK and JNK kinase cascades as well as NFκB pathway.
CD93	cg19010566	+4181	0.22	Membrane receptor that may play a role in phagocytosis in monocytes and macrophages.
CCR2	cg05900542	+2623	0.15	C-C chemokine receptor type 2 specifically mediates leukocyte chemotaxis leading to cell infiltration during inflammation processes.
CXC4R	cg07784959	+404	0.19	CXC chemokine receptor that mediates LPS-induced inflammatory responses.
STAT3	cg17833746	+50801	0.17	Signal transducer and transcription activator that mediates cellular responses to interleukins.
PRKCB	cg26562691	+3060	0.22	Serine-threonine protein kinase that mediates the activation of the canonical NFκB pathway.
IL1R2	cg06528771	-94321	0.41	Interleukin-1 receptor type II acts a decoy receptor preventing activity of IL1B or IL1A cytokines.
CD46	cg10501210	+71619	0.23	CD46 acts as a costimulatory factor for T lymphocytes promoting T-regulatory 1 cells which suppress immune response through IL-10.
HLA-A	cg25637655	+2506	0.27	HLA-A belongs to the Major Histocompatibility Complex (MHC) Class I and plays a central role presenting foreign antigens to the immune system.
ZAK	cg22093378	+75749	0.18	It is also known as mitogen-activated protein kinase kinase kinase 20 (MAP3K20). ZAK regulates JNK and p38 pathways through its kinase activity.
SOCS1	cg06601098	+30946	0.18	This gene encodes a member of suppressor of cytokine signaling (SOCS) family that is involved in negative regulation of JAK/STAT cytokine signaling.
HLA-C	cg13872627	+1827	0.16	It is a member of the MHC Class I molecules, responsible of antigen presentation during the immune response.
IL22	cg24596116	-17931	0.15	Interleukin 22 is a cytokine member of IL10 family involved in the inflammatory response through JAK/STAT and MAPKs pathways.
STAT4	cg04128669	-51040	0.20	Signal transducer and transcription activator that mediates cellular responses to interleukins.
S100A8	cg04681218	+6180	0.22	Member of the S100 protein family which has an important role in the regulation of inflammatory processes.

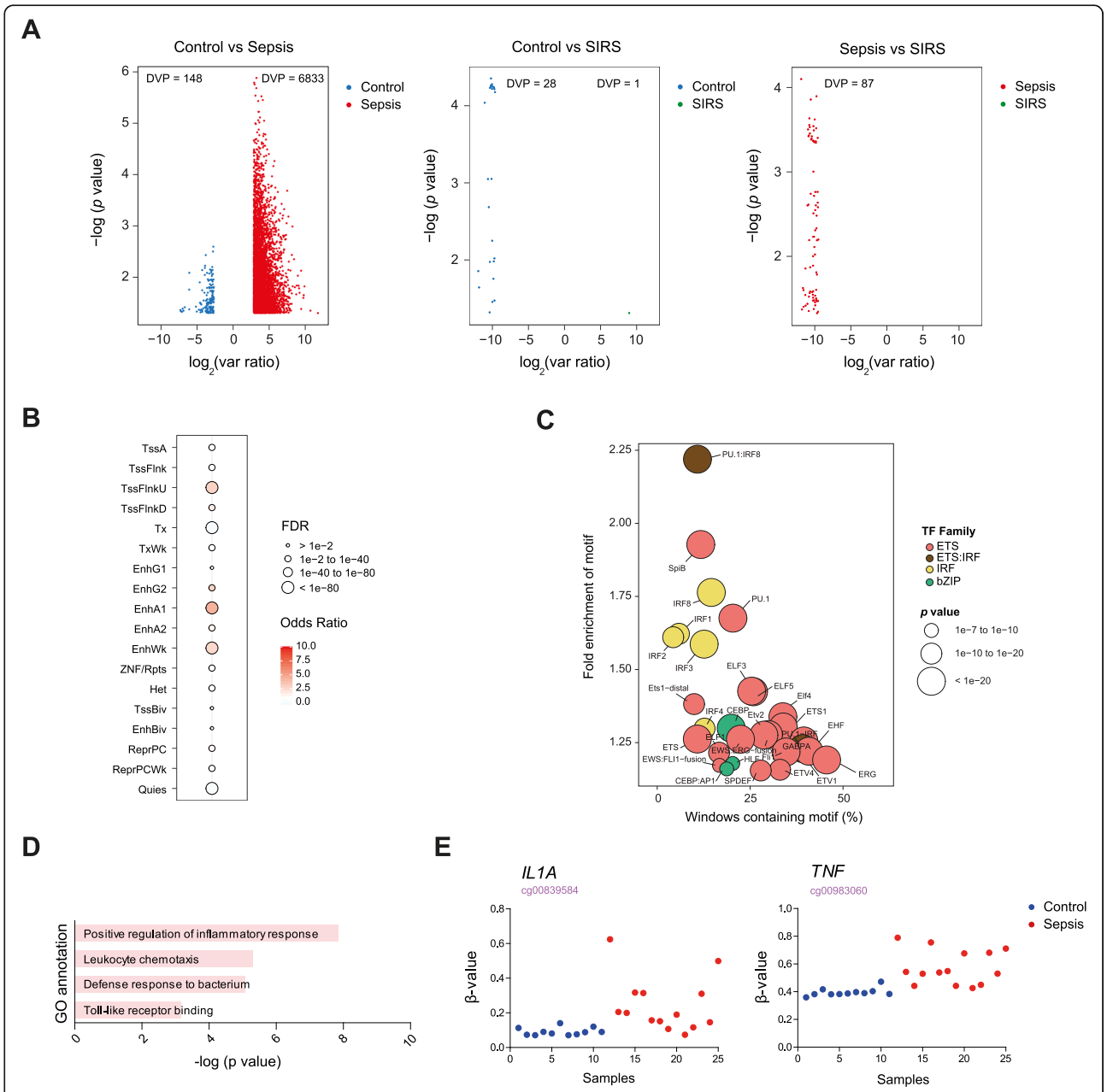

Fig. 2 Analysis of differentially variable CpG positions (DVPs) in monocytes from patients with sepsis. **a** Volcano plot showing the *p* value vs the variance ratio for healthy control, SIRS, and sepsis-associated DVPs. DVPs were identified using the algorithm iEVORA. **b** Enrichment of sepsis-associated DVPs at 18 distinct chromatin states using ChromHMM. **c** TF binding motif analysis for sepsis-associated DVPs. Bubbles are colored according to TF family. The *p* value is indicated by bubble size (selected TF with $p \leq 1e^{-07}$ for DVP regions). **d** GO categories resulted from GREAT analysis for sepsis-associated DVPs of section (**a**). **e** Representative examples showing beta values of DVPs

DNA methylation levels between septic and healthy controls (Fig. 3c), reinforcing the notion of the existence of differential methylation patterns between control and sepsis in addition to a contribution of IL-10 to the acquisition of such changes. Spearman's correlation between the DNA methylation data and the IL-6 levels identified 2492 CpG sites becoming hypermethylated and 909 CpG sites hypomethylated in relation to increasing levels of IL-6 ($r > 0.5$; $\Delta\beta \geq 0.1$) (Additional file 7: Figure S2A and Additional file 8: Table S6).

CpG sites displaying changes in methylation in relation to IL-10 levels enriched for specific chromatin features (Fig. 3d). Specifically, we found enrichment for enhancers in both the hypermethylated and hypomethylated sets. We also investigated for this set of CpG sites the enrichment in TF binding motifs for these differentially

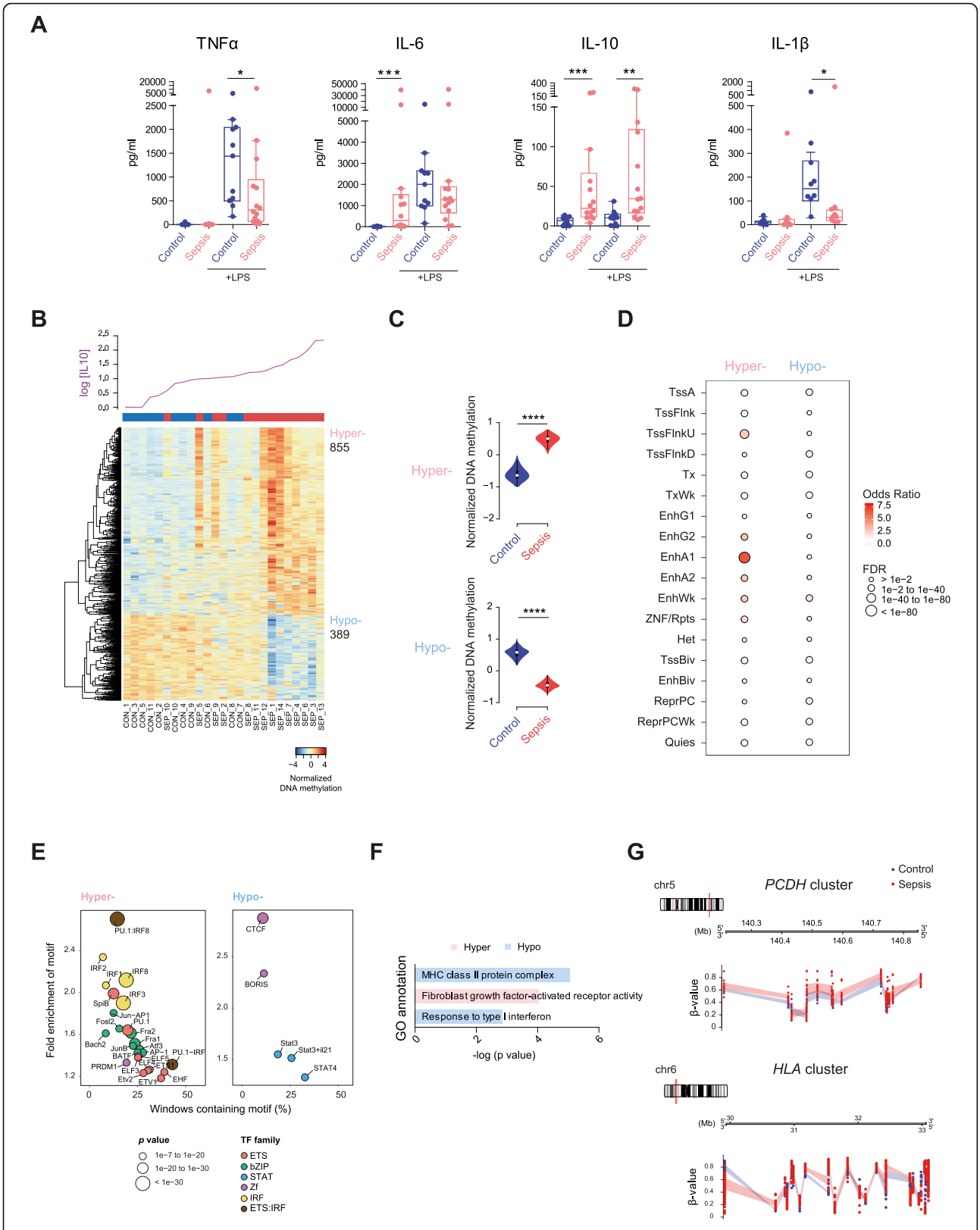

Fig. 3 (See legend on next page.)

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Fig. 3 DNA methylation changes in septic monocytes parallel the increase of IL-10 levels. **a** Cytokine measurement using cytometric bead array (CBA) from control and septic PBMCs before and after LPS stimulation ($t = 3$ h). Box and whisker plots show median values. Mann-Whitney test was used to determine significance (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). **b** DNA methylation heatmap of CpG changes in relation to IL-10 basal concentration (represented on the top of the heatmap as log scale). Spearman's correlation was used with $p < 0.01$, $r > 0.5$, and $\Delta\beta \geq 0.15$. A scale is shown at the bottom, wherein beta values range from − 4 (lower DNA methylation levels, blue) to + 4 (higher methylation levels, red). **c** Violin plots corresponding to the 5mC-normalized data for control and sepsis presented in the heatmap in the previous section. The median and the interquartile range are represented. **d** Chromatin state characterization of differentially methylated sites for section **b**. The relative enrichment of the different state assignments is representing using the odds ratio. FDR is represented by the size of the dots. **e** Bubble scatterplot of TF enrichment for hypermethylated and hypomethylated CpGs. The *x*-axis shows the percentage of windows containing the motif, and the *y*-axis shows the fold enrichment of the motif. Bubbles are colored according to TF family. *p* value is indicated by the bubble size (selected TF with $p < 1e^{-07}$ for hypermethylated and hypomethylation regions). **f** GO categories resulted from GREAT analysis of differentially methylated CpGs related to IL-10 concentration. **g** Genomic tracks representing the clusters of differentially methylated CpGs for protocadherins (*PCDH*, upper scheme) and human leukocyte antigen (*HLA*, lower panel). Blue and red lines represent the confidence intervals for each average values. A window of ± 50,000 bp was used

methylated CpG sites. We observed that approximately 25% of the hypermethylated CpG sites have motifs for bZIP TF factor family (Jun-AP-1) and 25% of the hypomethylated CpG sites display motifs for STAT family members (Fig. 3e), suggesting that methylation might be driven by the stimulation of TLR by bacteria and the increased levels of IL-10, stimulating its receptor IL-10R. Interestingly, 15% of the hypomethylated sequences displayed enrichment of CTCF binding motifs, whose global occupancy has been linked to differential DNA methylation [33]. Similar results were obtained when we analyzed the data for the Spearman's correlation with IL-6 levels (Additional file 7: Figure S2C and S2D).

GO analysis revealed distinct enriched biological processes for hypo- and hypermethylated CpG clusters in relation to IL-10 levels, including MHC class II protein complex and response to type I IFN, and fibroblast growth factor-activated receptor activity respectively (Fig. 3f). Of note, similar GO categories were also enriched for differentially methylated CpGs correlating to IL-6 levels (Additional file 7: Figure S2B).

Our data show that many members of Wnt signaling pathway (*WNT3A*, *WNT6*, and *AXIN2* among others) (Additional file 6: Table S5) display a gain of methylation in septic monocytes compared with their healthy counterparts, highlighting the potential link between aberrant DNA methylation and the Wnt pathway. In fact, cumulative evidence supports the role of the Wnt pathway in the regulation of the macrophage-mediated inflammatory response in sepsis [34], in which Wnt3a and Wnt6 reduce TNFα secretion and promote the differentiation towards an M2 anti-inflammatory phenotype attenuating the immune response [35]. Furthermore, we found a large genomic region that also displayed DNA hypermethylation in which it predominantly covered CpG sites in the three tandem gene clusters of protocadherin (*PCDHA*, *PCDHB*, and *PCDHG*) (Fig. 3g) (Additional file 6: Table S5). This region has previously been reported to undergo aberrant DNA hypermethylation in cancer and in other disorders [36, 37]. Moreover, recent studies have identified mechanisms by which PCDHs can regulate the Wnt pathway (reviewed in [38]), which further corroborates the Wnt pathway as a putative therapeutic target for the patient treatment.

Regarding the hypomethylated CpG sites, we found an enrichment in genes involved in the IFN-γ pathway, which is essential for antimicrobial defense and restoring monocyte deactivation in patients with sepsis [39]. Remarkably, among the CpG sites displaying changes in methylation in relation to IL-10 levels, we identified 23 CpG sites within the HLA cluster, which is also induced by the IFN-γ and JAK/STAT pathway [40] (Fig. 3g).

Monocytes exposed to LPS undergo DNA methylation changes in parallel with the acquisition of endotoxin tolerance

Our results suggest that TLR stimulation and the inflammatory environment generated in the context of systemic bacterial infection are able to induce DNA methylation changes in monocytes. First, the specific DNA methylation profiles of monocytes from patients with sepsis associate with IL-10 and IL-6 levels. Second, there is an enrichment of binding motifs for AP-1 and STATs. This suggests that both the stimulation of TLRs, the resulting inflammatory conditions, and subsequent anti-inflammatory signals, participate in shaping the generation of aberrant methylation profiles which might modulate and stabilize the phenotype of monocytes following a septic episode. We therefore explored the possibility of inducing in vitro DNA methylation changes observed in such conditions by exposing in parallel PBMCs and monocytes from healthy individuals to LPS, and compare it with PBMCs and monocytes without such stimulation (Fig. 4a). We cultured the cells for 4 days and measured the acquisition of tolerance. In the case of PBMCs, we isolated monocytes, sorted as CD14+CD66b− cells after these

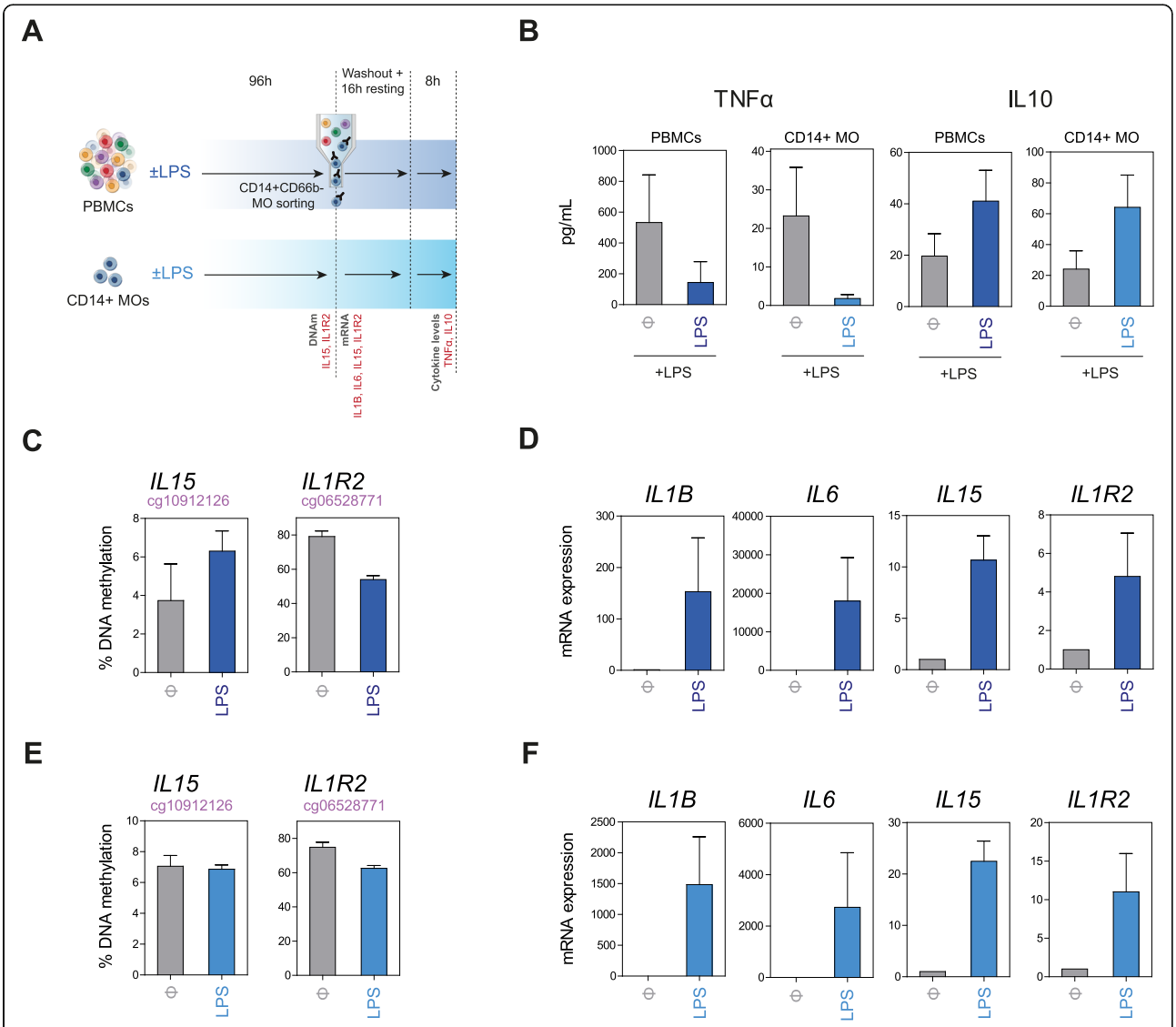

Fig. 4 In vitro exposure to LPS induces the acquisition of tolerance and DNA methylation changes similar to those observed in sepsis. **a** Schematic diagram depicting our in vitro model for sepsis. PBMCs were cultured with or without LPS during 4 days, and then, monocytes were sorted as CD14+CD66b− cells for subsequent analyses. In parallel, monocytes were isolated with magnetic CD14 antibody and cultured in the same conditions. **b** TNFα and IL-10 production, as determined by ELISA, from PBMCs or monocyte supernatants, following washout and resting following the 4 initial days (with/without LPS) and an 8-h exposure to LPS, as indicated in the scheme in **a**. **c** Bisulfite pyrosequencing of selected hypermethylated (*IL15*) and hypomethylated (*IL1R2*) genes in sorted CD14+CD66b− monocytes from PBMCs in the in vitro sepsis model. **d** *IL1B*, *IL6*, *IL15*, and *IL1R2* mRNA levels were analyzed by quantitative RT-PCR using *RPL38* as control in the same sorted monocytes. **e** Bisulfite pyrosequencing of *IL15* and *IL1R2* in CD14+ monocytes in the in vitro sepsis model. **f** *IL1B*, *IL6*, *IL15*, and *IL1R2* mRNA levels analyzed by quantitative RT-PCR in CD14+ monocytes

4 days (Fig. 4a, top). These experiments were performed in the presence of poly-2-hydroxyethyl methacrylate (poly-HEMA), an agent that restricts the attachment of the monocytes to the plates and therefore their differentiation to macrophages, to prevent the occurrence of vast changes in DNA methylation [19, 20]. Enzyme-linked immunosorbent assays (ELISA) revealed in both cases the acquisition of tolerance following the initial encounter with LPS. Specifically, these assays showed decreased levels of TNFα and increased levels of IL-10 in a second LPS exposure for those cells that had a first exposure to LPS (Fig. 4b). We then performed pyrosequencing of a selection of hypo- and hypermethylated genes in septic monocytes. Our analysis revealed that the in vitro stimulation with LPS is able to induce DNA methylation changes in these genes (Fig. 4c) with a similar trend to the changes observed in patients with sepsis when

PBMCs were exposed to LPS (followed by monocyte purification). In the case of monocytes directly exposed to LPS, we were able to stimulate only demethylation of the *IL1R2* gene, suggesting that perhaps signals from other cell types are necessary to induce gains of methylation following LPS treatment. Interestingly, changes in DNA methylation at the aforementioned sites occurred in association with changes in gene expression of these genes (Fig. 4d, e) in the two sets of samples exposed to LPS, and not in controls. In addition, we also observed an increase in mRNA levels of pro-inflammatory cytokines (IL-1β and IL6) in LPS-treated cells. These results proved that both gains and losses of DNA methylation and expression in septic monocytes are the result of TLR stimulation and the generation of an inflammatory environment and are associated with the acquisition of a tolerized state in monocytes.

Organ dysfunction associates with DNA methylation changes

We finally tested DNA methylation profiles in relation to SOFA, the main score used to assess organ dysfunction. When using Spearman correlation, we determined that there are 1890 CpG sites that become hypermethylated and 1536 CpG sites hypomethylated in relation to increasing SOFA (p value < 0.01; $r > 0.6$) (Fig. 5a and Additional file 9: Table S7). GO analysis revealed that DNA methylation changes in relation to SOFA affect inflammatory response and antigen presentation in a similar manner than previous comparisons (Fig. 5b). We also investigated for this set of CpG sites the enrichment in TF binding motifs for these differentially methylated CpG sites. We obtained similar sets of TF binding motifs (Fig. 5c) like the ones observed for the correlations with IL-10 and IL-6, suggesting the participation of these inflammatory cytokines in the acquisition of DNA methylation changes in relation to organic damage. Finally, individual inspection of the lists revealed the association of several relevant molecules including HLA-A, IL19, IL15, and IL27 (Fig. 5d). Altogether, this analysis suggested that the DNA methylation changes associated with organic damage involve similar changes to those observed in relation to inflammatory cytokines.

Discussion

Our study demonstrates for the first time the existence of DNA methylation alterations in human monocytes from individuals following a sepsis episode in relation to the acquisition of a tolerized phenotype, paralleling data obtained in a mouse model [41]. Most notably, changes occur in genes relevant to the function of these cells including the interferon-gamma-mediated pathway and MHC class II proteins. On the one hand, the observed methylation changes in patients with sepsis suggest their participation among the mechanisms leading to the generation of an aberrant phenotype of these cells. On the other hand, correlation analyses of the DNA methylation profiles in relation to IL-10 and IL-6 levels, which are increased in patients with sepsis, suggest a potential mechanism downstream to these cytokines participating in the defective generation of DNA methylation alterations. Furthermore, in vitro analysis of the influence of bacterial LPS and inflammatory context in determining the acquisition of DNA methylation alterations in monocytes also shows how these changes associate with aberrant transcriptional levels of dysregulated genes. Finally, our analysis shows increasing changes in DNA methylation in relation to organ dysfunction.

Epigenetic factors play a role in the acquisition of endotoxin tolerance. In fact, seminal studies by the teams of Netea, Logie, and Stunnenberg have shown that the transcriptional inactivity in response to a second LPS exposure in tolerized macrophages is accompanied by a failure to deposit active histone marks at promoters of tolerized genes [17]. It has also been reported that leukocytes of patients with sepsis have defects in important metabolism pathways and, interestingly, these immunometabolic defects were partially restored by therapy with recombinant IFN-γ [7].

The aforementioned studies paid less attention to DNA methylation changes, as they appeared to be less prevalent than those at occurring in histone modifications. However, DNA methylation is a relatively more stable epigenetic mark than histone modifications. That property makes this modification worth of study: firstly, because it might have a long-term contribution to the tolerized state of monocytes, and secondly, because it could potentially be used as a marker, if associations with patient prognosis and/or progression were found.

Many of the genes displaying differentially methylated CpG sites between patients with sepsis and controls occur within a limited number of pathways relevant to the stimulation associated with bacterial infection (Fig. 6). For instance, genes experiencing changes in methylation such as *IL1A*, *IL1R2*, *IL1R1*, *TAB2*, *TAB1*, *MAP 2K1*, and *MAP 3K1* occur within the same signaling pathway, which is also merging from the signals downstream to TNF. On the other hand, several of the genes encoding for cytokines like *IL27*, *IL23A*, *IL19*, and *IL22* also display CpG sites undergoing methylation changes, as well as genes encoding elements downstream to it, such as *TYK2*, *JAK1*, *STAT*3 and 4, and *SOCS3* and *SOCS5*. The relationship between all these genes suggests two possibilities: that DNA methylation changes at these sites have a causal effect in determining the activation or repression of the associated genes or, alternatively, that the monocyte methylome is acting as a sensor of the activation of these pathways through additional or alternative mechanisms.

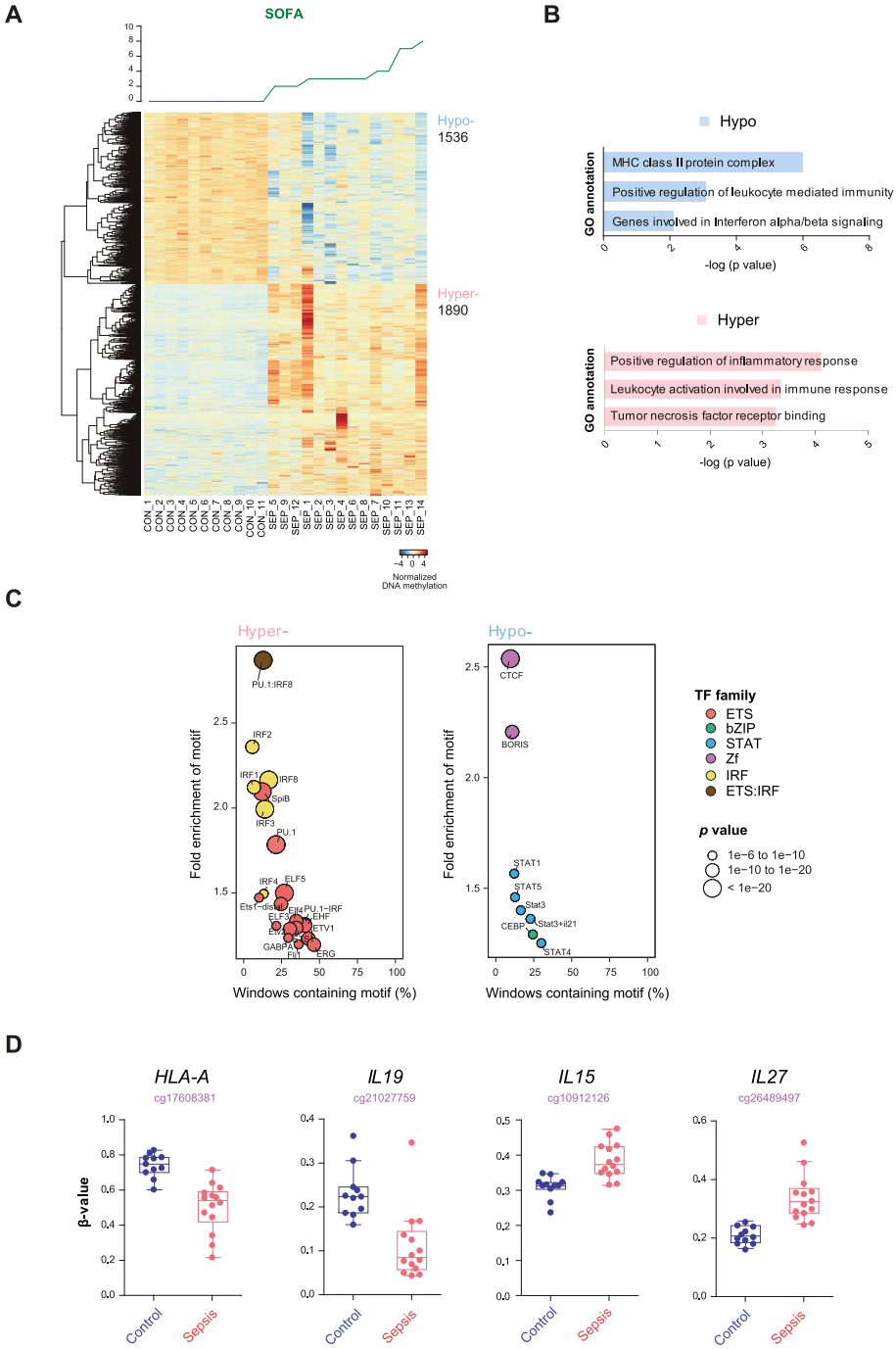

Fig. 5 DNA methylation changes in septic monocytes parallel organic damage. **a** DNA methylation heatmap of CpG changes in relation to the SOFA score (represented on the top of the heatmap). Spearman's correlation was used with $p < 0.01$, $r > 0.6$. A scale is shown at the bottom, wherein beta values range from − 4 (lower DNA methylation levels, blue) to + 4 (higher methylation levels, red). **b** GO categories resulted from GREAT analysis of differentially methylated CpGs related to SOFA. **c** Bubble scatterplot of TF enrichment for hypermethylated and hypomethylated CpGs. The *x*-axis shows the percentage of windows containing the motif, and the *y*-axis shows the fold enrichment of the motif. Bubbles are colored according to TF family. *p* value is indicated by bubble size (selected TF with $p \leq 1e^{-06}$ for hypermethylated and hypomethylation regions). **d** Box plots showing *β*-values obtained of genes significantly correlating with the SOFA score

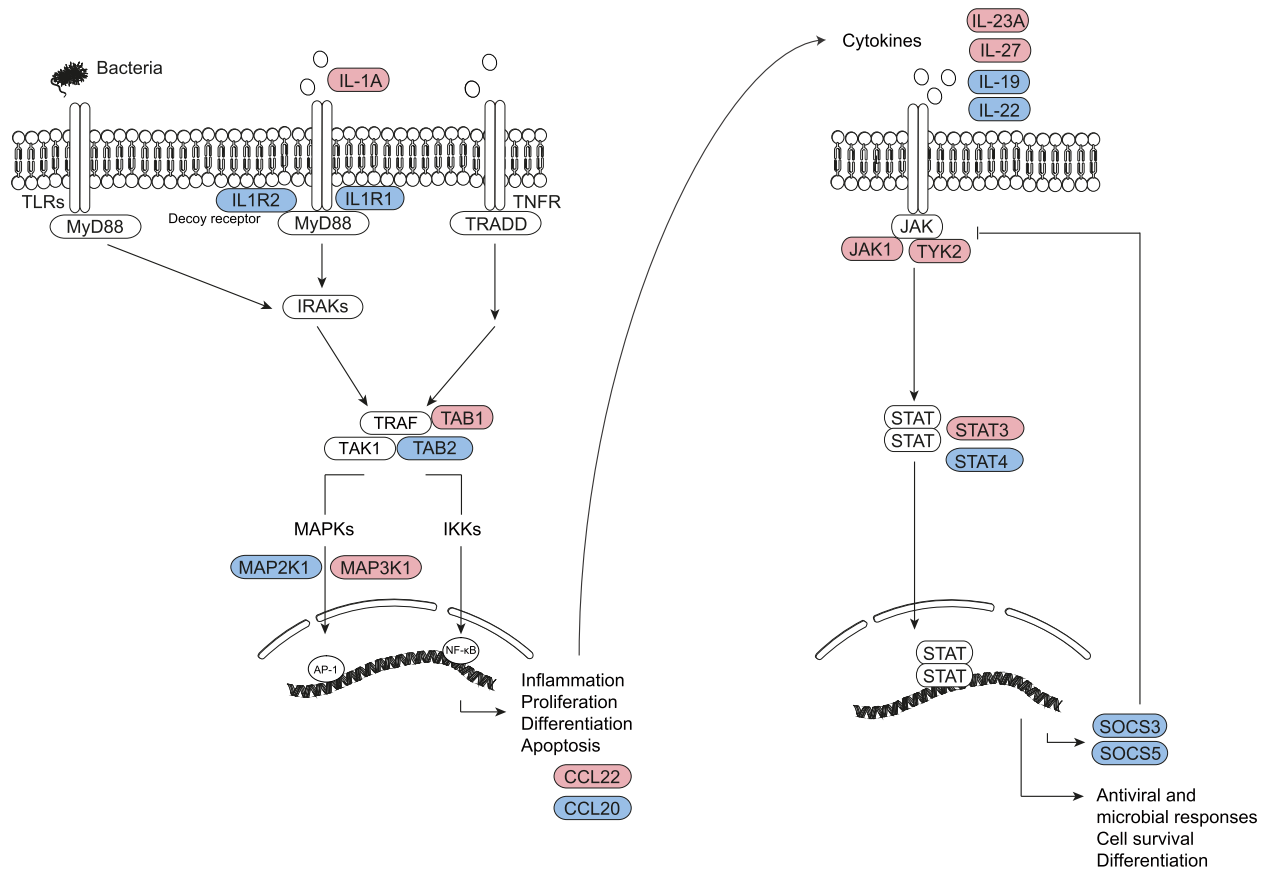

Fig. 6 Scheme depicting important signaling pathways related to immunity and sepsis. Molecules whose encoding genes displayed DNA methylation alterations in this study are shown in red and blue for hypermethylation and hypomethylation respectively. The following proteins/genes are represented in the figure: TLR, Toll-like receptor; MyD88, myeloid differentiation primary response 88; IRAK, interleukin-1 receptor-associated kinase; IL1R, interleukin-1 receptor; IL-1A, interleukin 1 alpha; TNFR, tumor necrosis factor receptor; TRADD, TNFR1-associated death domain; TRAF, TNF receptor-associated factor; TAK1, transforming growth factor (TGF) beta-activated kinase 1; TAB2, TGF beta-activated kinase 1 binding protein 2; MAPK, mitogen-activated protein kinase; IKK, IκB kinase; AP-1, activator protein 1; NF-κB, nuclear factor kappa B; CCL20, C-C motif chemokine ligand 20; CCL22, C-C motif chemokine ligand 22; IL-23A, interleukin 23A; IL-19, interleukin 19; IL-27, interleukin 27; IL-22, interleukin 22; JAK1, Janus kinase 1; STAT, signal transducer and activator of transcription; TYK2, tyrosine kinase 2; SOCS, suppressor of cytokine signaling. In this scheme, we have selected CpG sites/genes with a minimum 10% of differential of beta values, $p < 0.01$ and false discovery rate (FDR) < 0.05

In this respect, our results show that monocytes from patients with sepsis harbor an aberrant DNA methylation signature that is related with the abnormal environment derived from such inflammatory condition. Furthermore, we have determined that DNA methylation changes correlate with increased IL-10 and IL-6 levels and that those changes are functionally annotated to genes belonging to the Wnt and IFN-γ signaling pathways. In this respect, it has been demonstrated, as mentioned above, that IFN-γ-based therapy can partially restore the defective metabolic changes occurring in leukocytes from patients with sepsis [7].

Finally, our investigation demonstrates the sensitivity of monocytes to translate environmental changes into more stable changes at the transcriptional level through DNA methylation. Given that sepsis is associated with the generation of a particular cytokine environment [42], our results reinforce the notion that epigenetic changes are related to the maintenance of the dysregulated immune response following an episode of sepsis. However, with the existing data generated in this study, we cannot distinguish whether these epigenetic changes are a cause or a consequence: in other words, whether the DNA methylation changes are caused by these individuals' infection history, perhaps influenced by the environment, or whether environmental factors cause the generation of the aberrant DNA methylation signature, which is then accompanied by immune responses that are secondary to the sepsis. It is likely that the aberrant DNA methylation (and expression signature) generated as a result of the particular cytokine milieu generated under the sepsis episode contributes to perpetuating the

tolerized state of these monocytes. Whether those changes also affect bone marrow monocyte progenitors giving long-lasting reprogramming, as occurs with trained immunity [43], remains to be investigated. Interestingly, the observed changes appear to be reflective of the infection, as it suggests the data obtained for SIRS patients (even if it is a small cohort), which are also characterized by inflammation and organ dysfunction.

A potential limitation of our study is the size and characteristics of the cohort. In future studies, it would be necessary to use vaster cohorts, including patients with a representative number of Gram-positive and Gram-negative bacteria and patients at different stages following the sepsis episode. However, the size and features of our cohort, on the other hand, indicate that common changes in DNA methylation are associated with sepsis regardless of the infective bacteria. The identification of specific DNA methylation markers associated with the infecting organism of the clinical outcome of the patient will surely be useful for predicting the evolution of the patients and perhaps their clinical management.

Conclusions

In the present study, we have shown that patients with sepsis undergo widespread changes in the methylome of their circulating monocytes in parallel with the acquisition of endotoxin tolerance. Thousands of changes are associated with the aberrant levels of IL-10 and IL-6, as well as with organ dysfunction. Stimulation of the Toll-like receptor in monocytes induces similar changes in DNA methylation and expression, concomitant with the acquired tolerance that points to a major role in the stabilization of a tolerized phenotype through these alterations. Our results open up possibilities not only to use DNA methylation as a marker for disease but also for understanding its role in the acquisition of the aberrant phenotype of these cells.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13073-019-0674-2.

Additional file 1: Table S1. Clinical features of septic patients, SIRS and healthy controls included in this study.

Additional file 2: Table S2. Primers sequences.

Additional file 3: Figure S1. Purification and quality of monocytes. (a) Flow cytometry profiles indicating the sorting strategy and gates used in the study. (b) Cell type deconvolution of the hybridized samples using Houseman algorithm.

Additional file 4: Table S3. List of hypermethylated and hypomethylated genes in septic monocytes (p-value< 0.01; FDR < 0.05; $\Delta\beta \geq 0.15$).

Additional file 5: Table S4. List of differentially variable CpG positions (DVPs).

Additional file 6: Table S5. List of hypermethylated and hypomethylated CpGs related to IL-10 cytokine.

Additional file 7: Figure S2. DNA methylation changes in septic monocytes parallel the increase of IL-6 levels. (a) DNA methylation heatmap of CpGs changes in relation to IL-6 basal concentration. Spearman's correlation was used with $p < 0.01$, $r > 0.5$ and differential β-value ≥0.1. A scale is shown at the bottom, wherein beta values range from − 4 (lower DNA methylation levels, blue) to + 4 (higher methylation levels, red). (b) GO categories for differentially methylated CpGs of section (a). (c) Enrichment of differentially hyper- and hypo-methylated CpGs among chromatin states, defined using the 18-state ChromHMM model. (d) HOMER motif analysis for methylation changes. The x-axis shows the percentage of windows containing the motif and the y-axis shows the fold enrichment of the motif. Bubbles are colored according to TF family. p value is indicated by bubble size (TF with $p < 1e^{-15}$ for hypermethylated regions and $p \leq 1e^{-5}$ for hypomethylation were represented).

Additional file 8: Table S6. List of hypermethylated and hypomethylated CpGs related to IL-6 cytokine.

Additional file 9: Table S7. List of hypermethylated and hypomethylated CpGs related to SOFA.

Abbreviations

AP-1: Activator protein-1; CBA: Cytometric bead array; CTF/NF1: CCAAT box-binding transcription factor/nuclear factor I; DNMT: DNA (cytosine-5)-methyltransferase; DVPs: Differentially variable CpG positions; ELISA: Enzyme-linked immunosorbent assay; FDR: False discovery rate; GO: Gene ontology; HDAC: Histone deacetylase; HLA: Human leukocyte antigen; IKK: IκB kinase; IL: Interleukin; IFN-γ: Interferon-gamma; IRF: Interferon regulatory factor; JAK: Janus kinase; LPS: Lipopolysaccharide; MAPK: Mitogen-activated protein kinase; MHC: Major histocompatibility complex; MyD88: Myeloid differentiation primary response 88; NF-κB: Nuclear factor kappa B; PBMCs: Peripheral blood mononuclear cells; PCA: Principal component analysis; PCDH: Protocadherins; poly-HEMA: Poly-2-hydroxyethyl methacrylate; SNP: Single-nucleotide polymorphism; SOCS: Suppressor of cytokine signaling; STAT: Signal transducer and activator of transcription; TET2: Ten-eleven translocation methylcytosine dioxygenase 2; TF: Transcription factor; TGF: Transforming growth factor; TLR: Toll-like receptor; TNF: Tumor necrosis factor; TRAF: TNF receptor-associated factor; TSS: Transcription start site; TYK2: Tyrosine kinase 2

Authors' contributions

EB, CL-S, and DAE conceived the experiments; CL-S, AG-G, VT, and LC performed the experiments; AG-G and FC-M performed the biocomputing analysis; VT, JA-O, CM-E, AM-Q, MM-G, AR-S, CR-R, RF-R, AGC, and EL-C provided the samples; CL-S, AG-G, FC-M, VT, DAE, EL-C, and EB analyzed the data; EB wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

Methylation array data for this publication have been deposited in NCBI's Gene Expression Omnibus.
Lorente-Sorolla C, García-Gómez A, Català-Moll F, Toledano V, Ciudad L, Avendaño-Ortiz J, Maroun-Eid C, Martín-Quirós A, Martínez-Gallo M, Ruiz-Sanmartín A, García del Campo Á, Ferrer-Roca R, Ruiz-Rodriguez JC, Álvarez-Errico D, López-Collazo E, Ballestar E. Inflammatory cytokines and organ dysfunction associate with the aberrant DNA methylome of monocytes in sepsis. Methylation profiling by array of CD14+CD66bneg monocytes of 11 healthy donors, 4 SIRS, and 14 septic patients datasets. GEO Series accession number GSE138074.

Ethics approval and consent to participate

The Committees for Human Subjects of La Paz University Hospital (PIE2392) and Vall d'Hebron University Hospital (PR (ATR)122/2019) approved the study, which was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. All samples were in compliance with the

guidelines approved by the local ethics committee. All participants provided written informed consent to participate in the study.

Consent for publication
Written informed consent for publication was provided by the participants.

Competing interests
The authors declare that they have no competing interests.

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