SCUOLA DI DOTTORATO
UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

Dipartimento di Biotecnologie e Bioscienze

PhD in Biotechnology and Biosciences Cycle: XXX

Curriculum in Biotechnology

Toll-like Receptor 4 (TLR4) therapeutic modulation: a chemical biology approach

Surname: Facchini Name: Fabio Alessandro

Registration number: 713369

Supervisor: Prof. Francesco Peri

Co-tutor: Prof. Paola Fusi

Coordinator: Prof. Marco Ercole Vanoni

ACADEMIC YEAR: 2016/2017

TABLE OF CONTENTS

Abstract

Among the first receptors activated during host-pathogen interactions are Toll-like receptors (TLRs), which detect pathogen-associated molecular patterns (PAMPs) to induce innate and adaptive immune responses. TLR4 is the mammalian sensor of bacterial endotoxin, lipopolysaccharide (LPS). Dysregulated TLR4 activation is involved in acute systemic sepsis and in many disorders that involve inflammation, such as inflammatory bowel diseases (IBDs), rheumatoid arthritis (RA) and other neuroinflammatory and neurodegenerative disorders. Therefore, therapeutic modulation of the TLR4 signalling is of major interest. This PhD thesis is based on three papers (Chapter 1-3) and has the aim to study the capacity of synthetic small molecule TLR4 antagonists, alone or in combination with antimicrobial peptides (AMPs), to act as therapeutics in inflammatory diseases.

In Chapter I, starting from the assumption that opportunely designed cationic amphiphiles can behave as CD14/MD-2 ligands and therefore modulate the TLR4 signaling, we present the rational design and biological characterization of a panel of amphiphilic guanidinocalixarenes. The structure of these compounds was computationally designed and optimized to dock into MD-2 and CD14 binding sites. We found that some of these calixarenes were active in inhibiting, in a dose-dependent way, the LPS-stimulated TLR4 activation and TLR4-dependent cytokine production in human and mouse immune cells. Moreover, cationic guanidinocalixarenes also inhibited TLR4 signaling when TLR4 was activated by a stimulus different from bacterial LPS, the plant lectin PHA. While the activity of guanidinocalixarenes in inhibiting LPS toxic action has previously been related to their capacity to bind and neutralize LPS, the

results obtained in this chapter suggest a direct antagonist effect of calixarenes on TLR4/MD-2 dimerization: this suggests the use of the calixarene scaffold for the development of new TLR4-directed modulators.

In Chapter II is presented the effect of co-administration of antimicrobial peptides (AMPs) and a synthetic TLR4 antagonist (the glycolipid FP7) on TLR4 activation and signalling. The co-administration of two LPS-neutralizing peptides (a cecropin A-melittin hybrid peptide and a human cathelicidin) enhances by an order of magnitude the potency of FP7 in blocking the TLR4 signal. Interestingly, this potentiation effect also occurs when cells are stimulated with a non-LPS TLR4 agonist. Our data suggest a dual mechanism of action for the peptide/glycolipid combination, not exclusively based on LPS binding and neutralization, but also on a direct effect on CD14 and MD-2 binding. NMR experiments in solution show that peptide addition changes the aggregation state of FP7, promoting the formation of larger micelles. These results suggest a relationship between the aggregation state of lipid A-like ligands and the type and intensity of TLR4 response.

Chapter III describes a preclinical study in which the potent and selective TLR4 antagonist FP7 is used in an experimental model of inflammatory bowel disease (IBD). This study has the aim to evaluate a possible therapeutic strategy based on the use of small molecule that selectively targets TLR4/MD-2 complex to reduce IBD inflammation. The results obtained show that FP7 reduced the secretion of the main LPS-induced innate pro-inflammatory cytokines by peripheral blood mononuclear cells (PBMCs) and lamina propria mononuclear cells (LPMCs) isolated from IBD patients. FP7 anti-inflammatory effect is due to a reduced activation of the main myeloid differentiation primary response gene (88) (Myd88)-depedent pathway effectors normally

induced by LPS presence. We indicated that the mechanism of action of FP7 is related to its capacity to compete with LPS for the binding to MD-2 adaptor protein and to CD14 co-receptor. FP7 also reduced inflammation *in vivo* on a murine model of ulcerative colitis. Considering that IBD pathogenesis is associated to an abnormal innate immune response towards microbial antigens, TLR4 inhibition by chemical agents as FP7 emerged as a promising alternative approach to IBD treatment.

Riassunto

I recettori Toll-like (TLR) sono una famiglia di recettori attivati in molte interazioni ospite-patogeno. Tali recettori hanno il compito di riconoscere pattern molecolari associati a patogeni (PAMP) e di indurre la risposta immunitaria innata e adattiva nell'ospite. Il TLR4 permette ai mammiferi di rilevare l'endotossina dei batteri Gram-negativi, il lipopolisaccaride (LPS). L'attivazione incontrollata di questo recettore è alla base di sepsi sistemica acuta e di molti disturbi infiammatori, come le malattie infiammatorie intestinali (IBD), l'artrite reumatoide (RA) e altri disturbi neuroinfiammatori e neurodegenerativi. Pertanto, la modulazione terapeutica del TLR4 è di estremo interesse. Questa tesi di dottorato si basa su tre articoli (capitolo 1-3) e ha lo scopo di studiare il potenziale ruolo terapeutico anti-infiammatorio di piccole molecole antagoniste sul TLR4, testate da sole o in combinazione con peptidi antimicrobici (AMP). Considerando che molecole anfifiliche cationiche opportunamente disegnate possono modulare l'attivazione del TLR4, interagendo con i co-recettori CD14 e MD-2, nel capitolo I mostriamo la progettazione e la caratterizzazione biologica di una serie di calixareni anfifilici. La struttura di questi composti è stata progettata mediante studi computazionali in modo da poter interagire con i recettori MD-2 e CD14. Alcuni calixareni hanno dimostrato di poter inibire l'attivazione del TLR4 indotta da LPS in modo dose-dipendente e di ridurre la produzione di citochine mediata da TLR4 in cellule immunitarie umane e murine. Inoltre, i guanidinocalixareni cationici sono in grado di inibire l'attivazione del TLR4 indotta dalla lectina PHA. Sebbene l'attività inibitoria dei guanidinocalixareni nei confronti del TLR4 sia stata precedentemente correlata alla loro capacità di neutralizzare l'LPS, i risultati ottenuti in questo capitolo suggeriscono un

effetto antagonista diretto di tali molecole sul complesso TLR4/MD-2. Pertanto, la struttura dei calixareni potrebbe rivelarsi utile per lo sviluppo di nuovi composti terapeutici diretti sul TLR4.

Nel capitolo II sono riportati gli effetti della co-somministrazione di peptidi antimicrobici (AMP) e un antagonista sintetico del TLR4 (il glicolipide FP7) sull'attivazione del TLR4. La presenza di due peptidi che neutralizzano l'LPS (un peptide ibrido di cecropina A-melittina e una catelicidina umana) aumenta di un ordine di grandezza la potenza di FP7 nel bloccare il segnale mediato da TLR4. L'effetto di potenziamento si verifica anche quando le cellule sono stimolate con un agonista del TLR4 strutturalmente diverso dall'LPS. I nostri dati suggeriscono che tale effetto potrebbe essere dovuto ad un doppio meccanismo d'azione, non esclusivamente basato sulla capacità degli AMP di neutralizzare l'LPS, ma anche ad un effetto diretto di questi peptidi sui recettori CD14 e MD-2. Esperimenti NMR in soluzione mostrano che l'aggiunta degli AMP modifica lo stato di aggregazione di FP7, promuovendo la formazione di micelle più grandi. Questi risultati suggeriscono una relazione tra lo stato di aggregazione dei ligandi mimetici del lipide A e il tipo/intensità della risposta mediata dal TLR4.

Il capitolo III descrive uno studio preclinico in cui l'antagonista selettivo per il TLR4, FP7 è stato utilizzato in un modello sperimentale di malattia infiammatoria intestinale (IBD). Questo studio ha lo scopo di valutare una possibile strategia terapeutica basata sull'uso di piccole molecole dirette selettivamente sul complesso TLR4/MD-2 per ridurre l'infiammazione delle IBD. I risultati ottenuti mostrano che FP7 riduce la secrezione delle principali citochine proinfiammatorie indotta da LPS da parte di cellule mononucleate del sangue periferico (PBMC) e di cellule mononucleate della lamina propria

(LPMC) isolate dai pazienti con IBD. L'effetto anti-infiammatorio di FP7 è dovuto ad una ridotta attivazione della via di segnalazione indotta da LPS dipendente dal fattore myeloid differentiation primary response gene (88) (Myd88). Lo studio mostra che il meccanismo di azione di FP7 è correlato alla sua capacità di competere con l'LPS per il legame con i recettori MD-2 e CD14. Inoltre FP7 riduce l'infiammazione *in vivo* su un modello murino di colite ulcerosa. Considerando che la patogenesi delle IBD è associata a un'eccessiva risposta immunitaria innata verso antigeni microbici, l'inibizione del TLR4 da parte di agenti chimici come FP7 potrebbe rivelarsi un promettente approccio alternativo al trattamento delle IBD.

Acronyms

AIEC: adherent-invasive *E. coli*

ALS: amyotrophic lateral sclerosis

AMPs: antimicrobial peptides

AP-1: activating protein-1

CA: cecropin A

CD: Crohn's disease

CD14: cluster of differentiation 14

CMC: critical micellar concentration

DAMPs: Danger-associated molecular patterns

DCs: dendritic cells

DSS: dextran sulfate sodium

FA: fatty acid

GLA: Gifu lipid A

HEK: human embryonic kidney

HMGB1: High Mobility Group Box1

IBD: inflammatory bowel disease

IFN: interferon

IKKs: I kappa B kinases

IL-: interleukin-

IP: intraperitoneal

IRAKs: Interleukin-1 receptor-associated kinases

IRF-: interferon regulatory factor-

LBP: lipopolysaccharide binding protein

LRR: leucine-rich repeat

LOS: lipooligosaccharide

LPS: lipopolysaccharide

LPMCs: lamina propria monocuclear cells

LRR: leucine-rich repeat

LTA: lipoteichoic acid

M: melittin

MAPKs: mitogen-activated protein kinases

MD-2: myeloid differentiation factor-2

MTT: thiazolyl blue tetrazolium bromide

MyD88: myeloid differentiation primary response gene (88)

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NLRs: NOD-like receptors

OM: outer membrane

PAMPs: pathogen-associated molecular pattern

PBMCs: peripheral blood mononuclear cells

PMB: polimixin B

PRRs: pattern recognition receptors

RA: rheumatoid arthritis

RLRs: retinoic acid-inducible gene 1 (RIG-1)-like receptors

SAR: structure-activity relationship

SD: standard deviation

SEAP: secreted embryonic alkaline phosphatase

SEM: standard error of measurement

SMOC: supramolecular organizing center

TGF-β: transforming growth factor β

TLRs: toll-like receptors

TNF-α: tumor necrosis factor α

TNFR: tumor necrosis factor α receptor

TRAF6: TNF receptor-associated factor 6

TRAM: translocating chain-associated membrane protein

TRIF: Toll/IL-1 receptor (TIR)-domain containing adapter-inducing IFN-β

UC: ulcerative colitis

1. INTRODUCTION

1.1 INNATE IMMUNITY AND PATTERN-RECOGNITION RECEPTORS

In order to detect the presence of infectious agents and eradicate the threat without destroying self tissues, pluricellular organisms have evolved an efficient immune-recognition system. In vertebrate animals the immune system has been traditionally divided in innate and adaptive responses, which have different but complementary roles. The innate response provides the host a first fast line of defense against many common pathogens and plays a crucial role in controlling bacterial infections. However, to face the enormous molecular variety of pathogens and their high replication and mutation rates, innate response needs to be completed by a more versatile defense mechanism, the adaptive immunity. Adaptive immunity is based on the generation of a random and highly diverse pool of antigens-targeting molecules, followed by selection and expansion of the molecules able to target the invaders. Although adaptive immunity is an extremely potent defense mechanism able to increase protection against subsequent reinfection with the same pathogen (immunological memory), it presents two main limitations. First of all, the randomly generated antigens-targeting molecules are incapable to identify the source and the biological context of the antigen they have to recognize. Second, this immune response takes time and energy to be able to contribute to host defence (typically 4-7 days after infection). Therefore, the host needs innate immunity to rapidly counteract replicating pathogens and to guide and educate the initiation of the adaptive immune response.[1] The innate immune defense mechanism is based on the detection of constituent and conserved products of a large array of pathogens. Many of the microbial components recognized by the host are unique to microorganisms and essential for their survival. Considering that these pathogen-associated targets

are not always identical between different species of microbes, host innate immunity evolved in order to recognize the common molecular patterns highly conserved within this targets. These patterns are defined pathogen-associated molecular patterns (PAMPs) and the host receptors responsible for their detection are called pattern-recognition receptors (PRRs).[1] PRRs are specific receptor proteins strongly expressed in immune cells playing crucial roles in innate immunity and protection against pathogens in higher animals (Figure 1.1).[2] PRRs are generally classified into three families: Toll-like receptors (TLRs), NOD-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). TLRs are expressed on the cell surface or present in endosomes, and their activation by bacterial components lead to the recruitment of downstream adaptor and signalling molecules such as Myeloid Differentiation primary response 88 protein (MYD88) and/or TIR domain-containing adaptor protein inducing IFNβ (TRIF). TLRs activation results in the up-regulation of pro-inflammatory mediators that favours host immune responses.[3] NLRs are cytoplasmic receptors that regulate inflammatory and apoptotic responses. The NLRs nucleotide-binding oligomerization domain 1 (NOD1) and NOD2 recruit the same downstream adaptor molecule named receptor-interacting serine/threonine kinase (RICK), which initiates innate immunity through nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs). Moreover, a subfamily of NLRs receptors that includes NLRP3, NLRP1 and NLRC4 is involved in inflammasome formation, which activates caspase 1. Inflammasome-mediated caspase 1 activation leads to the secretion of mature IL-1β and IL-18, which are involved in host defence and also in the pathogenesis of several autoimmune diseases. The last PRR family are RLRs, which are cytoplasmic proteins involved in intracellular virus

recognition, gut immunity and disease pathogenesis.[4, 5] Among RLRs receptor, RIG-I and melanoma differentiation-associated protein 5 (MDA5) detect double-stranded RNA. Protein LGP2 lacks amino-terminal caspase-recruitment domains and acts as a regulator of RIG-I and MDA5 signalling.[2]

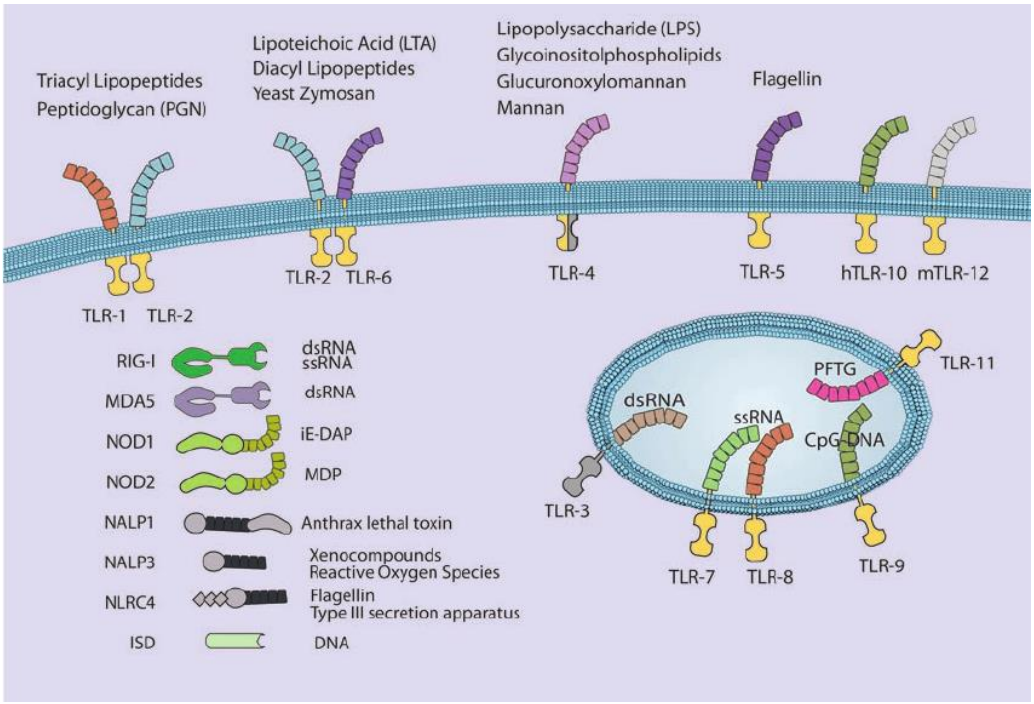


Figure 1.1. Graphic representation of pattern recognition receptors and their detected ligands. TLRs 3, 7, 8, 9 and 11 are represented in endosomal or intracellular compartment while NOD1, NOD2, RIG-I, MDA-5, NALP1, NALP3, NLRC4, and the intracellular DNA sensor (ISD) function in the cytoplasm. Only a partial list of ligands or classes of ligands for each receptor is given. The letter "h" or "m" before TLR indicates they are only present in human or mouse.[6]

1.1.1 TOLL-LIKE RECEPTORS

As mentioned above, TLRs are specific transmembrane receptors that belong to the PRRs family. TLRs have the important role to allow host cells to detect broad, but highly conserved microbial structures commonly named PAMPs, normally present on infectious agents such as bacteria, fungi, parasites and viruses.[7] TLR family includes 13 mammalian type I trasmembrane glycosylated receptor proteins (10 in humans and 12 in mice) (Table 1) composed of a highly variable extracellular leucine-rich repeat (LRR) ectodomain, a transmembrane portion and a highly conserved region in the short intracellular tail, called toll-interleukin-1 receptor (TIR) domain. The LRRs motifs present in TLR ectodomain commonly fold together to form a solenoid domain, giving this portion a characteristic horseshoe shape. TLRs LRR ectodomain has the evolutionarily conserved role to detect PAMPs. The molecular patterns recognized by this class of receptors include combinations of sugars, proteins, lipid-bearing molecules, and some nucleic acid motifs. In humans, ten TLRs have been described, each of them responsible to detect a specific PAMP: TLR2 associated with TLR1 or TLR6 (lipopeptides), TLR3 (viral dsRNA), TLR4 (lipopolysaccharide), TLR5 (bacterial flagellin), TLRs 7 and 8 (viral or bacterial ssRNA) and TLR9 (CpG-rich unmethylated DNA) (Table 1 and Figure 1.2).[7-9] TLR10 is not expressed in mice and it is the only human TLR with unknown ligands and biological function. However recent studies revealed that TLR10 is a modulatory PRR with mainly inhibitory and anti-inflammatory properties.[10] TLR11, 12 and 13 are TLRs present only in mouse. Studies showed that the first two receptors (TLR11 and 12) are responsible to detect profilin, a PAMP involved in many process of *toxoplasma* infections;[11] while there is very little known about the functions and ligands of TLR13 (Table 1). TLRs can be

classified into two subgroups based on their cell localization. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are normally present on the cell surface, with TLR2 forming heterodimers typically with TLR1 or TLR6. Otherwise, TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 are localized in the intracellular compartments within endosomes (Table 1 and Figure 1.2).[3]

Receptor	PAMP	Origin of PAMP	Human/Mouse	Localization
TLR2	Peptidoglycan	Gram-positive bacteria	Human/Mouse	Cell surface
	Lipoarabinomannan	Mycobacteria		
	Hemagglutinin	Viruses		
	GPI and GIPLs	*Trypanosoma*		
TLR2/TLR1	Triacyl lipopetides	*Mycoplasma*	Human/Mouse	Cell surface
TLR2/TLR6	Diacyl lipopetides	*Mycoplasma*	Human/Mouse	Cell surface
	Lipoteichoic acid	Gram-positive bacteria		
	Zymozan	Fungi (*S. cerevisiae*)		
TLR3	dsRNA	Viruses	Human/Mouse	Endosome
TLR4	Lipopolysaccharide	Gram-negative bacteria	Human/Mouse	Cell surface
TLR5	Flagellin	Bacteria	Human/Mouse	Cell surface
TLR7/TLR8	ssRNA	Viruses	Human/Mouse	Endosome
TLR9	CpG DNA	Bacteria	Human/Mouse	Endosome
	DNA	Viruses		
TLR10	N.D	N.D	Human	Cell surface
TLR11	Profilin	*Toxoplasma*	Mouse	Cell surface
	N.D	*Uropathogenic bacteria*		
TLR12	Profilin	*Toxoplasma*	Mouse	Cell surface
TLR13	N.D	N.D	Mouse	Endosome

Table 1. TLRs, TLR ligands and localization. GPI, glycosylphosphatidylinositol anchors; GIPLs, glycoinositolphospholipids; ssRNA, single strand RNA; dsRNA, double strand RNA; N.D, not described. See text for details and references.

Although TLRs ligands generally derive from pathogens, during “sterile” inflammation, autoimmune syndromes and hypertension, TLRs can be also bind and activated by endogenous signal molecules named DAMPs (danger-associated molecular patterns) derived from damaged or necrotic tissues.[12] Indeed, a large array of endogenous (host-derived) molecules that arise from injured and dying cells are able to activate TLRs signaling. Among these molecules are extracellular matrix components (e.g., fragments of hyaluronan), plasma membrane, nuclear, and cytosolic proteins (e.g., high-mobility group box protein 1), and elements of damaged/fragmented organelles (e.g., mitochondrial DNA).[12] DAMPs-mediated TLRs activation (in particular of TLR4) will be widely described in paragraph 1.2.4.

Multiple signaling pathways are activated by TLRs upon stimuli, and some of these pathways are unique to particular TLRs. These signaling events lead to the up-regulation of pro-inflammatory mediators like cytokines, chemokines, and adhesion molecules, through a MyD88-dependent pathway (TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9) or a MyD88-independent (or TRIF-dependent) pathway (TLR3 and TLR4).[13] The signaling pathway activated depends on the ligand and on which adaptor molecules are recruited to associate with the respective TLR cytosolic TIR domain.

The TIR domain represents an essential platform able to trigger interactions between homo- or heterodimeric TLR subunits and to recruit the cytosolic adapter proteins necessary to initiate the downstream signaling cascades. The TIR domain is not unique to TLRs but it is also shared by other receptors such as those of the IL-1R family.

The adaptors recruited are all TIR domain-containing proteins such as MyD88, TIR domain-containing adaptor protein (TIRAP), MYD88-adaptor-like protein (MAL), Toll/IL-1 receptor (TIR)-domain containing adapter-inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM) (Figure 1.2). Following ligand-induced TLRs dimerization, the cytosolic TIR domain of TLRs may engage MYD88 and MAL (MyD88-dependent pathway), or otherwise TRIF and TRAM adaptors (TRIF-dependent pathway). TLR4 possesses a peculiar activation mechanism as it moves from the plasma membrane to endosomes in order to switch signalling from MYD88 to TRIF, thus activating both pathways (Figure 1.2).[14, 15] TLR4 is the only TLR that signal through two distinct pathways. The first is the MyD88-dependent pathway that starts by MyD88-promoted activation of the mitogen-activated protein kinases (MAPKs) p38, ERK1/2 and JNK that induce the activation of activator protein 1 (AP1); and of I kappa B kinase (IKK) that induces activation of NF-κB and interferon regulatory factor (IRF)7.[3, 16] The second is the TRIF-dependent pathway which involves IRF3 activation that leads to the subsequent expression of type I IFNs and IFN-stimulated genes (ISGs).[14, 17] A major consequence of the TLR signalling is the induction of pro-inflammatory cytokines, and in the case of the endosomal TLRs, the induction of type I IFN (Figure 1.2).[3]

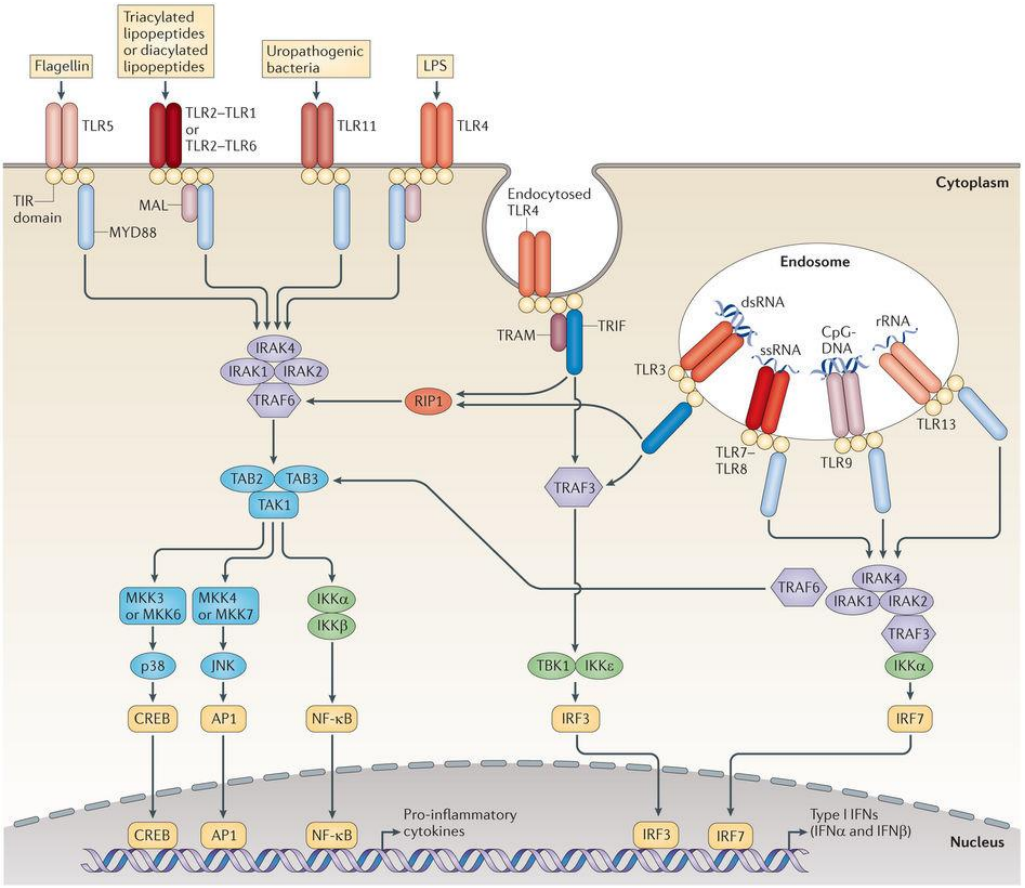


Figure 1.2. **Mammalian TLRs, their ligands and signalling pathways activated upon stimuli.** TLR5, TLR11, TLR4, and the heterodimers of TLR2–TLR1 or TLR2–TLR6 bind to their respective ligands at the cell surface, whereas TLR3, TLR7–TLR8, TLR9 and TLR13 localize to the endosomes, where they sense microbial and host-derived nucleic acids. TLR4 localizes at both the plasma membrane and the endosomes. TLRs signalling is initiated by ligand-induced dimerization of receptors and lead to the induction of pro-inflammatory cytokines, and in the case of the endosomal TLRs, the induction of type I interferon (IFN).[3]

1.2 THE TOLL-LIKE RECEPTOR 4 (TLR4)

TLR4 is the cell sensor of LPS, one of the major components of Gram-negative bacteria outer membrane. TLR4 plays a crucial role in initiating the innate immune response, triggering the production and secretion of pro-inflammatory cytokines.[18] TLR4 can also be activated by some endogenous factors such as heat-shock protein 70, fibronectin, oxidized phospholipids, and other molecules released by the host in certain situation of danger.[12] This receptor is located on the plasma membrane of the main cells of innate immunity: it is strongly expressed by monocytes, macrophages and dendritic cells, but also present in lymphocytes and epithelial cells.[19, 20]

TLR4 is fundamental for host immunity as it is involved in different key functions: as mentioned above, its main role is to sense and respond to minute (pM) amounts of LPS (also known as endotoxin), which is generally considered the most potent immunostimulant among microbial components. Secondly, TLR4 strongly promotes the recruitment of other immune cells to the infection site in order to potently neutralize the threat; and finaly this receptor is crucial to initiate the adaptive immunity.[21] TLR4 is a type I transmembrane protein composed of 839 amino acids, of which 608 residues in the extracellular domain (ectodomain), 20 in the transmembrane helical domain and 187 residues in the cytosolic tail.[22, 23] TLR4 belongs to LRR superfamily, indeed its ectodomain is composed of tandem copies of the LRR motif, which is typically 22–29 residues in length and contains specific hydrophobic amino acids spaced at distinctive intervals.[24, 25] This peculiar motif is found in many animals, plants and microorganisms proteins and it is often linked to the immune recognition process.[24] During protein assembling, LRRs inter-positioning and folding induces TLR4 ectodomain to assume the characteristic horseshoe-like

structure, whose concave surface is composed by parallel β-strands and whose convex surface is formed by loops (Figure 1.3).[24] Although TLR4 ectodomain is responsible for LPS sensing, the receptor needs an adaptor protein called myeloid differentiation factor 2 (MD-2) to bind edotoxin.[26] MD-2 is a 22 kDa protein which is anchored by several hydrogen bonds to the lateral and concave surface of the TLR4 ectodomain, contacting residues from the LRR2-LRR10 area (Figure 1.3).[22] MD-2 is essential for LPS signaling, since it binds directly to LPS and no physiological TLR4 activation in the absence of MD-2 has been observed.[22] MD-2 possess a β-cup fold with two antiparallel β sheets that contain three and six β-strands respectively, that form a large hydrophobic pocket able to receive the lipopolysaccharide (Figure 1.4).[27] The internal surface of the MD-2 wide cavity is completely covered of hydrophobic residues that favor the interaction with the lipophilic portion of LPS, while the positively charged residues positioned at the rim of the protein pocket interact with the phosphate groups of endotoxin stabilizing the bound LPS.[26] LPS recognition trigger TLR4/MD-2 dimerization with another TLR4/MD-2 ectodomain (TLR4*), thus forming the $(TRL4/MD\text{-}2/LPS)_2$ homodimer that initiates the intracellular signaling (Figure 1.3).[28]

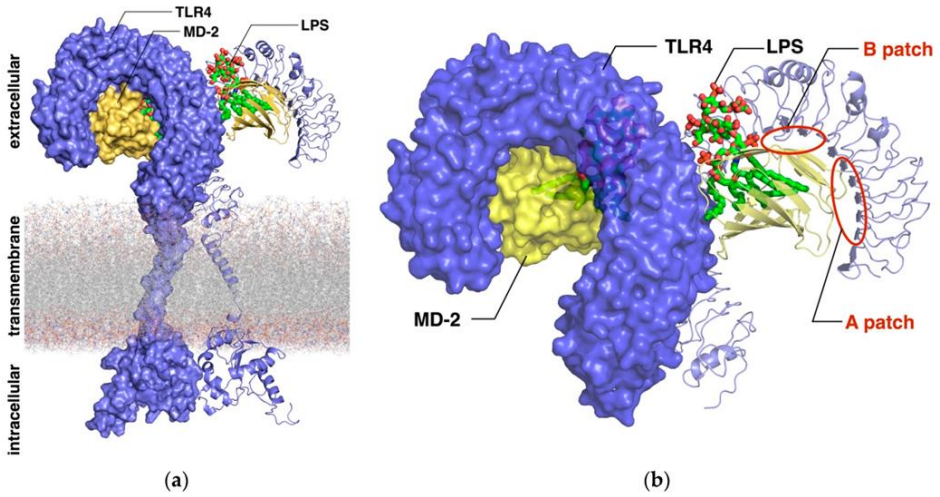


Figure 1.3. Representation of the 3D structure of TLR4/MD-2/LPS. (a) Intracellular, transmembrane and ectodomains of TLR4/MD-2 in complex with *E. coli* LPS. 3D structures correspond to the X-ray crystallographic structure for the extracellular domain (PDB ID 3FXI) and homology modeling for the transmembrane and intracellular domains. **(b)** Close-up look of TLR4 ectodomain (purple) in complex with adaptor protein MD-2 (yellow) and LPS (CPK colors with C atoms in green) from PDB ID 3FXI.[29]

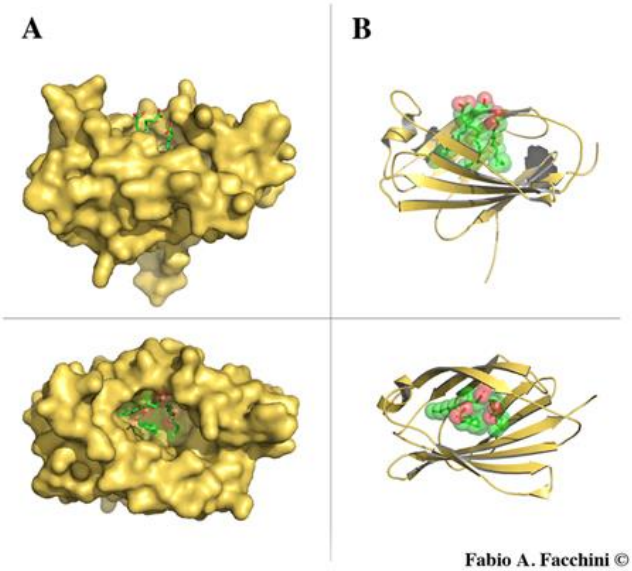


Figure 1.4. Representation of the 3D structure of MD-2 in complex with LPS. (A) Top panel: surface of MD-2; bottom panel: LPS accommodation in MD-2 hydrophobic cavity. **(B)** Secondary structure of MD-2 (cartoon representation) in complex with LPS (Spheres) in two different orientations. Structure from PDB ID 2E56.

1.2.1 THE LIPOPOLYSACCHARIDE

Lipopolysaccharides (LPS) groups a heterogeneous population of extremely heat-stable amphiphilic molecules composed of a predominantly lipophilic region, named lipid A, and a covalently linked hydrophilic poly- or oligosaccharide portion.[30] As mentioned above, LPS represents an essential component of the outer leaflet of the outer membrane (OM) of various Gram-negative bacteria (Figure 1.5), including commensal and human pathogenic bacterial species. LPS guarantees the viability and survival of bacteria, contributing to the correct assembly of the OM.[31] Moreover, endotoxin provides an extraordinary permeability barrier towards a large pool of molecules, including antibiotics, detergents and metals. Indeed, the peculiar OM low fluidity is due to the highly order structure of the LPS monolayer.[31]

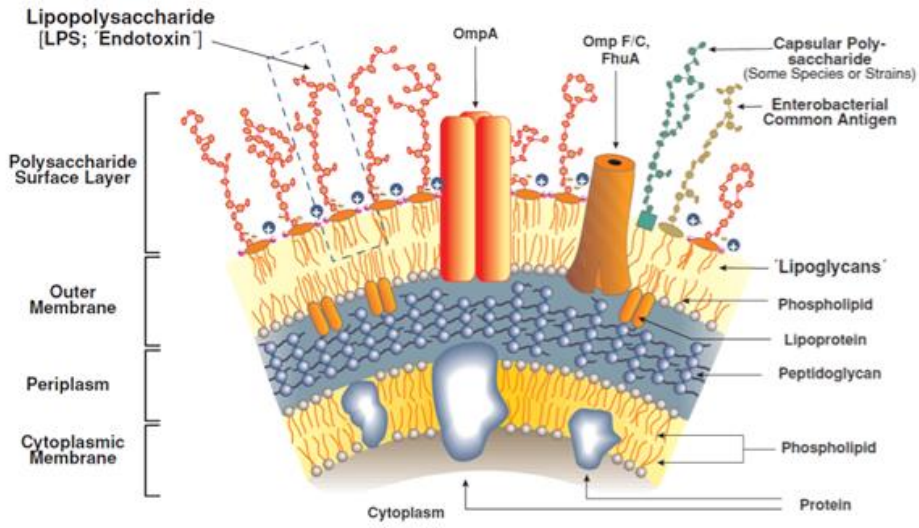


Figure 1.5. Wall architecture of Gram-negative bacteria.

The lipophilic lipid A is normally inserted into the phospholipid bilayer of the external leaflet of the OM. The oligo/polysaccharidic chains are covalently linked to this anchor and protrude in the extracellular compartment mediating many host-bacterium interactions including adhesion, colonization, virulence and symbiosis.[31] The polysaccharide region of LPS is commonly subdivided into different portions: the highly variable terminal O-specific chain (or O-antigen) and the core region, which is most proximal to lipid A. (Figure 1.6) In the majority of Gram-negative bacteria, the O-specific chain consists of up to 50 repeating oligosaccharide units formed of 2–8 monosaccharide moieties in a highly species- and strain-specific manner. In the vast majority of LPS structures, the O-specific chain is characterized by an extremely high structural variability even within a given bacterial strain.[32]

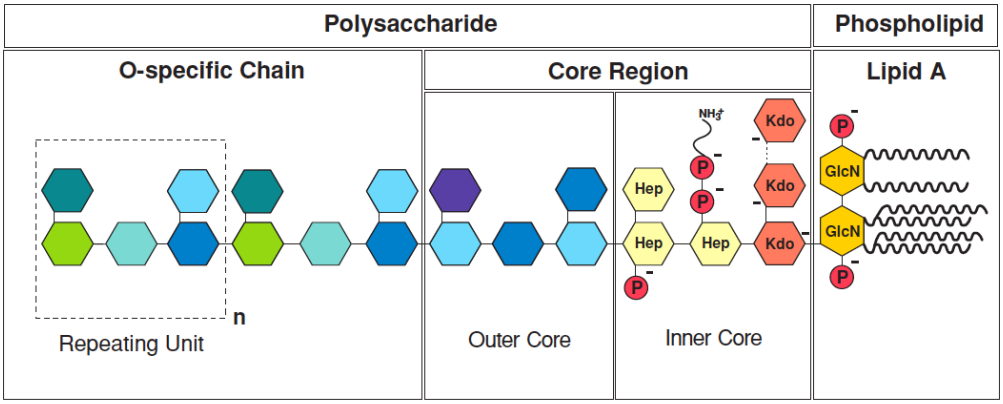


Figure 1.6. **General chemical structure of LPS from Gram-negative bacteria.** O-chain is extremely variable and gives serological specificity to the different LPS variants. The core region is more conserved than O-chain and is divided in outer and inner core. The outer core usually contain common sugars like hexoses and hexosamines, while the inner core is highly conserved and composed of unusual sugars such as kdo and heptose. The di-glucosamine backbone (Lipid A) is the most conserved portion of the LPS and the number and length of acyl chains are crucial determinants for endotoxicity.[31]

LPS is among the most potent pro-inflammatory and immunostimulant molecules and its presence is a strong indicator of Gram-negative bacteria infections for many eukaryotes.

LPS is an amphiphilic molecule with typically low sub-micromolar/nanomolar values of critical micellar concentration (CMC) in aqueous environment, hence it aggregates in the concentration range relevant for biological responses. The issue of the biologically active unit of endotoxins, whether large or small aggregates, or monomers, has been amply debated in the literature. Lipid A is considered the conserved primary immunoreactive center of LPS, because it is the smallest LPS unit effectively detected by TLR4 and able to trigger a potent inflammatory response.[26, 33, 34] The majority of lipid A structures so far identified presents a backbone structure that consists of a central β(1→6)-linked D-glucosamine (DGlcN) disaccharide unit. In most cases the disaccharide unit is phosphorylated in positions 1 and 4' and presents a variable number of acyl fatty acid (FA) chains.[35] An immunogenic lipid A, for example *Escherichia coli* lipid A, is generally composed of a glucosamine disaccharide core (β(1→6)-linked D-glucosamine disaccharide) with two negatively charged phosphate groups in C1 and C4' positions and six fatty acid (FA) acyl chains linked to C2, C3, C2' and C3' disaccharide positions (hexa-acylated) (Figure 1.7). In particular four (R)-3-hydroxymyristoyl residues are directly linked to the 1, 4'-bisphosphorylated disaccharide at positions 2, 3 and 2', 3' via amide or ester linkages. Both primary (R)-3-hydroxyacyl chains at positions 2' and 3' are esterified with lauric (C_{12}) and myristic (C_{14}) acid respectively, forming acyloxyacyl groups in both positions. Finally, the primary hydroxyl function at position 6' is covalently linked to the polysaccharide region (Figure 1.7).[36]

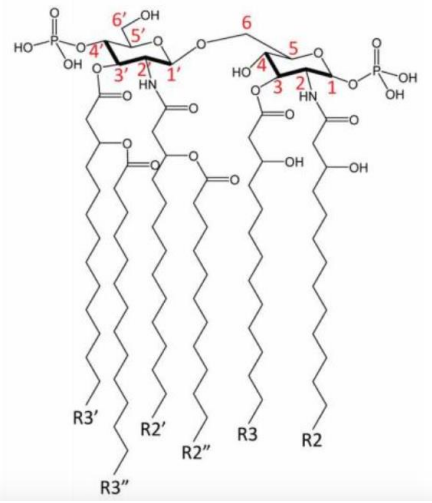


Figure 1.7 Chemical structures of *E.coli* lipid A.

Hexa-acylated lipid A forms with two phosphate groups and 12/14 carbons long acyl chains are the most potent stimulatory or agonistic structures for innate immunity activation in humans and other mammalian hosts.[37] The strong pro-inflammatory action of hexa-acylated lipid A is based on the specific interaction mode of the molecule with the host receptor TLR4 (mechanism explained in the next paragraph).[28]

1.2.2 LPS/TLR4 INTERACTION AND SIGNALLING

The mechanism of lipid A recognition is very complex and depends on the coordinated action of different LPS-binding proteins that work together to initiate the TLR4-mediated signalling.[38] LPS is normally attached to Gram-negative bacteria OM or released in the extracellular compartment. In the aqueous environment LPS is prone to form typical aggregates or micelles of various sizes due to its amphiphilic nature. The first LPS-binding protein to come into play is LPS binding protein (LBP), which is able to extract monomeric molecules of endotoxin from the bacterial membrane or from LPS micelles.[39] LBP transfers monomeric LPS to a second LPS-binding protein called cluster of differentiation 14 (CD14), which is soluble or anchored to the plasma membrane via a C-terminal glycosylphosphatidylinositol (GPI) anchor.[39-41] CD14 has the import role to present monomeric LPS to MD-2 protein, which is non-covalently bound to TLR4 ectodomain, and which is the final acceptor of LPS.[38, 39, 41, 42] In the absence of any of these accessory proteins, the concentrations of LPS needed to activate an inflammatory response increase by several orders of magnitude.[42] LPS binding to MD-2 co-receptor promotes the dimerization of TLR4 by forming the complex $(TLR4/MD\text{-}2/LPS)_2$ (Figure 1.8).[38] TLR4 and MD-2 are essential for LPS detection; the absence of one of these two proteins completely abolishes the activation of the LPS-triggered intracellular signaling.[43]

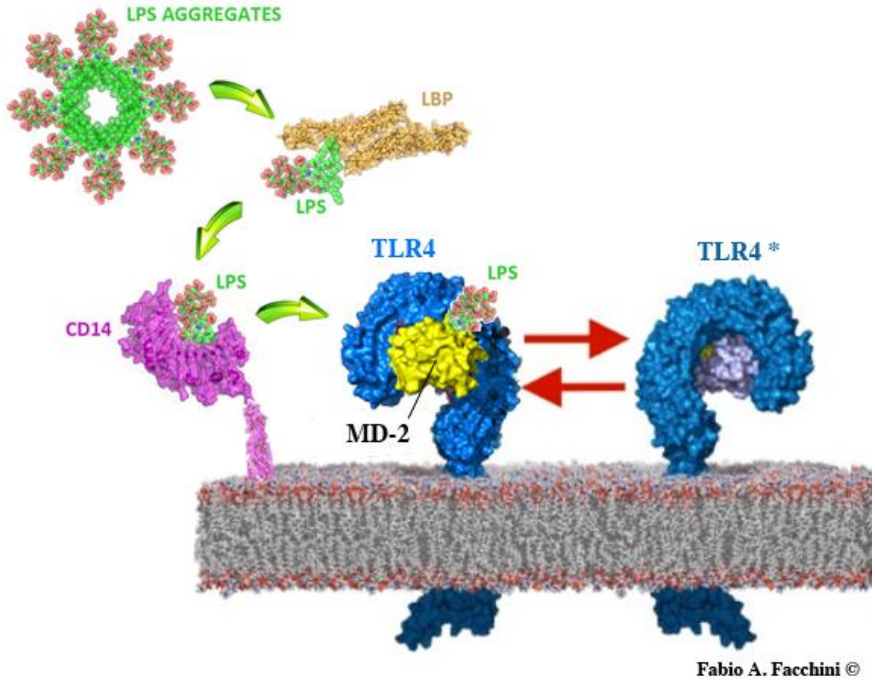


Figure 1.8. Schematic representation of *E. coli* lipid A detection process.

When LPS is presented to TLR4/MD-2 complex by CD14 co-receptor, five of the six lipid chains of lipid A accommodate inside the deep MD-2 hydrophobic pocket and the remaining chain (R2) is partially exposed to the MD-2 surface, forming the core hydrophobic interface necessary for interaction with a second TLR4 ectodomain (TLR4*).[22, 28] The two phosphate groups of the lipid A contribute to receptor dimerization by forming interactions with positively charged residues in TLR4, TLR4* and MD-2 (Figure 1.9 and 1.10).[22, 28]

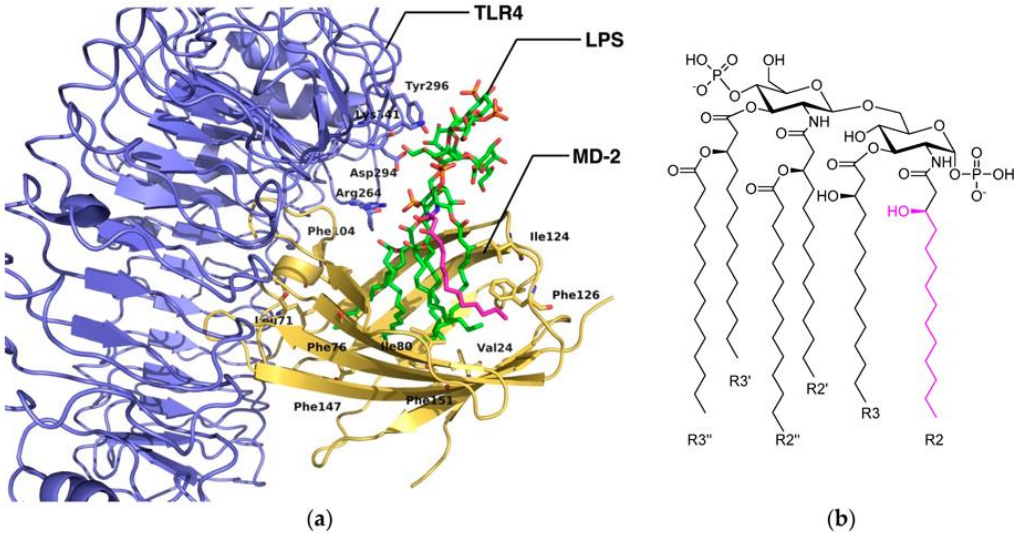


Figure 1.9. Crystal structure of LPS in complex with TLR4/MD-2. **(a)** Detail of the 3D structure of the complex between TLR4/MD-2 and E. coli LPS (CPK colors with C atoms in green and R2 C atoms in magenta) from the X-ray crystallographic structure (PDB ID 3FXI); **(b)** chemical structure of *E. coli* lipid A. The R2 FA chain (magenta) placed at the channel of MD-2 completes the dimerization interface.[29]

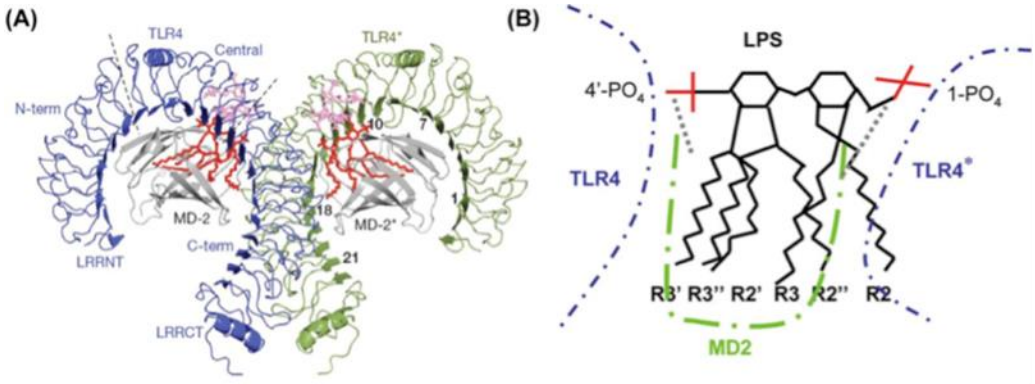


Figure 1.10. Crystal structure of (TLR4–MD2–LPS)$_2$ complex. **(A)** Secondary structure (cartoon) of (TLR4–MD2-LPS)$_2$ complex. The lipid A portion is in red.[28] **(B)** Particular of lipid A accommodation in MD-2 cavity: five of the six acyl chains of lipid a enter MD-2 pocket (in green), while the remaining chain (R2) is exposed to the surface interacting with the second TLR4 ectodomain (TLR4*) (in blue). Two phosphate groups of lipid A (1-phosphate and 4-phosphate highlighted in red) are key factor for to stabilize TLR4 dimer through binding to positively charged lysine residues and an arginine residue on both TLR4 and MD-2.[44]

Some studies indicate Phe126 as the "molecular switch" in endotoxin signaling. In particular, the entry of the five lipid A acyl chains into MD-2 hydrophobic cavity induces a local conformational change that involve the Phe126 side chain and the surrounding residues (the loop composed of residues 123–129) (Figure 1.11).[45]

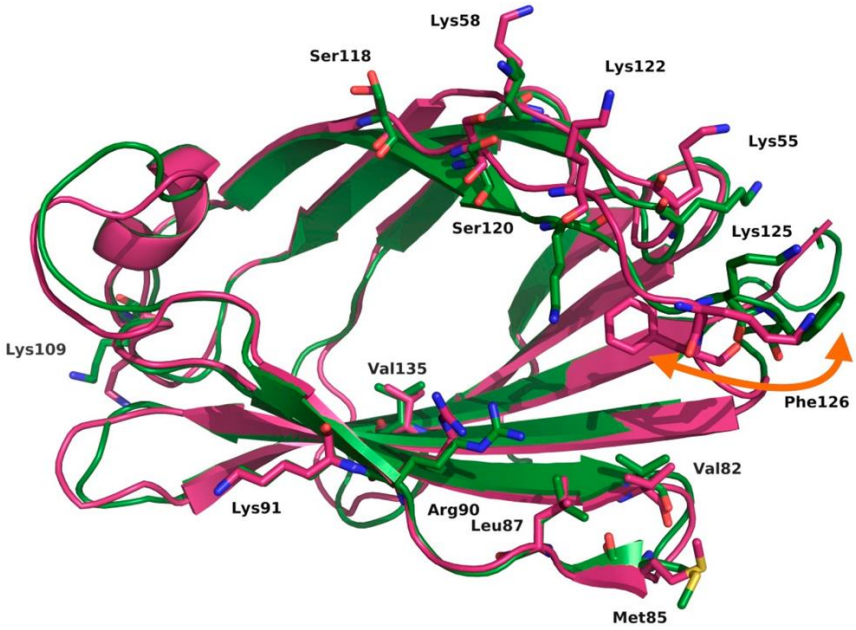


Figure 1.11. Superimposition of the X-ray crystallographic structures of the agonist (magenta) and the antagonist (green) conformations of MD-2 from, respectively, PDB ID 3FXI and 2E56. *E. coli* LPS have been hidden to remark the conformational change of the molecular switch Phe126.[29]

Phe126 loop switch would play a key role in supporting the formation of the hydrophobic interface to allow TLR4 dimerization to occur.[45]

LPS-induced TLR4 dimerization usually occurs in phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2)-rich regions of the plasma membrane. The first cytosolic event is the recruitment of the sorting adaptor TIRAP on the TLR4 cytosolic TIR domain,[46] which is necessary for the assembling of a higher-order

filamentous structure called myddosome.[47, 48] Myddosome is a supramolecular organizing center (SMOC) composed of the signaling adaptor MyD88 and several IRAK family kinases, which initiates signaling events leading to the activation of pro-inflammatory transcription factors such as AP-1 and NF-κB (p50/p65) (Figure 1.12).[49, 50] Transcription factors activation is the crucial event that leads to the production and secretion of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 and IL-8.

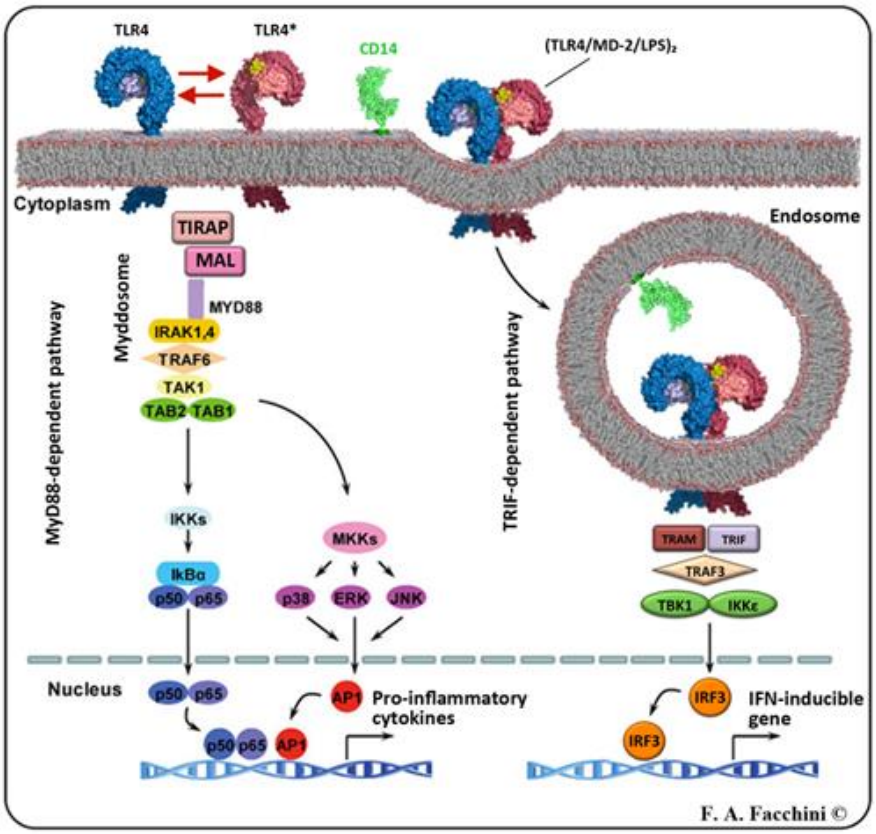


Figure 1.12. Schematic representation of TLR4 signaling pathways activated upon LPS stimulation.

Concomitant with TLR4 signaling from the plasma membrane, LPS binding to GPI anchored CD14 also promotes TLR4 endocytosis.[15] Recent studies have

shown that, in contrast to the endocytosis process of other transmembrane receptors, TLR4 internalization is entirely promoted by extracellular interactions.[51] In particular, LPS binding to CD14 is the crucial event that allows TLR4 ectodomain to be selected as a cargo for the endocytosis process and that adaptor protein MD-2 is the cargo-selection agent to accomplish the process.[51] Thus CD14 is not only an accessory protein able to increase the sensitivity of the LPS-detection process, but more precisely it possesses a dual function in ligand transport and receptor transport.[15, 51] Once internalized, TLR4 engages another sorting adaptor, called translocating chain-associated membrane protein (TRAM), and the signaling adaptor TRIF, which lead to the subsequent expression of IFNs and IFN-stimulated genes (ISGs) (Figure 1.12).[52] A recently proposed model suggests that CD14 is constantly cycling through the plasma membrane, surveying the extracellular environment for most variants of LPS. When the co-receptor recognizes a "pathogenic" LPS form, it transfers LPS to MD-2 to trigger TLR4 dimerization.[51] This event induces myddosome formation and signaling at the plasma membrane and converts the immunologically silent entry route taken by CD14 into an inflammatory endocytosis pathway.[51]

1.2.3 SPECIES-SPECIFIC LPS SENSING

Studies performed during the last two decades revealed that the lipid A biosynthetic precursor, lipid IVa (Figure 1.13), with four acyl chains, acts as TLR4 antagonist on human LPS-responsive cells, while, in contrast, it is able to induce TLR4 dimerization and activation in mouse and chinese hamster cells. This observation suggested that the capacity to detect lipid A (or in general LPS) may vary among species. At the base of the differential lipid IVa activity

among species there are important structural differences that involve proteins TLR4[53, 54] and MD-2.[55-57] In order to clarify the molecular basis for this discriminating mechanism, several amino acidic regions of TLR4/MD-2 complex were three-dimensional mapped.[58] The analysis of receptor structures highlighted a triangular interface or "wedge" in which molecular interactions between TLR4, MD-2 and ligand take place (Figure 1.14). The study identified two areas within the wedge linked to agonism or antagonism behavior that explained why lipid IVa possesses a species dependent dual activity. In particular, these areas allow lipid IVa to be more deeply buried into human MD-2 cavity. In this way only one phosphate group (P1) can occupy the conserved phosphate binding location (lower right corner of the wedge) necessary to have a LPS-like agonist effect (Figure 1.14). Indeed, the second phosphate (P2) cannot reach the phosphate binding site of agonist ligands (upper left, just below the higher angle of the wedge). Furthermore, the orientation of lipid IVa sugar-phosphate backbone in complex with human TLR4/MD-2 is inverted as compared to the agonistic binding pose of lipid IVa in murine TLR4/MD-2 (Figure 1.14). This inverted binding mode forms a "repulsion region" that prevents the association of the (TLR4/MD-2-Lipid IVa)$_2$ complex, which is essential for the activation of the signaling (Figure 1.14).[58] The agonistic action of lipid IVa in mouse is linked to a glutamate residue specifically present in murine MD-2 (Glu122). The anionic side chain of this residue generates a repulsive force that favors the upward shift of phosphate P1 into the bridging position formed by a cluster of residues of TLR4, MD-2 and TLR4*. Furthermore, the shift forces an alkyl chain to stand outside the pocket.[58]

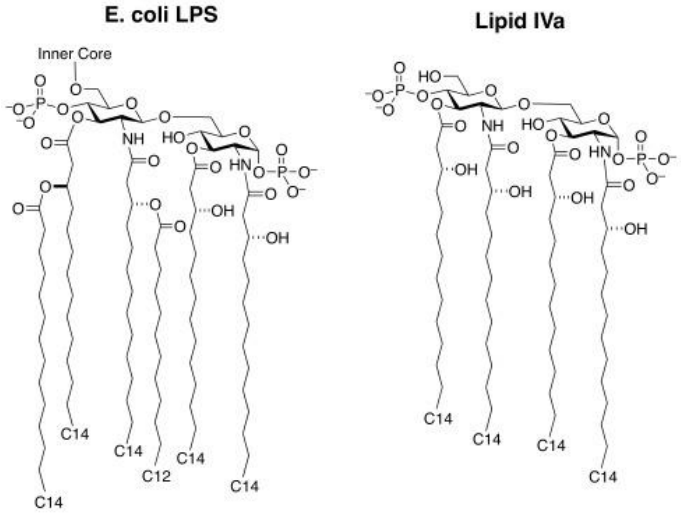


Figure 1.13. Comparison between the structure of *E.coli* lipid A and its biosynthetic precursor lipid IVa.

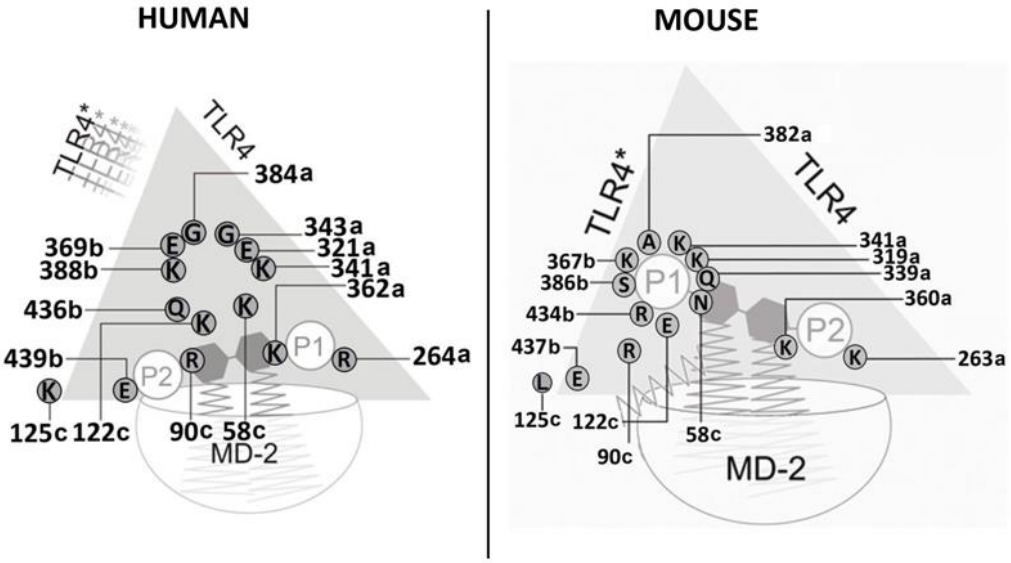


Figure 1.14. Schematic picture of TLR4 and MD-2 residues arrangement relative to bound lipid IVa determining its agonistic or antagonistic action respectively in mouse and human MD-2.[58]

1.2.4 DAMPs-INDUCED TLR4 ACTIVATION

As described in paragraph 1.1 innate immunity has the crucial role of sensing pathogens and eradicates the threat. However, danger signals can originate not only from external invaders but also from endogenous molecules released in case of cell injury end death.[59] In physiological conditions danger signals are hidden into living cells and during programmed cell death phagocytes sequestrate apoptotic cells in order to prevent the release of these molecules in the extracellular compartment. However in case of necrosis cells lose membrane integrity causing a massive release of cytosolic components. Host-derived molecules released in case of danger are generally named danger-associated molecular patterns (DAMPs). DAMPs are pro-inflammatory molecules able to trigger innate immunity by interacting with PRRs, including TLRs. The response mediated by DAMPs is also named “sterile inflammation” because it is triggered in response to trauma and tissue damage and not by pathogens. Several DAMPs are also secreted from activated leukocytes in response to PAMPs or pro-inflammatory cytokines.[60]

DAMPs are a heterogeneous group of molecules that include several intracellular proteins, nucleic acids and nucleotides. They are expressed in different cell types and play multiple roles important for cellular homeostasis. These molecules can be localized in both the nucleus and cytoplasm (HMGB1), only in the cytoplasm (S100 proteins), into the exosomes (heat shock proteins) or in extracellular matrix (small fragments of hyaluronan).[60] High-mobility group box 1 (HMGB1) is a nonhistonic chromatin-associated protein, which in physiological conditions is localized in the nucleus in order to favor the assembly of transcriptional complexes. HMGB1 is released in the extracellular compartment during cell necrosis or secreted by leukocytes in response to

multiple stimuli including LPS, INF-ɣ and TNF-α.[61] Outside the cell this molecule has a potent pro-inflammatory effect acting as a cytokine and interacting with PRRs. Indeed, it is known that HMGB1 binds to TLR4 inducing the initiation of the signaling cascade that lead to NF-κB activation and to the secretion of pro-inflammatory cytokines.[62] Also the calcium-binding proteins S100A8 and S100A9 are able to trigger a strong pro-inflammatory response interacting with TLR4 and they promote endotoxin-induced shock.[63] Furthermore S100A8 and S100A9 can induce the expression of serum amyloid A (SAA3), which is a chemoattractant protein able to recruit myeloid cells and in turn to activate TLR4 signal.[64] Studies conducted revealed that HMGB1 and S100A8/A9 play a crucial role in the acute inflammation phase of colitis-associated carcinogenesis through their capacity to bind and activate TLR4.[59] Also some extracellular matrix (ECM) components were described to modulate immune responses by interacting with TLRs. Fibronectin, for instance, one of the main components of ECM, is able to induce TLR4-mediated NF-κB activation in HEK 293 cells stably transfected with TLR4, but not in the parental cell line.[65] Fibronectin agonist activity was then confirmed in many other cell populations and *in vivo* models.[66] As for fibronectin, also fragments of ECM hyaluronan (HA) have been shown to play a crucial role in inducing inflammation by activating dendritic cells and macrophages through a TLR4-dependent response.[67] Controversial and still object of investigation is the DAMPs-mediated TLR4 activation during oxidative stress. In order to counteract infections phagocytes can generate and release in the extracellular compartment a range of reactive oxygen species (ROS) to damage invading microorganisms. However a massive production of ROS can overwhelm the host antioxidant defense mechanisms leading to the development of a harmful

state of oxidative stress. Increasing evidence supported the idea that oxidative stress plays a key role in the development and maintenance of inflammation, through its capacity to induce the release of DAMPs able to interact and activate many PRRs.[68] Indeed, high ROS levels can oxidize many host molecules, including lipids and lipoproteins, making them pro-inflammatory mediators. Membrane lipids with unsaturated fatty acid chains, for instance, are particularly prone to oxidation, generating oxidized species able to activate TLR4.[69, 70] In the same way, other studies showed that also oxidized low-density lipoprotein (oxLDL) triggers the production and secretion of pro-inflammatory cytokines in smooth muscle cells both *in vivo* and *in vitro*, and that this effect is highly TLR4-dependent.[71] Other research groups, however, observed an opposite behavior of these oxidized molecules on TLR4 and other TLRs. Erridge *et al.* for example showed in a report published in 2008 that some oxidized phospholipids, such as 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC), possess the capacity to inhibit LPS-induced TLR4 signalling and lipopeptide-induced TLR2 signalling; and that this capacity is mediated via interaction with accessory molecules including CD14, LBP, and MD2 co-receptors.[72]

A potential explanation for the dual effect of oxidized molecules on TLR4 is that the receptor can function as a sensor of oxidative stress, detecting the levels of oxidation of molecules. Indeed, Manček-Keber *et al.* demonstrated in 2015 that extracellular vesicles (EVs) isolated from patients with chronic inflammatory diseases contained oxidized phospholipids with a biphasic role on TLR4, depending on their oxidation level: partial oxidation led to a TLR4-stimulatory effect, while extensive oxidation led to a decreased activity, and to the generation of TLR4 antagonists as observed by Erridge *et al.*[73] At the base

of the difference between the only stimulatory LPS-mediated effect and the biphasic effect of EVs on TLR4 there is probably the capacity of innate immune system to differentially interpret pathogenic from endogenous sources of TLR4 agonists. Indeed, despite LPS and EVs activate TLR4 through a similar MD-2-dependent molecular mechanism the two ligands stimulate the expression of different downstream genes. Both types of stimulus induced the expression of pro-inflammatory cytokines; however, EVs also increased the expression of mediators involved in inflammation resolution, promotion of wound repair, and tissue healing (such as IL-4). Manček-Keber *et al.* speculated that the difference in signaling between LPS and EVs could be a result of different stabilities of the activated ligand-receptor complex, or could be due to interactions with additional co-receptors affecting different downstream signaling pathways.[73]

TLR4 is ubiquitously expressed also in the vasculature to modulate vascular function. In various cardiovascular diseases some vasoactive molecules like heat shock protein 60 and ANGII act as TLR4 agonists inducing a pro-inflammatory and proliferative phenotype. Furthermore, ANG II is also able to upregulate TLR4 mRNA expression and trigger TLR4-mediated myeloperoxidase secretion in macrophage cells. [74]

The table below summarizes some of the main host DAMPs able to trigger inflammation by interacting with TLRs.

DAMP	Receptor	Effect
ECM components		
Fibronectin	TLR4	Increase NF-*k*B, promote leukotriene synthesis and PMN migration, activate the adaptive immune system
Hyaluronan	TLR4 ± TLR2, NLR3	Induce proinflammatory cytokines, activate DCs and macrophages
Heparan sulphate	TLR4	Increase TNFα expression, activate DCs
Stress-response molecules		
Heat shock proteins	TLR2, TLR4	Induce cytokines and protein kinases, activate PMNs
HMGB1	TLR2, TLR4	Induce NF-*k*B nuclear translocation and cytokine expression
Nucleic acids	TLR3, TLR7, TLR9	Induce cytokine expression, activate DCs, stimulate recruitment of leukocytes and PMNs
microRNA	TLR8	Induce NF-*k*B and cytokines expression
Immunomodulatory proteins		
β-Defensins	TLR1/TLR2, TLR4	Induce NF-*k*B and cytokine expression, activate DCs and monocytes
Surfactant protein A	TLR4 ± TLR2	Reduce NF-*k*B and cytokine expression
Surfactant protein D	TLR2, TLR4	Inhibit cytokine production and recruitment of PMNs

Figure 1.15. Endogenous ligands of TLRs and their physiological effects

1.2.5 TLR4 EXPRESSION IN HUMAN CELLS AND TISSUES

In humans, TLR4 is predominantly expressed in cells of myeloid origin. Characteristic patterns of TLR4 and MD-2 expression are observed in monocytes, macrophages, immature and mature DC.[75] Furthermore, the exposure of monocytes and granulocytes to LPS or to other pro-inflammatory mediators is able to increase the expression of TLR4 on the cells surface.[76] Although, analyses performed on total RNA showed that TLR4 mRNA levels are low in resting or activated lymphoid cells, some works revealed that the expression of the receptor may be induced by specific stimuli (such as IL-4) in some lymphocytes.[77] Studies focused on investigate TLR4 expression in human tissues detected the highest levels of TLR4 mRNA in the spleen and in peripheral blood leukocytes (PBLs). Small intestine, colon, placenta, ovary and lungs express the receptor at moderate levels; whereas in the brain, heart, kidneys, liver, prostate, pancreas and muscles the expression is low.[78]

Studies revealed that TLR4 is expressed also in the central nervous system (CNS). In particular the receptor is mainly present in two non-neuronal cytotypes: CNS resident macrophages (also commonly named microglia) and in

macroglial cells like astrocytes.[79] Although TLR4 is generally expressed on the cell surface in order to recognize extracellular ligands, in microglial cells the receptor is localized within intracellular vesicles. On the contrary TLR4-containing intracellular vesicles are not detected in astrocytes, and the receptor is exclusively present on the cell surface. The difference in TLR4 subcellular localization may be related to the different role of phagocytosis and antigen processing respectively played by microglia and astrocytes in the CNS.[80] Although TLR4 is mainly expressed on supporting glial cells, several studies reported that the receptor is also present on neural cells, such as nociceptive neurons.[81, 82] Despite the role of TLR4 in these cells remains unknown, results obtained by different research groups suggest that neural cells can act as key sensors of infection to initiate CNS inflammation. TLR4 and CD14 expression by nociceptive neurons, for instance, allows also these cells to detect and respond to tissue levels of bacterial LPS and DAMPs.[82] Furthermore, TLR4 is present on adult neural stem cells (NSCs) and on neural progenitor cells (NPCs), where it exerts different and contrasting functions in NPCs proliferation and differentiation.[83, 84] TLR4 activation has been shown to correlate with increased proliferation of NSC/NPC after hippocampal ischemic injury.[83] Previous studies in murine cells or animal models have shown a multifaceted role played by TLR4 in neurogenesis,[85] but the lack of a human system to study the CNS and the paucity of data on human patients have represented a roadblock to the appropriate knowledge of some pathophysiological mechanisms and to plan possible therapeutic strategies. On the other hand, there is increasing pharmacological interest in TLRs targeting in CNS pathologies.[86] Interestingly, TLR4 is constitutively expressed in pancreatic insulin-producing β-cells[87] and in many cell types of insulin target

tissues, including epathocytes,[88] adipocytes of human fat tissue,[89] vasculature[90, 91] and skeletal muscle, suggesting a role of this receptor in these human districts. Several studies showed that TLR4 is also expressed in different subsets of intestinal epithelial cells.[92] Although TLR4 is present at low levels in this district due to gut lumen proximity, the expression of the receptor is up-regulated in many conditions that involves inflammation like inflammatory bowel disease (IBD) (see section 1.5.3). TLR4 is ubiquitously expressed also in the vasculature to modulate vascular function. For instance the receptor was found in human dermal microvessel endothelial cells (HMEC) and in human umbilical vein endothelial cells (HUVEC).[93] However, unlike other TLR4 expressing cells, endothelial cells lack CD14 co-receptor, suggesting that these cells needed higher amount of circulating LPS to be activated.

1.2.6 TLR4-RELATED PATHOLOGIES

The activation of TLR4 signaling pathway and the subsequent release of pro-inflammatory mediators is crucial for an optimal host immune response against invading Gram-negative bacteria. Inflammation is beneficial for the host provided it is rapid and short-lived in order to allow tissue repairing and healing after threat eradication. Persistent and excessive immune system activation may be deleterious and cooperate to the onset of a broad spectrum of disorders. In particular, excessively potent and deregulated TLR4 pathway activation is the main cause of severe septic shock and sepsis. Furthermore, aberrant TLR4 signaling is related to many other important syndromes such as inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC),[94] vascular inflammations,[95] obesity-linked type II diabetes,[88] atherosclerosis,[96] skin inflammations (dermatitis),[97] psoriasis,[98]

rheumatoid arthritis (RA),[99] and neuroinflammatory disorders such as neuropathic pain,[100] and amyotrophic lateral sclerosis (ALS).[101]

The term sepsis is used to indicate a potentially life-threatening condition that can arise from a massive and overwhelming host response towards infectious agents causing injury of host tissues and organs. Septic shock is the life-threatening complication of sepsis that occurs when severe sepsis causes serious host damage including insufficient blood flow, multiple organ dysfunction syndrome and consequently multiple organ failure. Many are the pathogens that can in principle trigger sepsis, including Gram-negative and positive bacterial cells, virus, fungi and parasites.[102] Bacterial sepsis is among the major causes of mortality worldwide, whose incidence has dramatically increased in the past two decades.[103] In order to cause the pathology, infective agents have to overcome the host anatomical barrier, evade host innate immune system and replicate in different organs.[104] The bacterial capacity to trigger sepsis is closely linked to the expression of virulence factors, which are related to the stage of infection. Among these factors there are toxins like the gram-negative lipopolysaccharide.[105] As described earlier LPS is the most potent immunostimulant among microbial products and, although other PAMPs are involved in sepsis onset, LPS is probably the most important toxin responsible for the pathology. The conserved lipid A moiety of LPS is sufficient to cause endothelial cell injury and apoptosis by promoting the expression of tissue factor and pro-inflammatory mediators.[106] LPS-triggered, TLR4-mediated signal play a crucial central role in sepsis pathogenicity, indeed studies conducted on murine models that lack TLR4 ($TLR4^{-/-}$ knockout mice) are incapable to develop septic shock upon massive LPS exposure.[107]

We previously described that besides LPS-triggered TLR4 activation, TLR4 can bind and be activated also by endogenous DAMPs initiating a pro-inflammatory response. Indeed many are the endogenous molecules released by cells in case of danger that can interact with TLR4. Therefore, this receptor plays a crucial role not only in LPS-related pathologies like septic shock, but also in many other inflammatory disorders mediated by DAMPs. Moreover, a growing amount of data reveled that TLR4 expression is significantly higher in these pathologies, increasing the patient's sensitivity towards DAMPs. For example, in RA patients different cell types including peripheral blood monocytes[108] and fibroblast[109] express higher levels of TLR4 compared to cells of healthy patients. This allow cells to strongly respond to the great amount of DAMPs present in the inflamed district of RA patients leading to a persistent state of inflammation.[110] As in the case of RA, studies revealed that the expression of TLR4 is increased in obesity-linked type II diabetic subjects, suggesting a pivotal role of this receptor in the pathogenesis of insulin resistance and diabetes. Moreover, chronic, low-grade inflammation is one of the main hallmarks of obesity that lead to an increased production of pro-inflammatory mediators thought to contribute to the onset of the disease. Studies conducted on mice lacking TLR4 showed that the absence of the receptor actually reduced diet-induced insulin resistance and inflammation.[111] TLR4 is expressed in many cytotypes of insulin target tissues, including liver, adipose tissue, skeletal muscle, vasculature, pancreatic β cells, and brain. Thus, the activation of TLR4 in these district can dampen insulin action both directly, through pro-inflammatory kinases activation and ROS production, and indirectly, via activation and release of pro-inflammatory, insulin-desensitizing factors.[112] In order to investigate the TLR4 expressing cells responsible to these

pathologic effects, Jia *et al.* generated two mouse models that are deficient in either hepatocyte ($Tlr4^{LKO}$) or myeloid cell ($Tlr4^{\Delta m\Phi}$) TLR4.[88] This strategy allowed the research group to surprisingly discover in 2014 that hepatocytes are important mediators of diet-induced inflammation via TLR4 pathway. Contrary to expectations the removal of TLR4 from macrophages did not reduce the levels of circulating inflammatory cytokine. This last result suggested a possible compensatory increased in TLR4 expression in other cell types, such as dendritic cells, B cells or endothelial cells, that contributed to the elevated inflammatory response.[88] Many studies focused to investigate the ligands responsible to promote TLR4 activation in diet-induced obesity (DIO) found that both type II diabetic patients and obese mouse models exhibited high plasma LPS levels.[113] Further analysis revealed that both high-fat and high-fructose diets are able to influence the amount of circulating endotoxin by altering the growth and composition of gut microbiota[114] and gut epithelial permeability.[115, 116] Although the mechanisms that increase plasma LPS levels in obese subjects are not yet completely clarified, it seems that diet-introduced lipids can promote endotoxin incorporation into chylomicrons, favoring LPS absorption by gut enterocytes.[117] This event would strongly contribute to postprandial endotoxemia resulting in a persistent and systemic pro-inflammatory stimulation of TLR4 singalling.[112]

In the central nervous system, TLR4 is localized on microglia in order to protect neurons from invading microorganisms. However the presence of typical protein amyloid aggregates of some neurodegenerative disease is able to trigger a potent TLR4-dependent inflammatory and neurotoxic response that exacerbates the disease.[118] This observation was confirmed by the fact that loss-of-function mutation in TLR4 gene strongly ameliorate neurotoxicity.[118]

Moreover, TLR4 involvement in various cardiovascular diseases has been widely described.[119] Indeed this receptor was the first TLR to be implicated in the etiology of vascular dysfunction and hypertension. Studies revealed that the expression of TLR4 is particularly high in mouse model of hypertension and that specific treatment based on the use of anti-TLR4 targeting antibodies ameliorated the pathological phenotype.[120] Furthermore, more recent works reported that mutations that inhibit TLR4 activity allow type II diabetic mice to be protected against endothelial dysfunction and hyperglycemia.[121]

An important role for TLRs (and in particular for TLR4) signalling also in IBD pathogenesis has been established through many studies over the last decade.[94] In order to maintain tolerance towards gut microbiota, the expression and activity of TLR4 and others TLRs is maintained at low levels in normal intestine.[92, 122-125] Indeed, when circulating monocytes leave the peripheral blood to become resident macrophages of the intestinal mucosa, they change their gene expression profile to become more tolerant to TLRs antigens.[126] However in IBD the down-regulation of TLR4 activity is lost contributing to the onset of the disease.[94] The role of TLR4 in IBD will be widely described in paragraph 1.5.

1.3 TLR4 THERAPEUTIC MODULATION

Growing evidences have recently pointed TLR4 as an emerging molecular target involved in a large number of disorders.[127]

Indeed, as described above, TLR4 plays a crucial role in many diseases that involve inflammation caused by bacterial LPS or endogenous factors (sterile inflammations). Considering that most of the pathologies listed in the previous

paragraph still lack a specific and efficient pharmacological treatment, the possibility to develop small molecules able to selectively modulate TLR4 activation has attracted increasing interest in a wide range of possible clinical settings. To date numerous are the compounds used to modulate the TLR4 signaling. On one hand there are TLR4 antagonists, which inhibit TLR4 signal by two different mechanisms: LPS sequestrants that avidly bind endotoxin to neutralize its toxic effect[128-130] and small-molecules that directly target the endotoxin receptor system, competing with LPS for CD14 and TLR4/MD-2 binding.[45, 131-134] While the use of LPS sequestrants is limited to the treatment of endotoxin-related pathologies (sepsis and septic shock), the use of TLR4-MD-2/CD14 targeting molecules can also be extended to the treatment of DAMP-related disorders such as neuroinflammations[135] and viral syndromes.[136] On the other hand there are also compounds with agonistic properties, which activate TLR4, and because of their activity are usually employed as vaccines adjuvants.[137] In the next sections some TLR4 modulators are presented and grouped into categories depending on their specific molecular target.

1.3.1 LPS SEQUESTRANTS

The first strategy to therapeutically modulate TLR4 is based on LPS neutralization by the formation of non-covalent adducts with cationic compounds, such as positively charged antimicrobial peptides (AMPs) including polymixin B, and synthetic dendrimeric polyamines which contain positively charged groups. The anionic amphiphilic nature of LPS enables ionic interaction with these positively charged agents, which form large LPS-

containing noncovalent complexes, preventing endotoxin from interacting with the receptors.

1.3.1.1 Antimicrobial Peptides as LPS sequestrants

Antimicrobial peptides (AMPs), also called host defense peptides (HDPs), represent an ancient host defense mechanism shared by all living organisms evolved to fight infectious agents, such as viruses, bacteria and fungi.[138] AMPs are oligopeptides generally composed of less than 100 amino acids and characterised by a high density of positive charges. The prototypic AMP is polymyxin B, a cationic, small cyclic lipopeptide, largely investigated for its endotoxin neutralizing property.[139] Further examples are cecropins,[140] magainins,[141] proline-arginine-rich peptides,[142] tachyplesin,[143] defensins,[144] and others.[145, 146] Structures of many of these peptides are known and include turn/loop, helix or □-sheet patterns. AMPs possess a broad-spectrum antimicrobial activity and were generally grouped using structure, biological target and mechanism of action as classification criteria.

Cecropins are AMPs that constitute a main part of the cell-free immunity of insects. The cecropin family is composed of 22-39 amino acids long lytic peptides described the first time in insects, but also present in pig intestine.[147] Cecropin A (CA) is composed of 37 amino acids and it was the first member of insect cecropins to be described (Figure 1.16).[148] The antimicrobial ability of CA is associated on its capacity to interact with bacterial cell membranes, forming large pores (ion channels) that cause cell death. The mechanism of action of this peptide is based on CA ability to avidly bind LPS and LTA.[149] Indeed, the two negatively charged phosphate groups of the lipid A moiety of LPS act as

binding site of CA molecules, allowing these peptides to interact with membrane and form pores. Data indicate that the interaction between CA and LPS is also due to hydrophobic interactions between the C-terminal domain of CA and the lipid A moiety of LPS.[149] In humans, two large families of AMPs have been identified: cathelicidins and defensins. The two AMPs families have little in common other than their strong microbial activity, however they share some features like a net positive charge and an amphiphilic structure with charged residues separated from hydrophobic residues. Cathelicidins are stored in cells as inactive pro-forms, generally contained in the peroxidase-negative granules of neutrophils.[150] In case of infection, the inactive pro-protein is cleaved generating a bioactive form, which is released in the extracellular compartment.[150] In humans, hCAP18/LL-37 is the only member of cathelicidins described (Figure 1.16).[151] Upon cleavage, hCAP18 *C*-terminal portion, named LL-37, is released exerting a variety of activities in order to protect the host. LL-37 was so named because its sequence begins with two leucine residues and is 37 amino acids long. This AMP possesses a broad antimicrobial activity,[152] it can modulate host immune response acting as chemoattractant[153] and it is a potent LPS-neutralizing factor.[154, 155] Defensins are a highly conserved family of AMPs generally released by keratinocytes and epithelial cells of different mucosal districts.[156] The peculiarity of this AMPs family is to contain 6 to 8 cysteine amino acids that are involved in the formation of characteristic disulfide bonds. The alignment of disulfide bridges is usually used as criteria to classify defensins into α-defensis, β-defensins (present in human) (Figure 1.16) and θ-defensins.[157] Similar to cathelicidins, α-defensis play crucial activities on both host and microbial cells. These peptides induce the production of pro-inflammatory cytokines like TNF-α and IL-1 β by

different leukocytes and are able to modulate host innate immune response against pathogens. Defensins are able to trigger chemotaxis of multiple host immune cells and to induce histamine release by mast cells. As for cecropins, the antimicrobial activity of this family of AMPs is linked to their ability to form pores on bacterial cells. [157]

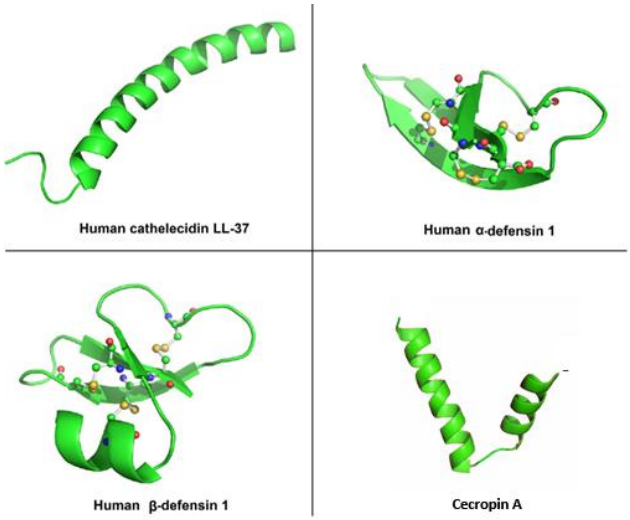


Figure 1.16. Cartoon representation of the four AMPs described above

Endotoxin-neutralizing activity

As introduced above, AMP are known to bind and neutralize LPS and interact with endotoxin.[128] Neutralization of LPS by AMPs involves a strong exothermic coulombic interaction between the two species, with ensuing fluidization of LPS acyl chains and a drastic change in LPS aggregate type from cubic into multilamellar. This interaction increases LPS aggregates size, disfavoring the binding of LBP and other mammalian proteins to the endotoxin.[158]

The cationic decapeptide polymyxin B (PMB) is known to possess high affinity for LPS,[159, 160] inhibiting many of its activity including its lethality *in vitro* and in animal models of endotoxemia (Figure 1.17).[161-163]

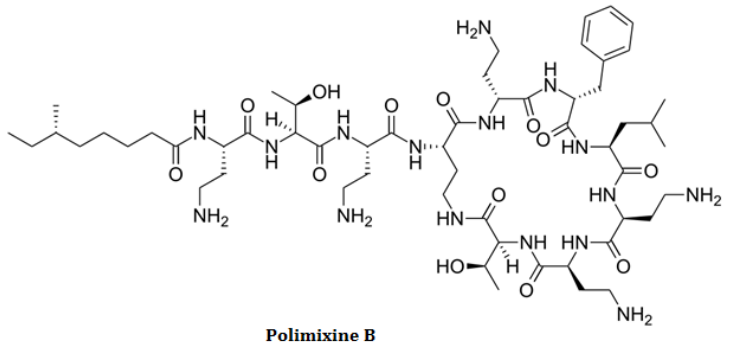


Figure 1.17 Chemical structure of the cationic cyclic decapeptide polymyxin B.

The addition of PMB to LPS-containing solutions results in the formation of a stable complex between these two molecules. Some studies revealed that PMB is able to recognize and bind the lipid A-KDO region of LPS with a stoichiometry of 1:1 (one molecule of PMB binds one molecule of lipid A). The interaction between these two molecules is reversible and most probably involves both ionic and hydrophobic interactions. It has been also demonstrated that the formation of PMB-LPS complexes does not abrogate all of the biological activities of PMB. The demonstration that PMB interacts with lipid A confirms the important role played by this LPS portion in many *in vivo and in vitro* biological activities. [164] In some models of endotoxin-induced tissue injury and experimental gram-negative septicemia, PMB has been found to be more effective than antibodies to core LPS.[165] Unfortunately, the higher PMB toxicity limits its use as a therapeutic agent in septic shock.[164, 166-168] Indeed, PMB is usually used as topic antibiotic because its toxicity precludes the systemic use. Non-toxic PMB derivatives have been developed, as well as

several classes of synthetic cationic amphiphiles including acyl and sulfonamide homospermines.[130, 169]Also LL-37, the unique member of human cathelicidins described above, is able to bind and neutralize LPS and LTA.[154, 155, 170, 171] This ability is associated to the linear amphiphilic α-helical conformation adopted by the peptide, which distributes positively charged amino acids one side and hydrophobic residues on the opposite side of the molecule (Figure 1.18).[157] Considering the enormous therapeutic potential of LL-37, many research groups focused their attention on the design and development of new synthetic variants of this peptide in order to increase its activity and stability and to reduce its susceptibility to enzymatic degradation.[154, 172, 173] New synthetic peptides for LPS and LTA neutralization in bacterial infections were developed and evaluated for their efficacy and safety as potential therapeutic drugs.[172] The first aim of these SAR studies was to obtain shorter peptides that maintain the strong endotoxin neutralizing activity of LL-37, but with lower ability to induce leukocytes chemotaxis.[172]

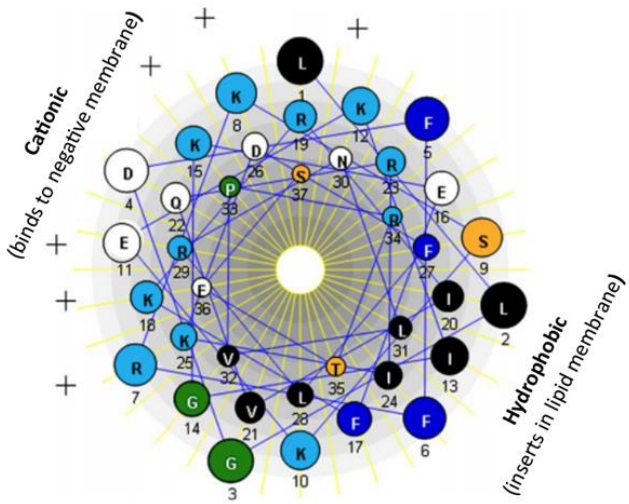


Figure 1.18. Schematic wheel plot of amino acid distribution of cathelicidin LL-37 in alpha-helical strcture.[157]

As human LL-37, also other AMPs possess the peculiar ability to bind and neutralize endotoxin. For instance, the cecropin CA described above is a small alpha-helical cationic peptide able to bind to the diphosphoryl lipid A moiety of LPS.[149] CA high affinity toward LPS allows this peptide to interact with the outer membrane of Gram-negative bacteria causing their disruption and consequently causing bacterial death. Considering the increasing resistance of a wide range of microbes towards antibiotics, AMPs represent potential novel therapeutic agents, whose safety and efficacy have to bee evaluated. LL-37 and CA possess a linear but relatively long sequence, which is not an ideal candidate for drug development. In order to obtain peptides with improved antimicrobial activity and low hemolytic effect, several research groups designed and synthesized AMPS hybrids. For instance, hybrids of CA and melittin (M) (the main component of bee venom) like CA(l-8) M(1-18) showed powerful antibacterial activities, exhibiting an improved potency relative to CA without the undesirable cytotoxic effects of melittin.[174, 175]

Although AMPs are able to neutralize LPS, the majority of AMPs are multifunctional molecules that play several important roles for the host. Despite the several studies, the capacity to estimate AMPs therapeutic effects is still difficult because of the discrepancy between the claims of the new AMPs as powerful drug candidates and the actual results of the clinical trials. The development of optimal AMPs formulations and the correct administration route for AMP-based drug are still considered the major barriers that hinder their practical use. Most of the new AMP-based drug candidates failed in the preclinical testing or at the discovery phase owing to their proteolytic degradation or unpredicted toxicity *in vivo*. For these reasons,

scientists have focused on the development of new topical AMPs as a safer and cost-effective option.

Other AMPs activities

As described above AMPs possess a broad-spectrum antimicrobial activity towards bacterial cells, viruses and fungi. Furthermore this effect seems to be synergistic with peptides that belong to other families.[176] The AMPs ability to kill pathogens may involve a variety of mechanisms that include membrane depolarization and permeabilization, induction of hydrolases activity, disruption of membrane functionalities and inactivation of intracellular effector proteins.[177] The crucial event that allows AMPs to take part to all this processes is the selective interaction with microbial membranes. The prokaryote cell wall is composed of many highly anionic molecules directed toward the extracellular compartment, such as LPS, in case of Gram-negative bacteria) or lipoteichoic acids (in case of Gram-positive bacteria). AMPs strongly bind to these components forming large aggregates in the lipid microbial bilayer. This event physically damages bacterial wall, allowing AMPs to enter the prokaryote cell and bind cytosolic target.[178-180]
Although AMPs are usually categorized by their antimicrobial activities, there is evidence that the majority of AMPs are multifunctional molecules involved in many important roles (Figure 1.19). Some AMPs, for instance, may act as immune-modulators mediating many process of host immune response. Some studies, for example, demonstrate that AMPs may act as opsonins causing bacteria opsonisation[181] or recruit several leukocytes behaving as chemoattractants.[182] In addition they can recruit leukocytes in an indirect fashion triggering the release of pro-inflammatory cytokines and mediators

like IL-8, IL-6 and MCP-1.[177] On the other hand AMPs may also exhibit an anti-inflammatory effect, inducing the production of anti-inflammatory mediators like IL-10 and TGFβ[177] or reducing TLRs activation. Indeed, some studies described that some AMP like cathelicidin are able to inhibit TLRs-mediated production of cytokines, to modulate components of TLR signaling[177] and to prevent dendritic cells maturation.[183] AMPs also play a crucial role in pro-resolution of immune response protecting the host against tissue damages caused by the inflammatory process. Indeed, during inflammation, phagocytic cells release many proteases to degrade ingested pathogens,[184] however and excess of these enzymes can seriously injures host tissue. In order to contain proteases activity, host responds sereting antiprotease molecules including many AMPs.[185] In addition, AMPs play a central role in many other intracellular processes, such as angiogenesis, arteriogenesis, cell signaling and wound healing responses.[186, 187]

At the base of AMPs "multifunctionality" there is probably their amphiphilic character that enables this molecules to be soluble in aqueous environment and also to enter lipid-rich membranes.[157] Indeed, although AMPs amino acid composition and molecular size are extremely diverse, these molecules share structural characteristics, which are essential for their multiple activities. First of all, AMPs present a net positive charge that enables tight interactions with the negatively charged membrane of microbial agents. This peculiarity allows AMPs to selectively act on bacterial cells, whereas mammalian cell membranes are neutrally charged.[186] Furthermore, AMPs hydrophobicity enables them to intercalate into the cell membrane causing the formation of pores responsible for cell lysis.[188] An increase in the hydrophobicity of the amino acid sequence of AMPs correlates with its low selectivity and toxicity toward mammalian

cells. This versatility makes them interesting candidates in the research and development of new drugs.

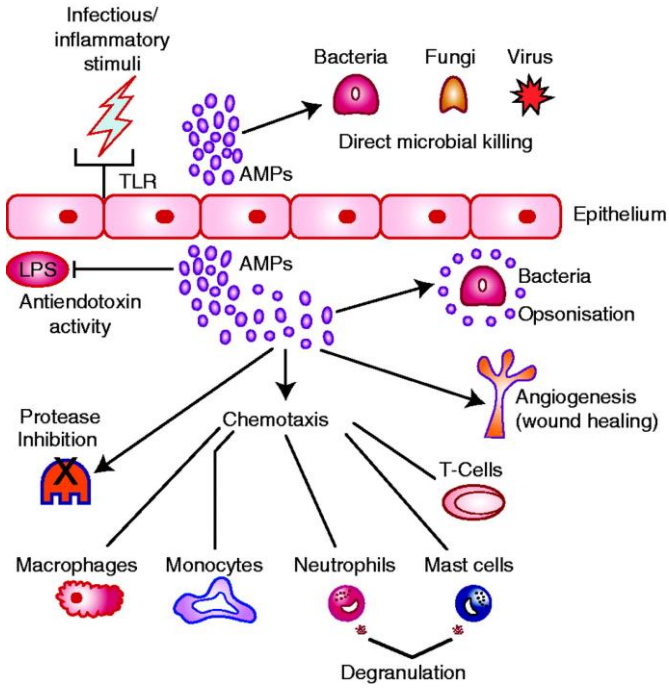


Figure 1.19. Schematic representation of epithelial AMPs main function.[189]

1.3.2 MOLECULES THAT TARGET LPS RECEPTORS

While the use of LPS neutralizing agents is limited to sepsis and septic shock, TLR4 antagonists that directly bind CD14 and TLR4/MD-2 complex have potential also as therapeutics to treat disorders caused by DAMP-TLR4 signaling.

1.3.2.1 Lipid A mimetics

The classic approach used to obtain antagonist small-molecules able to target the endotoxin receptor system, is the so-called "ligand-based drug design". This approach is based on the rational design of new small-molecules using the natural TLR4 ligand structure, the lipid A, as starting point. The main modification that can be maid to obtain a TLR4 antagonist, thus a molecule able to bind MD-2 without triggering TLR4 dimerization, is the removal of two or more FA chains, to give the so-called underacylated derivatives (for example, Eritoran).[131] The rational design of potentially new TLR4 inhibitors takes advantage from the availability of the crystal structure of human $(TLR4/MD\text{-}2/LPS)_2$ complex. Indeed X-ray crystallographic structure allowed diverse computational techniques to decipher some of the molecular basis that regard the ligand recognition processes involving TLR4/MD-2 system.[29] Some natural lipid A variants (e.g., lipid IVa, *Rhodobacter sphaeroides* lipid A, etc.) are underacylated and are not detected by the TLR4/MD-2 complex, allowing highly infectious bacteria to evade the innate immune system.[51] The synthesis of lipid A is an extremely complex process for organic chemists. Indeed the synthesis of the two glycosidic units, the donor and the acceptor, needs the use of a set of protecting groups, which increase the number of synthetic steps lowering the final yield. Furthermore, the two glycosylation

steps must be stereo-controlled reactions in order to obtain the selective formation, of β-(1→6) glycosylic and 1-α-phosphate bonds respectively.[190] To make the synthetic process less complicated, the design of new TLR4 modulators may also be perform simplifying the structure of lipid A to reduce the number of synthetic steps.[190, 191] There are many strategies to simplify the chemical structure of the natural ligand while preserving its biological activity and many of these have been described by F. Peri research group:[191]

- one of the two monosaccharides could be substituted by another chemical moiety such as an aminoacid;
- the di- glucosamine scaffold could be replaced by a monosaccharide;
- the whole disaccharide structure could be replaced by another polifunctional scaffold;
- phosphate groups could be replaced by bioisosteres groups such as carboxylic acids or a sulfates;
- the number of acyl chains can be reduced from 6 to a minimum of 2.

The variations listed above, in particular the number, type and length of the acyl chains, as well as the number of phosphate groups, are key determinant elements to get molecules with agonistic or antagonistic properties.[191] Indeed by varying one of more of these elements, a compound can switch from potent TLR4 antagonist to agonist by simply varying the length or number of the lipid chains.[58] Furthermore, as described in paragraph 1.2.3, because of the structural differences between human and murine TLR4/MD-2 receptors, some compounds which act as antagonist on human cells, show agonist properties in mice.[58]

Disaccharidic lipid A mimetics: E5531 and Eritoran (E5564)

Compound E5531 is an analogue of the lipid A from *Rhodobacter capsulatus* developed by Eisai laboratories (Boston).[192] E5531 showed potent inhibition of LPS-induced toxicity *in vitro* and *in vivo*, however its beneficial effect dramatically dropped over time because of the interaction with plasma lipoproteins.[193] A second-generation LPS antagonist, Eritoran (E5564) (Figure 1.20) was then developed by Eisai. Eritoran derived from the structure of non-inflammatory lipid A of *R. sphaeroides.* E5564 resulted as a potent *in vitro* endotoxin antagonist able to compete with LPS for the direct binding to MD-2 cavity, thus preventing TLR4 dimerization.[131] Eritoran was co-crystallized with TLR4/MD-2 complex thus becoming a valuable source of information for defining antagonistic properties of TLR4 ligands.[22, 28] Eritoran possess four acyl-chains (it is an underacylated lipid A variant), thus lacking the sufficient number of chains needed to trigger the dimerization process described in paragraph 1.2.2. In particular, the structure formed by the four acyl chains of Eritoran complements the shape of MD-2 hydrophobic pocket and occupies almost 90% of the solvent accessible-volume of the cavity. R2 and R3 acyl chains adopt a fully extended conformation, while R2' and R3' are bent in the middle (Figure 1.21).[22]

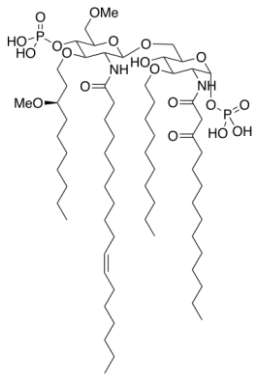


Figure 1.20. Eritoran structure.

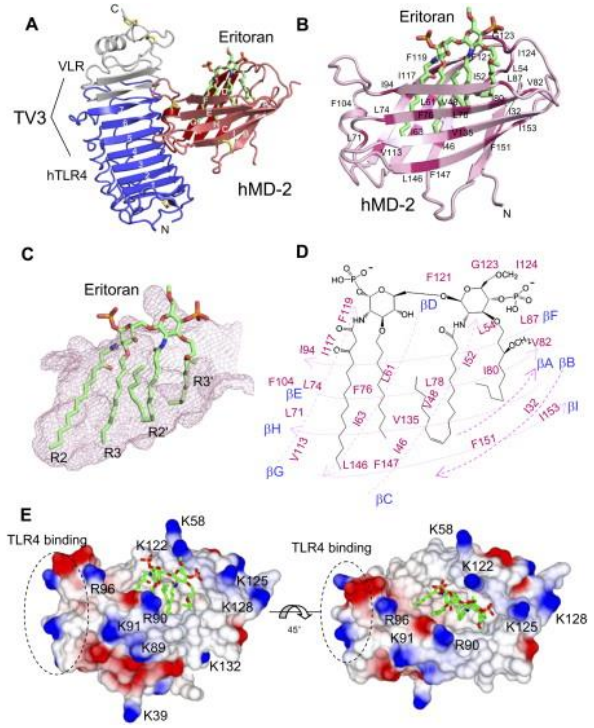


Figure 1.21. (A) Overall structure of the TLR4-MD-2-Eritoran complex. **(B)** Close-up view of the human MD-2 and Eritoran complex. The carbon, oxygen, and phosphorous atoms of Eritoran are green, red, and orange, respectively. MD-2 residues interacting with the hydrophobic acyl chains of Eritoran are colored magenta and labeled. **(C)** Shape of the Eritoran-binding pocket. The surface of MD-2 is drawn in purple mesh. The four acyl chains of Eritoran are labeled. **(D)** Chemical structure of Eritoran. MD-2 residues interacting with Eritoran are labeled. The β strands are shown schematically as broken arrows. **(E)** Surface representation of MD-2. Positively and negatively charged surfaces are colored blue and red, respectively. Lysines and arginines interacting ionically with Eritoran are labeled.[22]

Although potent activity showed, Eritoran failed to pass clinical Phase III. Eisai explained that the drug was discontinued because it did not meet the primary endpoint of reduction in 28-day all-cause mortality in patient with severe sepsis.[194]

“Pseudo”-disaccharidic lipid A mimetics

Aminoalkyl glucosaminide phosphates (AGPs) are lipid A mimetics developed by Corixa (and also called CRX compounds), in which the reducing glucosamine residue has been replaced by an acylated amino acid or another acylated function (Figure 1.22). These compounds generally retain significant activity as TLR4 modulators, having a simplified structure with a reduced number of stereogenic carbons, and can be therefore obtained by a simpler synthesis than lipid A. Many homologues have been synthesized, by changing the length of the alkyl chains and a systematic structure-activity relationship study has been conducted on these molecules.[195] Among AGPs, compound CRX-527 (Figure 1.22) with C_{14} primary and C_{10} secondary lipid chains has agonist activity and for this reason it is currently in use as vaccine adjuvants and in cancer immunotherapy. In contrast, CRX-526 (Figure 1.22) with C_{14} primary and C_6 secondary fatty acid chains has potent antagonistic activity and can block the induction of pro-inflammatory cytokines by LPS both *in vitro* and *in vivo*.[196]

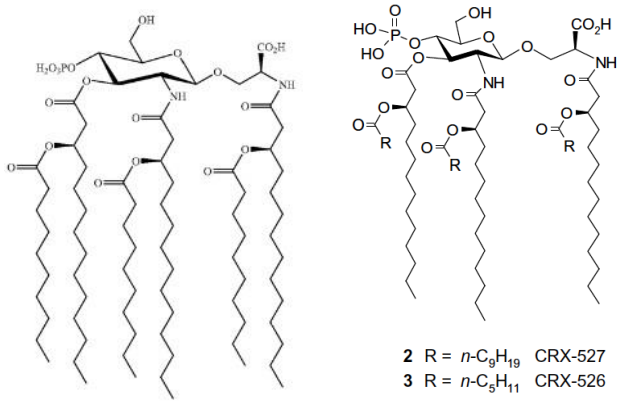


Figure 1.22. On the left AGP structure, on the right CRX-527 and CRX-526 structures.

Monosaccharidic lipid X mimetics

The rational design of new TLR4 antagonist can be made from the structure of the lipid A biosynthetic precursor, the lipid X (Figure 1.23). Lipid X corresponds to the reducing N-acetyl-glucosamine monosaccharide with different acylation patterns and it is able to block LPS-induced septic shock and priming of TLR4-dependent human neutrophils.[197, 198] For these reasons, lipid X has often been considered the simplified monosaccharide scaffold for the development of new TLR4 modulators.

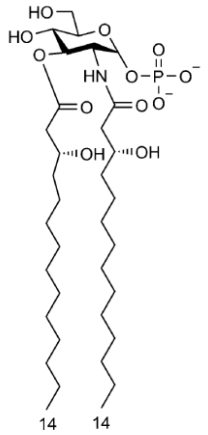


Figure 1.23. Structure of lipid X.

TLR4 modulators obtained using the approach of "mimicking" Lipid A and simultaneously by simplifying its structure are generally amphiphilic molecules. Among lipid X analogues, a large array of compounds named Gifu lipid A (GLA) have been synthesized by systematic variation of FA chains types (linear or branched) and lengths. The FA chains are linked to the sugar core as esters, amides, ethers, and amines. GLA have one or two phosphate groups in positions C1 and/or C4 and a variable number of acyl linear chains (2,3 and 4). Compounds with two and four C_{14} FA acyl chains showed TLR4 antagonistic

activity in human cells, otherwise compounds with three fatty acid chains are agonists both in murine and human cells monocytes. Among these molecules, GLA-47 (Figure 1.24) is a monophosphorylated monosaccharide with four acyl chains of 14 carbon atoms. GLA-47 only weakly induce TNF-α release in murine macrophages, but is able to reduced cytokine TNF-α and IL-6 release in human U937 cells.[199]

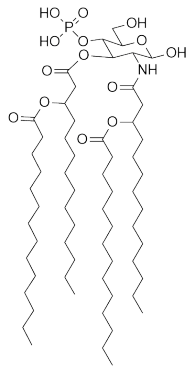


Figure 1.24. GLA-47 structure.

1.3.2.2 Cationic amphiphilic TLR4 modulators

Although the majority of TLR4 modulators are lipid A variants and synthetic lipid A mimetics, negatively charged at neutral pH, studies have found that also some amphiphilic molecules with a net positive charge can modulate the TLR4 receptor system. For instance, several cationic lipids composed of positively charged head-groups (tertiary or quaternary ammonium salts or polyamines) and a hydrophobic portion (alkyl chains or steroids) showed to be TLR4 modulators, acting both as agonists and antagonists.[200] Other studies, in fact, revealed that some positively charged liposomes formed by cationic amphiphiles are able to induce the production of pro-inflammatory mediators. It has been described that liposomes made up by diC14-amidine (Figure 1.25) trigger the secretion of a cytokine pattern very

similar to that induced by LPS-TLR4/MD-2 through the activation of both MyD88-dependent and TRIF-dependent pathways.[201] As for lipid A mimetics, minimal changes in the structure of the molecules can cause the switch from agonistic to antagonistic activity, as in the case of dioleoyl trimethylammonium propane (DOTAP) (Figure 1.25), able to inhibit TLR4 signaling by competing with LPS for the binding to LBP and CD14.[202]

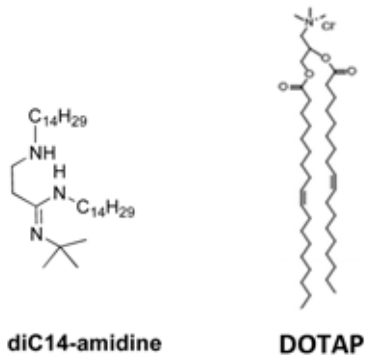


Figure 1.25. Structure of cationic amphiphilic TLR4 modulators.

Also the commercial transfection reagent, Lipofectamin™ showed antagonistic properties when administered with LPS on TLR4-expressing cells. Indeed, the positive charges of cationic Lipofectamin™ are able to interact with LPS forming large complexes that co-localize with surface and cytosolic CD14, but not with TLR4/MD-2 complex. These data suggest that the inhibition mechanism of Lipofectamin™ is probably based on its capacity to uncouple CD14 and TLR4 signaling.[203] In 2008 F. Peri research group synthesized a small library of positively charged small-molecules from the further simplification of lipid A structure. The compounds were named IAXO and were positively charged glycolipids in which two C_{14} alkyl chains are linked to the C2 and C3 positions of a methyl-α-D-glucopyranoside with a protonatable amine group on C6 (IAXO 101 and 102) or to an aromatic ammonium salt as in the case of

IAXO 103 (Figure 1.26). The compounds were active in inhibiting LPS-induced, TLR4-mediated inflammation by competing with LPS for specific CD14 binding.[204, 205] Among IAXO series, IAXO-102 and 103 resulted the most promising TLR4 inhibitors both *in vitro* and *in vivo*, with IC_{50} values of 5.5 and 1.7 μM, respectively, and high antagonist activity in *in vivo* models of sepsis.

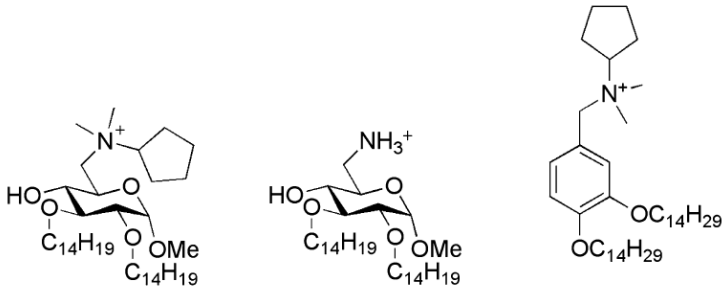


Figure 1.26. The IAXO compounds.

The carbohydrate scaffold in amino glycolipids is probably important to prevent possible random conformations and to favor an optimal, lipid A-type orientation of lipid chains. Indeed, the main structural feature of TLR4 modulators is the "facial" arrangement with positive/negative charges and lipophilic chains disposed in spatially well-defined regions. Therefore the carbohydrate core can be used as a platform to obtain amphiphilic molecules by the functionalization with cationic or anionic groups on one side and hydrophobic moieties on the others. In this context in 2014 F. Peri research group designed and synthesized a library of new cationic glycoamphiphiles using the monosaccharide methyl α-D- glucopyranoside and the disaccharide α,α'-trehalose as sugar cores.[206] Some of the compounds obtained (Figure 1.27) were active in preventing LPS-triggered inflammatory responses *in vitro* and *in vivo,* with a potency in the same order of magnitude of the best synthetic TLR4 antagonists so far tested by us[207] and other groups.[208] In

particular, the results obtained by biological test allow us to extrapolate some insight regarding the structure–activity relationship (SAR) of this class of compounds: first of all, the presence of acyl lipophilic chains in the hydrophobic portion seems to be a primary requisite for activity; secondly, the well-ordered facial amphiphilic character, ensured in particular by trehalose scaffold, seems to be a fundamental requirement for a high *in vitro* and *in vivo* antagonist activity.[206]

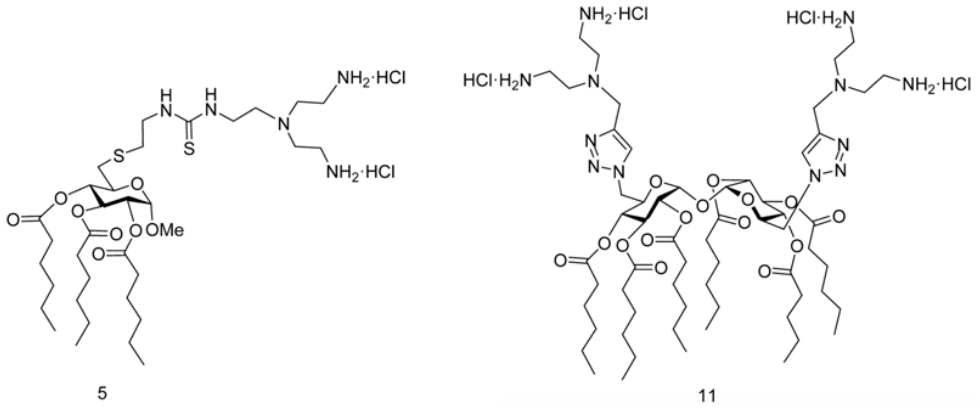


Figure 1.27 Structure of trehalose- and glucose-derived glycoamphiphiles.

1.4 THE DIPHOSPHRYLATED GLYCOLIPID FP7

1.4.1 RATIONAL DESIGN

As described above, the total synthesis of lipid A and of lipid A analogues with a disaccharidic scaffold (like Eritoran or others) [195, 209] is very complicated, because it implies the orthogonal protection of the many hydroxyl groups present on precursor sugars and a glycosylation step which generally has low yields or is hardly reproducible in different laboratories. Thus lipid A mimetics with monosaccharidic backbone represent valid solutions to simplify the synthesis process. With the goal of "keeping it simple" branched chains were not inserted in the structure. Moreover lipid A acyl chains, composed by either branched or not (R)-3-hydroxymyristic acid, are replaced in analogues by simple, linear chains of myristic acid (C_{14}).

F. Peri research group synthesized mono- and di-phosphate monosaccharidic lipid X analogues.[207] Mono-phosphate mimetics possess a unique phosphate group linked to the anomeric C1 or to the C4 position, while the di-phosphate mimetic possess a phosphate group on both positions. The two mono-phosphate mimetics showed a weak antagonist activity in HEK-Blue hTLR4 cells and murine macrophages, while in contrast the di-phosphate monosaccharide, named FP7 (Figure 1.28), proved to be a potent TLR4 antagonist. In particular FP7 is able to inhibit in a dose-dependent way LPS-triggered NF-κB activation in HEK-Blue hTLR4 cells and to reduce LPS-induced TNFα production in murine macrophages.[207] FP7 antagonist activity is observed in cells at concentration values below the critical micelle concentration (CMC) of 9 μM: FP7 IC_{50} is comprised between 1.5 to 3.5 μM in HEK-Blue hTLR4 cells; in this concentration range, FP7 is mainly in the monomeric form in solution.[207] The results obtained by F. Peri research group confirmed once more that the

number and position of phosphates are important molecular determinants for the biological activity of the molecules. The presence or absence of the (R)-3-hydroxyl groups on the fatty acid chains of Lipid X seems to be not relevant for activity, as neither is the presence of an ester or an amide linkage in C-3 position.

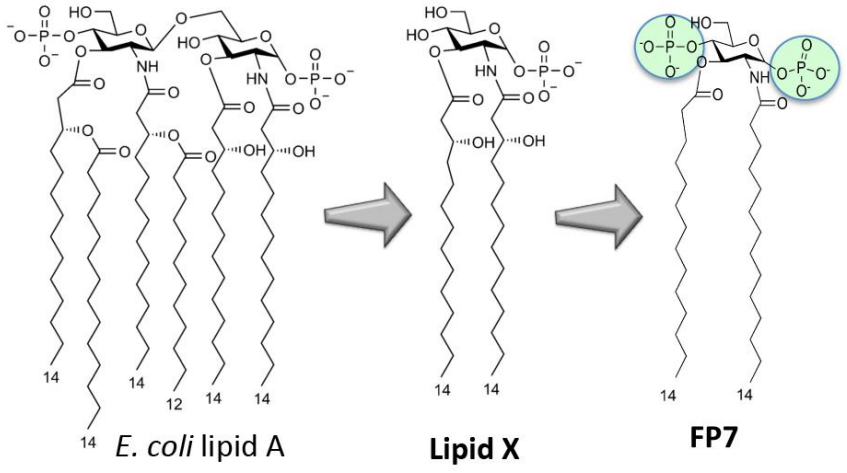


Figure 1.28. Molecular simplification of lipid A and rational design of FP7

1.4.2 MOLECULAR DOCKING STUDIES

FP7, as other lipid A mimetics, is designed to bind and occupy the hydrophobic cavity of MD-2 protein in order to prevent LPS-induced TLR4 dimerization. FP7 capacity to bind both MD-2 and CD14 receptors was evaluated through molecular docking studies. The resulted obtained and published by F. Peri research group in 2014,[207] showed reasonable binding poses predicted for FP7 in both proteins. In particular, AutoDock and Vina predicted that FP7 bind MD-2 cavity in two different fashions, with close predicted binding energies. The best of docked solutions corresponded to a binding pose in which the two FA chains of FP7 are deeply confined inside the

MD-2 pocket, similarly to what happens with lipid IVa and Eritoran. One of the FA chains establishes hydrophobic contacts with Leu74, Phe76, Phe104, and Ile117, in a similar way to the equivalent FA chain present in lipid IVa (Figure 1.29, violet structure). The second FA chain is directed into the region delimited by Ile52, Leu54, Phe121, Ile124, Tyr131, and Ile153, a subpocket also occupied by a FA chain in the complex with lipid IVa. However, in few cases, results from docking showed a second binding mode, in which one FA chain extending towards Val82 and placed over Ile124 (Figure 1.29, blue structure). Polar interactions were also identified in some of the docked binding poses. One phosphate group participates in hydrogen bonds with Ser118, for instance and is always located in the vicinity of Lys58 and/or Lys122, similarly to one of the lipid IVa phosphates. The second phosphate group is found in the vicinity of positively charged side chains or exposed to the outside. In addition, in some of the docking results either the amide CO group or an ester CO group from FP7 was found to establish a hydrogen bond with the Ser120 CO group. These predicted binding poses are in agreement with the NMR experiments and provide a 3D model for the interaction of the FA chains with MD-2 protein, as well as for putative polar interactions involving the phosphate groups.[207]

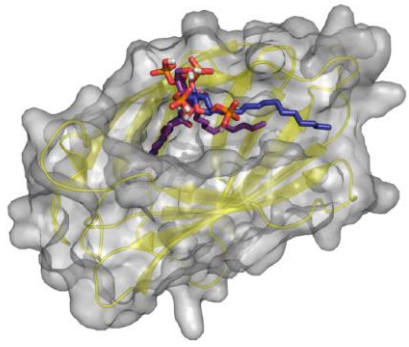


Figure 1.29. Superposition of the AutoDock binding poses of FP7. The two binding poses are characterized for having two (violet) or only one (blue) FA chain oriented inside the lipophilic MD-2 pocket.

Binding poses were calculated also for FP7 into CD14 co-receptors. CD14 also has a highly lipophilic wide pocket, but with fewer charged residues in the opening portion. The calculated binding poses showed that FP7 introduces both FA chains inside the pocket, with the polar phosphate groups and sugar placed at the entrance of the cavity (Figure 1.30). Polar interactions were also identified in some of the predicted binding poses. AutoDock calculations led to the observation of two hydrogen bonds involving one of the phosphate groups (with NH group of Lys122 and CO group of Ser120). The other phosphate establishes an electrostatic interaction with the nearby OH group of Tyr102. The docked poses calculated by Vina also predicted a hydrogen bond between one phosphate group and the Lys122 NH group, while the second phosphate group is exposed to the outside side of the protein. Regarding the second predicted binding mode, the two FP7 FA chains are again inside the lipophilic pocket, but only Vina modeling indicated in a few poses a possible hydrogen bond between one phosphate group and the amide group of Gln62 side chain.

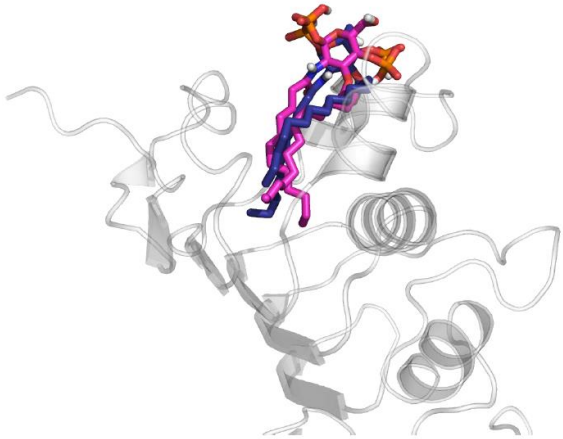


Figure 1.30. Superimposition of docked binding solutions of FP7 in CD14 from AutoDock 4.2 (depicted in blue) and Vina (depicted in violet).

1.4.3 POTENTIAL THERAPEUTIC APPLICATIONS

As described previously, LPS- and DAMPs-induced TLR4 signalling is strongly involved in many inflammatory diseases, many of which are described in section 1.2.6. The fact that TLR4 pathway can be activated by both pathogens-related and endogenous danger-associated molecules makes this receptor an interesting therapeutic target to be modulated to reduce inflammation in different context. Considering that LPS neutralizing agents are limited to sepsis and septic shock treatment; the use of CD14- and TLR4/MD-2-targeting compounds, as FP7, may be a successful strategy to treat a wider range of disorders, including DAMPs-associated diseases. Secondly, the use of small-molecules that selectively target TLR4 and its co-receptors, allow us to investigate the actual role of this receptor in different disorders and in different phases of the same pathology.

FP7 is currently being tested in different context in order to investigate its potential use to reduce inflammation. For instance, growing evidence indicates that deregulated inflammatory responses could play a crucial role in the pathogenesis of motor neuron injury in amyotrophic lateral sclerosis (ALS).[135] In particular, considering that abnormal TLR4 signaling in pro-inflammatory microglia cells had been related to motoneuron degeneration, FP7 anti-inflammatory effect was investigated on *in vitro* ALS models. The experiments conducted revealed that FP7 compound efficiently protected motoneurons from LPS-induced lethality in spinal cord cultures.[135] Furthermore, recent studies showed that FP7 is able to selectively prevent TLR4-mediated cell activation in DCs (IC_{50} < 1 μM) and to prevent DC maturation upon LPS stimulation. Moreover, FP7 showed to protect mice from influenza virus-

induced lethality. In this immunopathology TLR4 hyper-activation is caused by several DAMPs including HMGB1 and oxidized phospholipids.[210]

Good water solubility, the lack of cytotoxicity, and selective TLR4 targeting make FP7 and other recently developed compounds promising therapeutic candidates to be tested in different conditions that involve inflammation. On the other hand studies on the SAR of this molecule will allow to perform the so called “lead optimization”, in order to further improve FP7 activity.

Of particular interest is also to study the role of TLR4 modulators in inflammatory bowel disease (IBD). Although the causes that lead to the onset of IBD are still unclear, growing evidences indicate that the prolonged inflammation that characterized this group of intestinal disorders is the result of an abnormal immune response towards commensals in genetically susceptible individuals. Thus, one possible strategy to ameliorate IBD-associated inflammation may be to reduce the ability of the mucosal immune system to excessively respond to bacterial antigens via PRRs. The use of TLR4 antagonists could be beneficial to reduce the pro-inflammatory signal activated by the host, restoring the tolerance of gut mucosal immune cells towards microbiota components. Furthermore, as told above, the use of molecules that selectively target TLR4 may shed light on TLR4 role in IBD-associated inflammation.

Here mention IBD chronic inflammation as one of the potential clinical settings of FP7 and other TLR4 antagonists.

1.5 INFLAMMATORY BOWEL DISEASE (IBD)

Inflammatory bowel disease (IBD) represents a group of intestinal debilitating disorders that cause prolonged inflammation of the digestive tract. The two major forms of IBD in humans are Crohn's disease (CD) and ulcerative colitis (UC), both characterized by intolerance towards antigens contained in the intestinal lumen.[211, 212] The chronic inflammatory process in IBD leads to the appearance of lesions along the gastrointestinal tract that may compromise the normal and physiological functions covered by the digestive system.[213] This class of disorders has a chronic-intermittent clinical course with a high symptomatic recurrence rate in patients undergoing bowel resection.[214] Although the etiology of IBD is still unknown, there is evidence that the pathologic process results from the interaction of different factors, including environmental changes, the genetic background, gut dysbiosis and host immunity.[213, 215, 216] The current knowledge of IBD emerged from a combination of gene association studies, clinical investigations, and laboratory experiments on mice. In particular, studies conducted on experimental models of colitis suggest that the inflammation-driven tissue damage is the result of an abnormal immune response against an altered microbiota in genetically susceptible individuals.[211, 212] Moreover in IBD patients the uncontrolled host response is not offset by the physiological counter-regulatory mechanisms normally activated by the organism to end the inflammatory process.[217, 218]

1.5.1 EPIDEMIOLOGY

CD and UC are modern age disorder, whose spread gradually increased since the second half of the twentieth century.[219, 220] IBDs have a strong social

impact, which can strongly affect the relational and working life of patients. Epidemiology supports the important role played by the environment in IBD pathogenesis. Indeed the recent increase of IBD incidence in geographic regions with formerly low prevalence, such as Latin America and Asia,[221, 222] suggests the greatly impact of industrialization and 'westernization' phenomena in IBD geographical distribution.[223, 224] In some countries like USA and Canada, IBD prevalence follows gradients that reflect difference in population density, urbanization, genetic background and exposure to multiple environmental factors.[225]

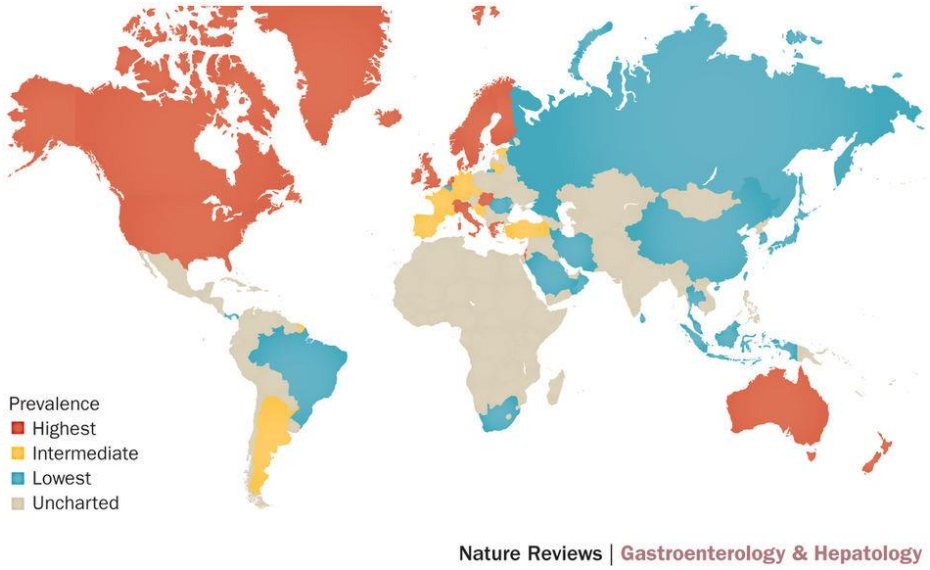


Figure 1.31 The global burden of IBD: from 2015 to 2025. Data from Molodecky et al.[220] Adapted from an image provided by PresenterMedia.[226]

1.5.2 AETIOLOGY

1.5.2.1 Role of genetic

Although the causes that lead to the onset of IBDs are not still clarified, multiple observations support the idea that genetic and environmental factors strongly interact with each other to foster the pathogenic process: firstly, the

strong family recurrence and second the IBD tendency to manifest in combination with other rare genetic disorders. However IBDs are more to be considered as polygenic pathologies with multifactorial etiology, rather than mendelian inheritance diseases. Indeed they are familial in only 5-10% of cases against the 90% of remaining sporadic cases.[227] The availability of new quantitative and qualitative genetic techniques has allowed to investigate the main genetic factors involved in IBD pathogenesis,[228, 229] leading to the identification of about 160 loci involved in CD and UC susceptibility.[230, 231] Prominent for CD are genomic regions containing nucleotide oligomerization domain 2 (NOD2),[215] autophagy genes (ATG16L1, IRGM)[232-234] and components of the interleukin-23–type 17 helper T-cell (Th17) pathway;[215] while genes with regulatory functions, such as IL-10 and ARCP2, E3 ubiquitin ligase loci and genes involved in intestinal epithelium functions resulted to be related to UC.[232] Studies showed that the majority of genes listed above reside in a particular chromosomal region called IBD-1 (inflammatory bowel disease-1, 16q12 chromosome), which is estimated to be responsible for at least the 15% of CD susceptibility.[215] A key step in the study of this genetic region was the identification of NOD2/CARD15 gene, a factor that belongs to the CARD (Caspase Recruitment Domain) family and whose role is linked to the immune response towards intestinal bacteria.[235] Indeed NOD2 protein is an intracellular sensor that allows monocytes and macrophages to detect bacterial peptidoglycan and consequently to promote the NF-κB-mediated production of pro-inflammatory cytokines.[235] Although the genetic background is important to increase IBD susceptibility, many studies conducted on twins suggest that genetic cannot be the only component to promote IBD.[215, 236] Indeed studies conducted on experimental models of colitis support the fact

that another important factor such as gut microbiota contributes to the pathogenesis of IBD, probably by augmenting host pro-inflammatory immune responses.[2] The strongest evidence for the role of microbiota in the development of IBD comes from the many mouse models of colonic inflammation. The fact that germ-free mice do not develop colitis, demonstrate that the presence of enteric bacteria is necessary to initiate the inflammatory process.[237] The table below summarized the main gene associated with UC and DC pathologies.

Table 1. **Genetic Associations with Crohn's Disease and Ulcerative Colitis.***

Gene	Genomic Region	No. of Genes in Region†	Associated with Crohn's Disease	Associated with Ulcerative Colitis	Function
Innate immune responses					
NOD2 (nucleotide-binding oligomerization domain 2)	16q12	1	Yes	No	Senses bacterial peptidoglycan to activate cell signaling
ATG16L1 (autophagy-related, 16-like)	2q37	1	Yes	No	Component of autophagy complex
IRGM (immunity-related GTPase M)	5q33	3	Yes	Equivocal	Role in autophagy; required for interferon-γ–mediated clearance of intracellular pathogens
Interleukin-23–Th17 pathway					
IL23R (interleukin-23 receptor)	1p31	1	Yes	Yes‡	Unique component of heterodimeric interleukin-23 receptor
IL12B (interleukin-12B, p40 subunit)	5q33	1	Yes	Yes‡	Component of interleukin-23 cytokine; common to interleukin-12
STAT3 (signal transducer and activator of transcription 3)	17q21	4	Yes	Yes‡	Major STAT downstream of various cytokines, including interleukin-6, 10, 17, 21, 22, and 23
CCR6 (chemokine [C-C motif] receptor 6)	6q27	3	Yes	No	Cell-membrane protein mediating migration and recruitment of inflammatory cells
Other genes in association regions					
PTGER4 (prostaglandin E receptor 4)	5p13	0	Yes	No	One of the receptors for the inflammatory mediator *PGE2*
ZNF365 (zinc finger protein 365)	10q21	1	Yes	No	Reported role in mitosis
SLC22A4 (solute-carrier family 22, organic-cation transporter)	5q31	7	Yes	Equivocal	Plasma membrane polyspecific organic cation transporter
PTPN2 (T-cell protein tyrosine phosphatase)	18p11	1	Yes	No	Multiple interactions with STAT proteins; also associated with type 1 diabetes
Major histocompatibility complex (MHC)	6p21	—	Yes‡	Yes	Distinct MHC class II associations between ulcerative colitis and Crohn's disease
NKX2-3 (NK2–transcription-factor–related, locus 3)	10q24	1	Yes	Yes‡	Homeodomain-containing transcription factor affecting lymphoid and spleen development
MST1 (macrophage stimulating 1)	3p21	35	Yes	Yes‡	Involved in macrophage chemotaxis and activation following proinflammatory signals
PLA2G2E (secretory phospholipase A_2)	1p36	0§	No	Yes	Releases arachidonic acid from membrane phospholipids
IL10 (interleukin-10)	1q32	1¶	Equivocal	Yes	Immunosuppressive cytokine with a central role in regulating intestinal inflammation
IFNG (interferon-γ)	12q15	2§	No	Yes	Critical cytokine in innate and adaptive immunity against intracellular pathogens

Figure 1.32. Genetic associations with CD and UC[212]

1.5.2.2 Role of the gut microbiota

Gut microbiota is the term usually used to indicate the large community of microorganisms that normally inhabit the animal gut. Between the

microbiota and the host exists a symbiotic mutualism in which both partners have benefits, that is the result of at least half a billion years of co-evolution.[238] The host provides niches and nutrients for microbial survival and regulates microbiota composition,[239-241] in return, commensals contributes to many host physiological processes; among them there are the digestion/fermentation of carbohydrates, the modulation of energetic metabolism, the production of vitamins, the prevention against pathogen colonization and the development/maturation of the mucosal and systemic immune system.[241-243] In order to maintain this beneficial homeostasis, the host has to adopt multiple mechanisms to be tolerant towards commensals and to minimize potentially dangerous immune responses.[244, 245] However, perturbations of different nature may disrupt the homeostatic equilibrium making these beneficial interactions harmful and consequently causing or contributing to the onset of disease.[246, 247] The key role played by the microbiota in the IBD onset is supported by results obtained on a huge number of experimental models of colitis. Indeed, all mice knockout (-/-) or transgenic (Tg) for specific regulatory cytokines (IL-2, IL-10, TGF-β) or for their receptors, or for antigen recognition molecules, develop spontaneous human IBD-like colitis only when commensal flora was present (Figure 1.33).[2] The fact that germ-free mice do not develop or develop very mild colitis, demonstrate that the presence of enteric bacteria is necessary to initiate the inflammatory process.[237]

Commensal bacterium	Host	Genotype	Disease	Receptors	Possible mechanism
Commensal microbiota that promote the development of IBD					
E. coli	Mouse	*Il10*$^{-/-}$	Spontaneous colitis	Unknown	Monocolonization of germ-free mice induces colitis
E. coli	Mouse	*Il2*$^{-/-}$	Spontaneous colitis	Unknown	Monocolonization of germ-free mice induces colitis
Enterococcus faecalis	Mouse	*Il10*$^{-/-}$	Spontaneous colitis	Unknown	Monocolonization of germ-free mice induces colitis
B. vulgatus	Rat	*HLA-B27–B2m* transgenic	Spontaneous colitis	Unknown	Monocolonization of germ-free rats induces colitis
B. vulgatus	Mouse	*Il10r2*$^{-/-}$*Tgfbr2*$^{-/-}$	Spontaneous colitis	Unknown	Colonization of antibiotic-treated mice triggers colitis
Bacteroides thetaiotaomicron	Mouse	*Il10r2*$^{-/-}$*Tgfbr2*$^{-/-}$	Spontaneous colitis	Unknown	Colonization of antibiotic-treated mice triggers colitis
B. thetaiotaomicron	Rat	*HLA-B27–B2m* transgenic	Spontaneous colitis	Unknown	Monocolonization of germ-free rats induces colitis
Bacteroides unifirmatis	Mouse	*Il10r2*$^{-/-}$ *Tgfbr2*$^{-/-}$	Spontaneous colitis	Unknown	Colonization of antibiotic-treated mice triggers colitis
*Klebsiella pneumoniae**‡	Mouse	*Tbx21*$^{-/-}$*Rag2*$^{-/-}$	Spontaneous colitis	Unknown	Other commensal bacteria are required for the induction of colitis
*Proteus mirabilis**‡	Mouse	*Tbx21*$^{-/-}$*Rag2*$^{-/-}$	Spontaneous colitis	Unknown	Other commensal bacteria are required for the induction of colitis
*Helicobacter typhlonius**	Mouse	*Tbx21*$^{-/-}$*Rag2*$^{-/-}$	Spontaneous colitis	Unknown	Transmissible to non-colitogenic TRUC mice
*Prevotellaceae**‡	Mouse	*Nlrp6*$^{-/-}$, *Asc*$^{-/-}$ or *Casp1*$^{-/-}$	DSS colitis	Unknown	Impaired IL-18 signalling promotes pathobiont expansion
TM7*‡	Mouse	*Nlrp6*$^{-/-}$, *Asc*$^{-/-}$ or *Casp1*$^{-/-}$	DSS colitis	Unknown	Impaired IL-18 signalling promotes pathobiont expansion
Bilophila wadsworthia	Mouse	*Il10*$^{-/-}$	Spontaneous colitis	Unknown	Consumption of a diet composed of milk-derived fat induces pathobiont expansion

B2m, gene encoding β2-microglobulin; *Casp1*, caspase 1; DSS, dextran-sulphate sodium; IBD, inflammatory bowel disease; IL, interleukin; MYD88, myeloid differentiation primary-response protein 88; NA, not applicable; *Nlrp6*, NOD-, LRR- and pyrin domain-containing 6; PBMCs, peripheral blood mononuclear cells; *Rag2*, recombination-activating gene 2; *Tbx21*, T-box 21 (encodes T-bet); *Tgfbr2*, transforming growth factor-β receptor 2; TLR, Toll-like receptor; TNBS, 2,4,6-trinitrobenzene sulphonic acid; T_{Reg} cell, regulatory T cell; TRIF, TIR domain-containing adaptor protein inducing IFNβ; TRUC, *Tbx21*$^{-/-}$*Rag2*$^{-/-}$ mice. *Transmissable to mutant hosts. ‡Transmissable to wild-type hosts.

Figure 1.33. Role of the gut microbiota in the induction of IBD.[2]

In particular, one of the key elements linked to the onset of intestinal and extra-intestinal disorders seems to be the alteration of gut commensals composition, a condition recently termed "gut dysbiosis".[238] Gut dysbiosis is thought to be caused by a shift in relative bacterial abundances, triggered by events of different nature, such as diet changes, inflammation, immune deficiency, or exposure to antibiotics or toxic agents.[2] The combined effect of these environmental factors with the genetically determined high IBD susceptibility would be crucial in the onset of the disease.[2, 94, 238] In genetically immuno-incompetent hosts, for example, commensal composition may shift and turn pathogenic, causing tissue-destructive host responses responsible for

colitis.[248, 249] The human gut microbiota is composed of more than 500 species that belongs to 4 dominant phyla: Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria.[250, 251] Bacteroidetes (Gram negative) and Firmicutes (Gram positive) are the most represented phyla (more than 90% in the colon), whereas the remaining 10% consist of Actinobacteria and Proteobacteria, which are scarce in the colon. Recent studies shown that gut dysbiosis may favour the blooms of potentially harmful bacteria normally present at low levels in the gut (for example Proteobacteria), which can impose on commensals and contribute to disease (Figure 1.34).[238] In particular, bacterial that belongs to *Enterobacteriaceae* (Proteobacteria) are particularly prone to bloom during gut dysbiosis in various contexts involving gut inflammation; patients affected by IBD (both CD and UC), celiac disease, colorectal cancer or treated with antibiotic showed a drastic increase of these Gram-negative bacteria in the colon.[252-256] A possible explanation is that dysbiosis-imposed environmental and nutritional changes may provide a favourable environment for *Enterobacteriaceae* expansion, giving these bacteria an advantage over other microbial components.[238] An emblematic example is represented by the high prevalence of adherent-invasive *E. coli* (AIEC) in patients with CD and UC.[257-261] Although *E. coli* overgrowth appears to be a consequence rather than a cause of IBD inflammation, it is proven that these bacteria play a crucial role in exacerbating the disease and in enhancing the host susceptibility to other pathogens.[262] For example, multiple studies on experimental mice models showed that *E. coli* pathobionts that increased in number upon dextran sulfate sodium (DSS)-treatment caused bacteremia and ultimately mouse mortality.[256, 263]

The capacity of *Enterobacteriaceae* to promote and exacerbate intestinal injury is due to specific conserved microbial components (pathogen associated molecular pattern, PAMPs), such as lipopolysaccharides (LPS), which are potent immunostimulant able to produce strong inflammatory responses.[238, 264, 265]

Species	Source of inflammation	Impact of Blooms
Mucosa-associated symbiotic *E. coli* strains	Patients with inflammatory bowel disease	Monocolonization increased incidence of invasive carcinoma in the IL-10-deficient colorectal cancer mouse model
Symbiotic *E. coli*	*C. jejuni*-colonized infant mice	Reduced colonization resistance against *C. jejuni*
Enterobacterial species	Mouse oral *C. rodentium* infection	Unknown
Proteobacterial species	Toll-like receptor 5-deficient mice	Transient instability of the gut microbiota
Adherent and invasive *E. coli* (AIEC)	Ileitis in patients with Crohn's disease	Unknown
E. coli isolates with heightened virulence	Isolated from patients with Crohn's disease or ulcerative colitis	*In vitro*, enhanced capability to activate NLRP3 inflammation and resistance to macrophage killing.
Blooms of *Klebsiella pneumoniae* and *Proteus mirabilis*	*T-bet*$^{-/-}$ × *Rag2*$^{-/-}$ ulcerative colitis (TRUC) mouse model	Oral infection with these strains elicited colitis in *Rag2*$^{-/-}$ and WT adult mice
Salmonella and *E. coli*	Mouse oral *Salmonella* model	High densities of *Salmonella* and *E. coli* lead to horizontal gene transfer of the colicin-plasmid p2 from *Salmonella* to *E. coli*
E. coli pathobiont	Ampicillin and neomycin-treated mice	Multidrug-resistant *E. coli* capable of inducing lethal NAIP5–NLRC4 inflammasome in systemic infection
Enterobacterial species	Clindamycin-treated mice	Enhanced susceptibility to *Clostridium difficile*-induced colitis

Abbrevations: C. *jejuni*, *Campylobacter jejuni*; *E. coli*, *Escherichia coli*; IL-10, interleukin-10; WT, wild type.

Figure 1.34. Enterobacterial blooms in human disease and mouse disease models.[238]

1.5.2.3 Role of host immunity

The constant exposure of the gastrointestinal mucosa to food and microbial-derived antigens induced the host to develop specific strategies to prevent tissue injury, without triggering potentially harmful immune responses. The synergistic action of an effective epithelial barrier guarded by an organized resident immune system allows the host to remove pathogens and to be tolerant towards commensal flora and food supplements.[266] However, dysfunction in one or both of these components may lead to uncontrolled immune and inflammatory responses, as is the case of IBDs.[266] The gut epithelium provides a protective barrier towards commensals and

pathogens and secretes antimicrobial peptides-containing mucus in order to hinder microbial translocation in the underlying layers.[267] When pathogens succeed in overcoming these anatomical impediments through tissue damage or infection, immune cells come into play in order to neutralize the breach.[245] Innate immunity represents the first-line defense against pathogens and it is mediated mainly by resident macrophages, dendritic cells (DCs) and natural killer cells, but also by epithelial cells and myofibroblasts. This defense mechanism is fast (within minutes) and specifically directed towards conserved microbial structures, such as foreign carbohydrates, lipids, lipopeptides and exogenous nucleic acids, commonly named PAMPs, generally present in infectious agents.[245] Host cells detect PAMPs through specific receptors called PRRs, such as transmembrane TLRs and cytosolic NOD-like receptors, which trigger complex signalling pathways in order to remove the threat.[268, 269] Among the variety of innate immune cells in the gut mucosa, resident macrophages play a crucial role in performing immunological surveillance to protect the host tissues. These cells normally exhibit avid phagocytic activity towards debris and stronger bactericidal action than peripheral blood monocytes.[270] However, despite what happens in blood monocyte, whose phagocytic activity triggers a strong pro-inflammatory cytokine release, resident macrophages perform host surveillance without inducing any inflammatory response. In contrast to macrophages present in other tissue, mucosal macrophages show a certain inflammation anergy to avoid potentially life-threatening immune responses in a district closed to myriad of bacteria.[124, 245, 270, 271] Resident macrophages peculiar ability is probably the result of millions years-long co-evolution between primates and colonizing microorganisms. This cohabitation allowed humans to become

more tolerant towards commensals favouring the establishment of the beneficial mutualistic symbiosis present in the gastrointestinal tract today.[245] The mechanisms underlying the inflammation anergy of resident macrophages seem to be related to the fact that these cells are potently down-regulated for several innate immunity molecules, including PRRs.[124, 270, 272, 273] Indeed, intestinal macrophages are incapable to produce TLRs-triggered pro-inflammatory cytokines (such as TNFα, IL-1β, IL-6, IL-8, etc.) upon stimulus, due both to the strong reduction of key signal proteins involved in TLRs signalling pathways (e.g. CD14, MD-2, MyD88, TRIF and TRAF6)[270, 273-275] and to the constitutive expression of negative signalling regulators (e.g. IκBα, IRAK-M, IL-10 and TGF-β).[245, 273, 274, 276, 277] Therefore, when circulating monocytes enter the healthy intestinal mucosa to replace senescent and apoptotic macrophages, the gut environment probably induce them to change their gene expression profile in order to become tolerant to the microbiota.[245, 275, 278] However IBD-associated gut dysbiosis may considerably change this delicate balance, triggering an excessive host immune response. In many CD and UC patients, for instance, there is a continuous influx of blood monocytes in the gut that results in an abundant accumulation of pro-inflammatory macrophages in the inflamed mucosa.[126, 245, 279] This macrophage population can be distinguished from the resident cells for the high expression of the LPS co-receptor CD14 ($CD14^{hi}$).[124, 280, 281] Despite mucosal macrophages, these cells show a typical inflammatory phenotype, characterized by the ability to produce large amounts of pro-inflammatory mediators such as TNFα, IL-1β, IL-6, IL-8, reactive oxygen intermediaries and nitric oxide.[275] Because of their inflammation-prone behaviour, these cells are among the main mediators responsible for the persistent inflammatory condition in IBDs. Considering that

the phenotype of $CD14^{hi}$ cells is quite distinct from the one of resident macrophages, many studies focused on the targeting of the monocyte-macrophage lineage in order to develop therapeutic IBD treatments.[275]

1.5.3 TLRs IN IBD

In paragraphs 1.5.2.2 and 1.5.2.3 the role of gut microbiota and innate immunity in IBD onset has been described respectively. In particular, we explained that resident macrophages tolerance towards gut microbiota is related to the low expression of several innate immunity molecules, including PRRs; and that this down-regulation is lost in IBD inflamed intestine. Among PRRs, an important role for TLRs signalling in the pathogenesis of IBD has been established through many studies over the last decade.[94] TLRs are expressed in different combinations by a broad range of cell types throughout the whole gastrointestinal tract.[124, 271, 282, 283] In particular they are expressed on intestinal epithelial cells (IEC) like absorptive enterocytes, Paneth cells, goblet cells, and enteroendocrine cells,[92, 123, 284-287] on myofibroblasts,[288] and on several immune cells within the intestinal lamina propria such as monocytes and macrophages,[124, 289] dendritic cells,[290, 291] and CD4+ T lymphocytes.[292, 293] Furthermore different cell types show specific expression patterns of TLRs depending on their role and their location in the intestine. For instance, lamina propria CD11c+ dendritic cells do not express TLR4 in order to be tolerant to omnipresent LPS in the gut lumen.[291] In the healthy intestine the expression of several TLRs, in particular of TLR2 and TLR4, is generally maintained at low levels to allow mucosal cells to be hyporesponsive towards commensals.[92, 122-125] Indeed as already described earlier, many are the strategies adopted by cells to dampen TLRs expression and activation. When circulating monocytes

leave the peripheral blood to become gut resident macrophages, they change their gene expression profile. For instance, they reduce the expression of surface CD14 co-receptor in order to be less sensitive to LPS presence.[126] Furthermore, different molecular mechanisms, such as receptors compartmentalization and negative regulation, were described to attenuate or abrogate TLRs activation in gut mucosa.[94, 284] However, IBD-associated gut dysbiosis and inflammation may alter TLRs expression and signalling.[94, 248, 249] First of all, the TLRs inhibitory mechanisms may be switched off, triggering downstream signal activation and consequently initiating immune responses against commensals.[244] Persistent TLRs hyper-activation may be the cause or contribute to inflammation in IBD. In IBD-susceptible hosts, for example, aberrant TLRs signalling may contribute to destructive host responses and chronic inflammation, leading to many different clinical phenotypes (Figure 1.35).[294] Other studies showed that the expression of TLR4 and its co-receptors CD14 an MD-2 is significantly increased in IEC and in lamina propria mononuclear cells (LPMCs) collected from the lower gastrointestinal tract of patients with CD and UC,[92, 280, 295] thus maximizing sensitivity to microbial antigens.

TABLE 1. Toll-like Receptors (TLRs)

TLR structure and signaling

- 13 mammalian, type I transmembrane glycoreceptors (10 in humans; 12 in mice) with divergent LRR-ectodomain and conserved intracellular TIR domain
- recognition of alarm patterns or signals:
  a) microbiota/viral-associated (commensal/pathogen)
  b) damage-associated (endogenous/exogenous)
- downstream activation of pro-/antiinflammatory cytokines and chemokines and link to adaptive immunity through at least 5 different adaptor proteins

Regulatory dichotomy in TLR expression and function between health and IBD

- constitutively or inducibly expressed throughout the whole GI tract by a wide variety of cell types, including IEC lineages, myofibroblasts, monocytes/macrophages, DCs and T cells
- healthy intestine:
  a) TLRs are present only in small amounts
  b) negative regulators maintain basal state of activation and prevent prolonged and excessive TLR signaling
- diseased intestine:
  a) distinct TLRs are significantly upregulated in certain cell subsets in intestinal mucosa
  b) positive regulators initiate aberrant state of activation and allow uncontrolled TLR signaling

TABLE 2. (Patho)physiology of TLRs in the Intestinal Mucosa

Physiological effects of normal TLR signaling in the healthy GI tract

- Integrity of commensal composition and commensal tolerance
- Protection of intestinal epithelial/mucosal barrier function, accelerated wound healing
- Control of Treg↔Teff – balance in the intestinal mucosa

→ *Maintenance of commensal and mucosal homeostasis*

Pathophysiological effects of aberrant TLR signaling in IBD

- Changes in commensal composition and commensal intolerance
- Impairment of intestinal epithelial/mucosal barrier function, delayed wound healing
- Promotion of Treg↔Teff – imbalance in the intestinal mucosa

→ *Disturbance of commensal and mucosal homeostasis*

Figure 1.35. Toll-like receptors (TLRs) expression, function and (patho)physiology in the intestinal mucosa.[94]

Furthermore some researcher showed that TLR4 and MD-2 up-regulation could also be due to other "non-canonical stimuli" from other ligands. For example, it has been described that T-cell-derived IFNɣ and TNF-α, which play critic pathophysiological roles in IBD onset, are able to up-regulate TLR4 expression on intestinal epithelial cells.[125, 296] Although aberrant TLR4 hyper-responsiveness towards commensals may be the result of microbiota composition variations in genetically susceptible hosts, receptor up-regulation may also reflect functional loss of immune responses.[94]

1.5.4 THERAPEUTIC TREATMENTS

Although IBD etiology is still unknown, modern therapeutic approaches allow to the majority of IBD patients to lead a good quality life. Current medical therapies have the goal to induce and maintain remission and to prevent post-surgical recurrences. The type of pharmacological treatment is dependent on the stage of the disease, the condition of the patient and the body ability to respond to a specific drug.[297] Patients with mild-moderate IBD are firstly treated with aminosalicilates, while corticosteroids and antibiotics are preferably prescribed in case of moderate-severe disease.[298, 299] Despite the cost of these treatments is generally low, these drug can cause multiple side effects and in many case do not lead to clinical remission.[300] Therefore many new therapies are currently in experimentation with the aim to improve more and more patients' life quality.[297] For example, the biological therapy is specifically designed to target pro-inflammatory mediators particularly upregulated in the gut lamina propria of IBD patients. Among these treatments, infliximab (IFX) and adalimumab (ADA) are therapies based on the use of monoclonal antibodies that specifically target and neutralize TNF-α, a cytokine that plays a key role in maintain a persistent and prolonged state of inflammation in the gut mucosa.[301] Besides cytokines-targeting therapies there are also novel treatments based on agents able to selectively block lymphocyte-endothelial interactions. Monoclonal antibodies like natalizumab (for CD) and vedolizumab (for UC and CD), for example, prevent leukocytes migration and recruitment to inflamed districts.[302] Although monoclonal antibody-based therapy represents a valid alternative to classical approaches with a specific and directed mechanism of action, the high cost of antibodies prevents this therapy to be widely used. In addition long-term biological

molecules-based treatments may cause side effects (for instance renal complications) and trigger immunogenicity by causing the production of anti-drug antibodies.[303, 304] Figure 1.36 presents and compares the main IBD therapies, their mechanisms of action and their adverse effects.

Treatment type	Related drugs	Mechanism of action	Features	Potentials adverse effects
Aminosalicylates	Mesalamine Olsalazine Balsalazide Sulfasalazine	Inhibition of IL-1, TNF-α, and platelet activating factor (PAF), decreased antibody secretion.	Locally immunosuppressive, nonspecific inhibition of cytokines; medium cost.	Headache, dizziness, dyspepsia, epigastric pain, abdominal pain, nausea, vomiting, and diarrhea.
Immunomodulators	Azathioprin 6-mercaptopurin Methotrexate	Blockage of *de novo* pathway of purine synthesis.	Antiproliferative effects, reduction of inflammation.	Black, tarry stools, bleeding gums, chest pain, fever, chills, swollen glands, pain, cough, and weakness.
Corticosteroids	Prednisone Methylpred-nisolone Hydrocortisone Budesonide	Blockage of phospholipase A2 in the arachidonic acid cascade altering the balance between prostaglandins and leukotrienes; stimulation of apoptosis of lamina propria lymphocytes; suppression of the transcription of cytokines.	High immunosuppression, risk of potential infections, adverse effects with long periods of use, low cost.	Full moon face, difficulty of healing, acne, sleep and mood disturbances, glucose intolerance, osteoporosis, osteonecrosis, subcapsular cataracts, myopathy, infections, acute adrenal insufficiency, myalgia, malaise, arthralgia or intracranial hypertension, and pseudorreumatism syndrome.
Biologicals: anti-cytokine drugs	Infliximab Adalimumab Certolizumab-pegol Golimumab Ustekinumab (phase 3 trial)	Induction of apoptosis in proinflammatory cells; binding specifically to TNF-α, blockage of the interaction the receptor.	Specific inhibition of cytokine, immunosuppression, high cost, advanced technology required.	Abdominal or stomach pain, chest pain, chills, cough, dizziness, fainting, headache, itching, muscle pain, nasal congestion, nausea, sneezing, weakness, vomiting, bloody urine, cracks in the skin, diarrhea, pain, fever, abscess, back or side pain, bone or joint pain, constipation, falls, facial edema, general feeling of illness, hernia, irregular heartbeat, unusual bleeding, weight loss, increased risk of reactivation of latent tuberculosis, and increased risk for developing infections and lymphoma.
Biologicals: anti-cell adhesion molecule	Vedolizumab Natalizumab	Inhibition of migration.	Specific inhibition of cell adhesion molecules high cost, advanced technology required.	Nasopharyngitis, headache and abdominal pain, increased risk of infections, serious infections, and progressive multifocal leukoencephalopathy (natalizumab).

Figure 1.36. IBD treatments: drugs in use, mechanisms of action, and side effects.[297]

2. LIST OF PUBLICATIONS and AUTHOR'S CONTRIBUTION

The thesis is based on the following papers, which are referred to as **Chapter I-III** in the text.

- **Chapter I**

  Sestito, S. E.; Facchini, F. A.; Morbioli, I.; Billod, J.-M.; Martin-Santamaria, S.; Casnati, A.; Sansone, F.; Peri, F. **Amphiphilic Guanidinocalixarenes Inhibit Lipopolysaccharide (LPS)- and Lectin-Stimulated Toll-like Receptor 4 (TLR4) Signaling.** *Journal of Medicinal Chemistry* **2017**, 60, 4882-4892. DOI: 10.1021/acs.jmedchem.7b00095.

  I contributed to the designed of the study and I performed the experimental work, with the exception of molecular docking analysis and synthetic process. I analyzed the biological data and I wrote the biological section of the manuscript.

- **Chapter II**

  Facchini, F. A.; Coelho, H.; Sestito, S. E.; Delgado, S.; Minotti, A.; Andreu, D.; Jiménez-Barbero, J.; Peri, F. **Co-administration of Antimicrobial Peptides (AMPs) EnhancesToll-like Receptor 4 (TLR4) Antagonist Activity of Synthetic Glycolipid FP7**. *Manuscript ahead of print by the journal ChemMedChem. DOI: 10.1002/cmdc.201700694.*

  I designed the study and performed the experimental work, except for NMR and TEM analysis. I analyzed the biological data and I wrote most of the manuscript.

- **Chapter III**

  Facchini, F. A.; DI Fusco, D.; Barresi, S.; Zaffaroni, L.; Minotti, A.; Jerala, R.; Granucci, F.; Monteleone, G.; Peri, F.; Monteleone, I. **TLR4 synthetic antagonist FP7 inhibits LPS-induce production of pro-inflammatory cytokines in mononuclear cells of IBD patients and shows anti-Inflammatory effects in dextran-sulfate-sodium (DSS)-induced colitis model.** *Manuscript in preparation.*

  I designed the entire study and I performed all the experimental work presented. I analyzed the data and I am currently writing the manuscript.

Paper published but not included in the thesis.

Grasselli, C.; Ferrari, D.; Zalfa, C.; Sonicini, M.; Mazzoccoli, G.; Facchini, F. A.; Marongiu, L.; Granucci, F.; Copetti, M.; Vescovi, A. L.; Peri, F.; De Filippis, L. **Toll-like receptor 4 modulation influences human neural stem cell proliferation and differentiation.** *Manuscript accepted for publication on Cell Death and Disease*.

3. PURPOSE OF THE WORK

The main aim of this thesis is the study of the therapeutic potential of small organic molecules active in inhibiting the TLR4 signal. These molecules are studied with the double aim to develop new drug hits and to study TLR4 signalling in different biological contexts.

In CHAPTER I, new positively and negatively charged, calixarene-derived amphiphiles were designed as potential TLR4 modulators, were synthesized and their activity on TLR4 was assessed in cells. Starting from the assumption that opportunely designed amphiphilic molecules can behave as CD14 and TLR4/MD-2 ligands and therefore modulate the TLR4 signaling, we first designed and synthesized a small library of cationic and anionic calixarenes. Calixarenes are synthetic molecules with a three-dimensional shape that resemble a calix, with a hydrophobic pocket and two chemically functionalizable rims. The activity of functionalized calixarenes was first screened on HEK-Blue hTLR4 cells, a cell system precisely designed to study the activation of TLR4 pathway. The first screening led to the identification of active compounds, whose mechanism of action was then investigated. The activity of the two most potent calixarene-based amphiphiles was finally evaluated on human and murine white blood cells, leading to the identification of a promising candidate for *in vivo* test.

In CHAPTER II, we aimed at developing an innovative strategy to inhibit TLR4 signaling based on the co-administration of TLR4 targeting molecules with LPS-neutralizing peptides. For this purpose we used synthetic FP7 antagonist in combination with synthetic cationic AMPs, previously described as LPS-neutralizing agents. Co-administration of FP7 with a cecropin A-melittin hybrid peptide and human cathelicidin LL-37 has been studied in the same cell system described above. In order to investigate the contribution of the LPS-

neutralizing effect of AMPs we stimulate cells with a TLR4 agonist structurally different from LPS. The results obtained in this section suggest a dual mechanism of action for AMPs, not exclusively based on LPS binding and neutralization, but also on a direct effect on LPS-binding proteins of the TLR4 receptor complex (namely MD-2 and CD14).

Based on the information on the TLR4 activity of FP7 and on the knowledge of its mechanism of action, in CHAPTER III a preclinical study in which FP7 is used in an experimental model of inflammatory bowel disease (IBD) is described. Although the aetiology of IBDs is still not fully understood, growing evidences suggest that IBD-associated tissue damage results from an excessive immune response directed against a perturbed microbiota. Indeed, immune cells that inhabit the gut mucosa of IBD patients are generally less tolerant towards bacterial antigens. Considering the key role played by TLRs in bacterial PAMPs recognition and the importance of TLRs dysfunction in IBD pathogenesis, modulation of TLRs activity may represents a promising approach to reduce inflammation. CHAPTER III has the aim to evaluate a possible therapeutic strategy based on the use of small molecule that selectively targets TLR4/MD-2 complex to reduce IBD inflammation. For this purpose the synthetic lipid X mimetic FP7 was used. The anti-inflammatory property of FP7 was first evaluated in PBMCs. The mechanism of action of FP7 was investigated *in vitro* using an optimized cell-free binding assay and in cells monitoring the activation of TLR4 signalling at different levels. FP7 capacity to reduce gut mucosal inflammation was then evaluated on lamina propria mononuclear cells (LPMCs) and *in vivo* on a mouse model of colitis.

CHAPTER I

Amphiphilic Guanidinocalixarenes Inhibit Lipopolysaccharide (LPS)- and Lectin-Stimulated Toll-like Receptor 4 (TLR4) Signaling

Stefania E. Sestito,† Fabio A. Facchini,† Ilaria Morbioli,‡ Jean-Marc Billod,§ Sonsoles Martin-Santamaria,§ Alessandro Casnati,‡ Francesco Sansone,*,‡ and Francesco Peri*,†

†Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza, 2, 20126 Milano, Italy

‡Dipartimento di Scienze Chimiche, della Vita e della Sostenibilità Ambientale, Università di Parma, Parco Area delle Scienze 17/a, 43124 Parma, Italy

§Department of Chemical and Physical Biology, Centro de Investigaciones Biologicas, CIB-CSIC, C/Ramiro de Maeztu, 9, 28040 Madrid, Spain

Abstract

We recently reported on the activity of cationic amphiphiles in inhibiting TLR4 activation and subsequent production of inflammatory cytokines in cells and in animal models. Starting from the assumption that opportunely designed cationic amphiphiles can behave as CD14/MD-2 ligands and therefore modulate the TLR4 signaling, we present here a panel of amphiphilic guanidinocalixarenes whose structure was optimized to dock into MD-2 and CD14 binding sites. Some of these calixarenes were active in inhibiting, in a dose-dependent way, the LPS-stimulated TLR4 activation and TLR4-dependent cytokine production in human and mouse cells. Moreover, guanidinocalixarenes also inhibited TLR4 signaling when TLR4 was activated by a non-LPS stimulus, the plant lectin PHA. While the activity of guanidinocalixarenes in inhibiting LPS toxic action has previously been related to their capacity to bind LPS, we suggest a direct antagonist effect of calixarenes on TLR4.MD-2 dimerization, pointing at the calixarene moiety as a potential scaffold for the development of new TLR4-directed therapeutics.

Adapted from: *Journal of Medicinal Chemistry* **2017** 60 (12), 4882-4892[305]

1. INTRODUCTION

We recently observed that glycoamphiphiles with a sugar core (trehalose or glucose) functionalized with lipid chains and positively charged ammonium groups are able to inhibit LPS-stimulated TLR4 signal in vitro with IC_{50} values ranging from about 5 to 0.2 μM and to reduce TLR4-dependent production of inflammatory cytokines in vivo.[206] The main structural feature of these molecules is their "facial" arrangement with positive charges and lipophilic chains disposed in spatially well-defined regions. Therefore, we hypothesized that calixarene-based facial amphiphiles could also be suitable as scaffolds to obtain TLR4 ligands with antagonist activity. Recently, amphiphile calixarenes actually showed remarkable properties also in a biological context significantly related to this feature.[306] The calixarene scaffold represents a very versatile structure to build amphiphilic compounds due to the possibility to variably and selectively functionalize both its upper (aromatic *para* positions) and lower (phenolic oxygens) rims. Moreover, the possibility to link to the macrocyclic platform several active moieties and binding units, resulting in pre-organized arrays, gives rise to systems that, exploiting a multivalent effect, frequently show improved biological activity with respect to corresponding monovalent models.[306, 307] From this point of view, also the tight compaction of hydrophobic chains located at one of the rims can result in the enhancement of some properties such as (self)assembling capabilities in an aqueous environment.[306-309] We present here a study on the inhibition of TLR4·MD-2 signaling by a series of positively and negatively charged calixarene-based amphiphiles (compounds **1**-**6** and **7-9** in Figure 1, respectively) and the investigation of their mechanism of action. In the series we included calixarene **1** and **2** as reference compounds whose activity in this biological context has

been previously reported[310] and associated to its capacity to bind and neutralize LPS as topomimetic of LPS-binding peptides. However, the binding mode of the direct interaction between LPS and calixarenes was not investigated, leading us to question this proposed mechanism.[310] Since we hypothesized that calixarene derivatives could directly bind to human and murine MD-2 and CD14 in a similar fashion than LPS, we preliminarily performed docking calculations to support this mode of interaction. Moreover, we aimed here to verify if the TLR4 antagonist activity is a rather general property of positively charged amphiphilic calixarenes, and if the antagonist effect also derives from the direct interaction of calixarenes with the receptors, and not exclusively from LPS neutralizing action, as suggested for calixarenes **1** and **2**.

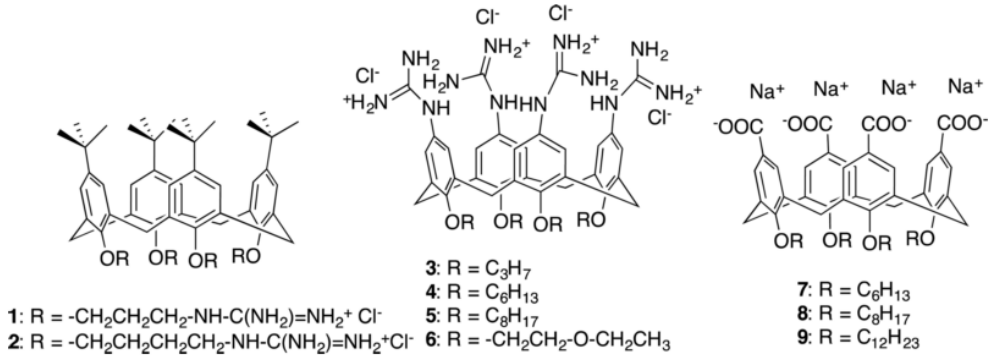


Figure 1. Positively charged guanidinocalixarenes 1−6 and negatively charged carboxy calixarenes 7−9.

2. RESULTS

2.1. Rational design of amphiphilic calixarenes as CD14/MD-2 ligands

We were inspired by the hypothesis that the calixarenes could be TLR4 modulators similar to lipid A variants and to trehalose or glucose-based

glycoamphiphiles developed and described by our research team in a previous study.[206] Positively charged guanidinocalixarenes **1-6** and negatively charged carboxylate calixarenes **7–9** were designed in order to investigate the suitability of this macrocyclic scaffold to build CD14 and TLR4/MD-2 ligands (Figure 1). These calixarene derivatives have an amphiphilic character due to the presence of lipophilic tails on one rim and charged polar groups on the other. Only compound **6**, having ethoxyethyl chains at the lower rim, has a reduced amphiphilicity and was included in the library precisely to verify the possible relevance of this property in the biological activity. More precisely: calixarenes **1** and **2** present lipophilic upper rims bearing four *tert*-butyl groups and polar lower rims with positively charged guanidinium groups linked through, respectively, propyl and butyl chains. These two compounds were described in literature to possess LPS-neutralizing activity[310] and were included in the study as references. Calixarenes **3–6** are completely new and present a reversed arrangement of lipophilic and charged groups: guanidinium groups are directly linked to the scaffold on the upper rim, and hydrocarbon chains of different length (C_3, C_6, and C_8 for compounds **3**, **4**, and **5**, respectively), or an ethoxy ethyl chain in the case of compound **6**, are linked at the lower rim. Finally, anionic calixarenes **7–9** were designed with the purpose of studying the influence of negatively charged groups. Thus, these anionic calixarenes present carboxylate groups at the upper rim, aiming to mimic the phosphate groups of LPS, and hydrocarbon chains of variable length (C_6, C_8, and C_{12}) at the lower rim (Figure 1 and Table 1).

Table 1. Calixarene-based amphiphilic molecules.

Entry	Charge	Polar groups	Hydrocarbon chain length	Ref.
Compound **1**	cationic	guanidinium	tert-butyl groups	[310]
Compound **2**	cationic	guanidinium	tert-butyl groups	[310]
Compound **3**	cationic	guanidinium	Linear C3 hydrocarbon chains	New
Compound **4**	cationic	guanidinium	Linear C6 hydrocarbon chains	New
Compound **5**	cationic	guanidinium	Linear C8 hydrocarbon chains	New
Compound **6**	cationic	guanidinium	ethoxy ethyl chains	New
Compound **7**	anionic	carboxylate	Linear C6 hydrocarbon chains	New
Compound **8**	anionic	carboxylate	Linear C8 hydrocarbon chains	New
Compound **9**	anionic	carboxylate	Linear C12 hydrocarbon chains	New

Three-dimensional (3D) structures of compounds **1–9** were built and optimized by means of computational techniques. We superimposed the 3D structures of compound **2** and **3** with that of lipid IVa, a natural underacylated MD-2 ligand with activity as (h)TLR4 antagonist. When comparing lipid IVa (3D structure from the X-ray crystallography structure) with compound **3** (Figure 2-right), the oppositely charged groups (phosphate *vs* guanidinium) aligned perfectly, and also did the disaccharide over the aromatic calix backbones, and the acyl over the alkoxy chains. This preliminary result prompted us to further study calixarenes **1**-**9** as putative TLR4·MD-2 and CD14 ligands.

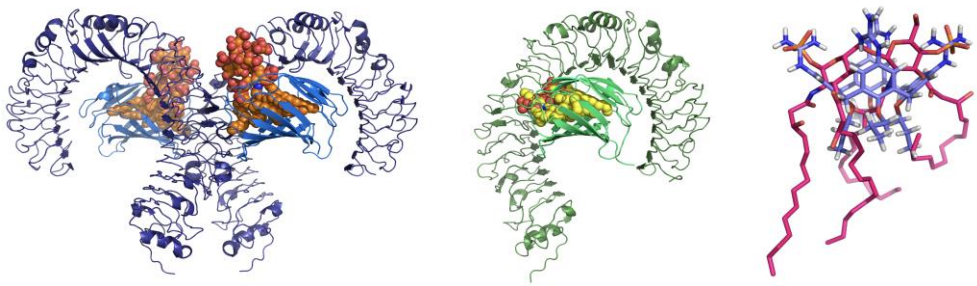


Figure 2. Left: 3D structure of human TLR4.MD-2/LPS dimer from PDB ID 3FXI. Middle: 3D structure of TLR4.MD-2/Lipid-IVa from PDB ID 2E56. Right: Superimposition of lipid IVa (from PDB ID 2E56, magenta) and calixarene **3** (purple).

Firstly, compounds **2**, **3** and **4**, as representative derivatives, were docked into the binding site of the human CD14 protein (PDB ID 4GLP). For all these three compounds, docking calculations predicted favorable binding poses inside the human CD14 protein (Figure 3), where the guanidinium moieties are placed at the rim of CD14 and the hydrophobic chains are inserted into the hydrophobic pocket.

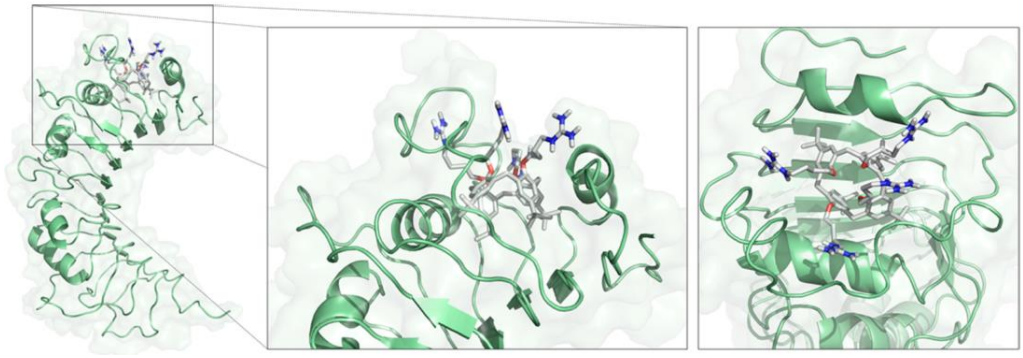


Figure 3. Docked pose for compound **3** inside CD14 (PDB ID 4GLP). Left: full perspective. Middle: side view. Right: top view.

Docking calculations were also performed with compounds **1–9** into four different structures of the TLR4/MD-2 system: human and mouse, in agonist and antagonist conformations of MD-2 (Figure 4 and Figures S1, S2, and S3). Overall, all the ligands were predicted to bind inside the different TLR4/MD-2 structures, with the guanidinium/carboxylate moieties placed at the rim of MD-2, where polar interactions predominate, and the lipophilic groups (alkoxy or tert-butyl chains) inside the MD-2 pocket. These docked poses are in agreement with calculations reported by us of compounds binding both CD14 and MD-2 proteins. Although MD-2 is more specific in the ligand recognition, both MD-2 and CD14 binding pockets share some similarities regarding volume and accessible surface area.[207] Docking calculations were also performed on the 3D structures of mouse TLR4.MD-2 (PDB IDs 3VQ2 and 2Z64, Figure S2

Supp. Info.) leading to similar conclusions: the calixarene derivatives are able to bind inside the MD-2 protein with the guanidium/carboxyl moieties establishing electrostatic interactions with the polar groups at the rim of MD-2, and the lipophilic groups (alkoxy or *t*-butyl chains) inserted into the MD-2 pocket. Regarding reported compound **2**, in the docked poses in both agonist and antagonist conformations of human MD-2, the guanidinium groups establish H-bonds with the side chains of Glu92, Tyr102, and Ser118, and the backbone of Lys122 (Figures 4A, S1 and S2 at Supp. Info.), while one of the aromatic rings of the macrocycle is engaged in a π-p-stacking interaction with Phe119. In details, the guanidinium groups at the upper rim of compounds **3**-**5** establish H-bonds with the backbone of Ser120, and the side chains of Glu92 and Tyr102 (Figure 4B). The longer alkyl chains of compounds **4** and **5** occupy deeper regions of the MD-2 pocket. Interestingly, when comparing the best predicted docked poses for compounds **2** and **3**, it was observed that they are half turn rotated one from another in regards to the calixarene moiety (Figures 4A and S2, Supp. Info.). In both cases, the guanidinium moieties are accommodated at the entrance of the pocket while the hydrophobic groups (*tert*-butyl and propyl for compound **2** and **3**, respectively) are buried inside the MD-2 hydrophobic pocket.

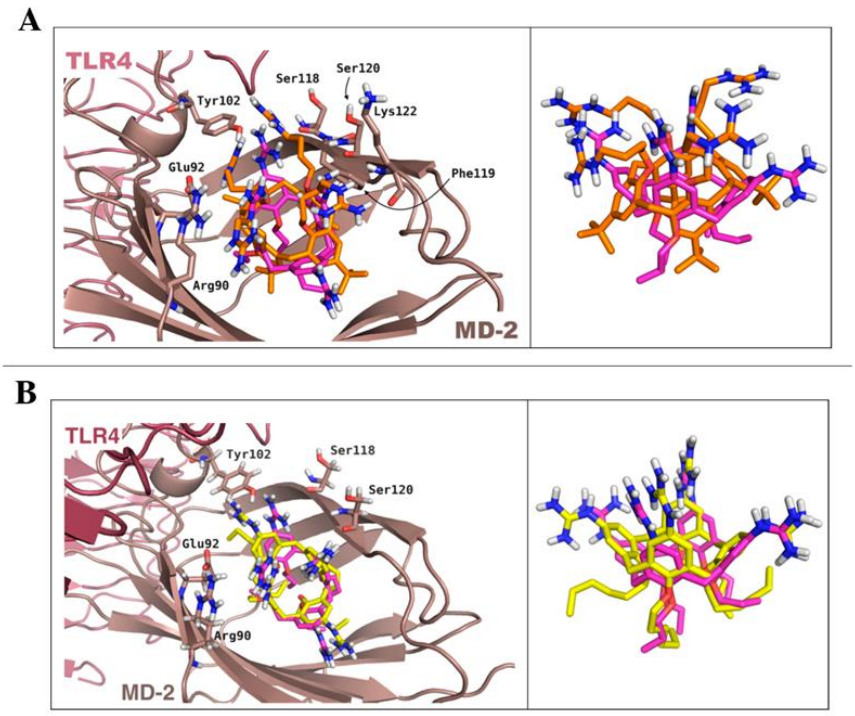


Figure 4. A) Superimposition of the best docked poses for compound **2** (orange) and **3** (magenta) in TLR4.MD-2 (PDB ID 2Z65). A 90º rotated view is shown on the right (TLR4.MD-2 has been hidden for the sake of clarity). **B)** Superimposition of the best docked poses for compounds **3** (magenta) and **4** (yellow) in (h)TLR4.MD-2 heterodimer (PDB ID 2Z65). A 90º rotated view is shown on the right (TLR4.MD-2 has been hidden for the sake of clarity).

Regarding compounds **7**-**9**, they presented similar docked poses where the alkyl chains were also buried inside the hydrophobic MD-2 pocket and the carboxylate moieties were establishing polar interactions with the resides at the MD-2 rim. Compounds **8** and **9** presented docked poses protruding slightly more than compound **7**, probably due to the longer alkyl chains, although the difference was very subtle (Figure S3, Supp. Info.). To ensure the stability of the docked poses of compound **3** with TLR4.MD-2 and to gain insights on the interactions that take place, we performed 90 ns molecular dynamic

simulations of the (h)TLR4.MD-2/**3** complex starting from the docked geometries for both the antagonist and the agonist conformations of (h)TLR4.MD-2. In the simulation starting from the agonist conformation of MD-2 we could observe that compound **3** rotates of almost 90 degrees around its plan of symmetry (a partial rotation happens at 5 ns of simulation and the full rotation at approximately 38 ns) to find a more stable bound conformation that was maintained stable for the rest of the simulation (Figure S4, Supp. Info.). This rotation forced the MD-2 pocket to adopt an antagonist-like conformation (characterized by, inter alia, great motion of residue Phe126). In this new binding mode, two guanidinium groups of compound **3** continued to interact through hydrogen bonds with the side chains of Glu92, and Ser120, a third guanidinium group formed a new hydrogen bond with the CO group of Pro88, and the fourth guanidinium group was involved in polar interactions with the solvent. Moreover, later in the simulation (starting at 42 ns), the loop made by residues 80 to 90, undergoes a considerable deformation (Figure S5, Supp. Info.). In contrast, in the simulation of the TLR4.MD-2/**3** complex starting from the antagonist conformation, the geometries of both compound **3** and MD-2 were stable during the 90 ns run (Figure S5, Supp. Info.), not experiencing important conformational changes. These results clearly indicated that in complex with calixarene **3** MD-2 in agonist conformation is less stable than in the antagonist one, therefore providing explanations for the antagonist activity later observed (see below).

2.2 Inhibition of LPS-Stimulated TLR4 Signal in HEK-Blue hTLR4 Cells

Compounds **1–9** were first screened for their capacity to interfere with LPS-stimulated TLR4 activation and signalling on HEK-Blue hTLR4 cells. Compounds **1–5** were able to inhibit in a dose-dependent way the LPS-triggered TLR4

signal, while compound **6** with oxygenated ethylene glycol chains instead of hydrocarbon chains showed weak antagonistic activity. In contrast, negatively charged amphiphilic calixarenes **7–9** showed no or very weak inhibition of LPS-TLR4 signal (Figure 5).

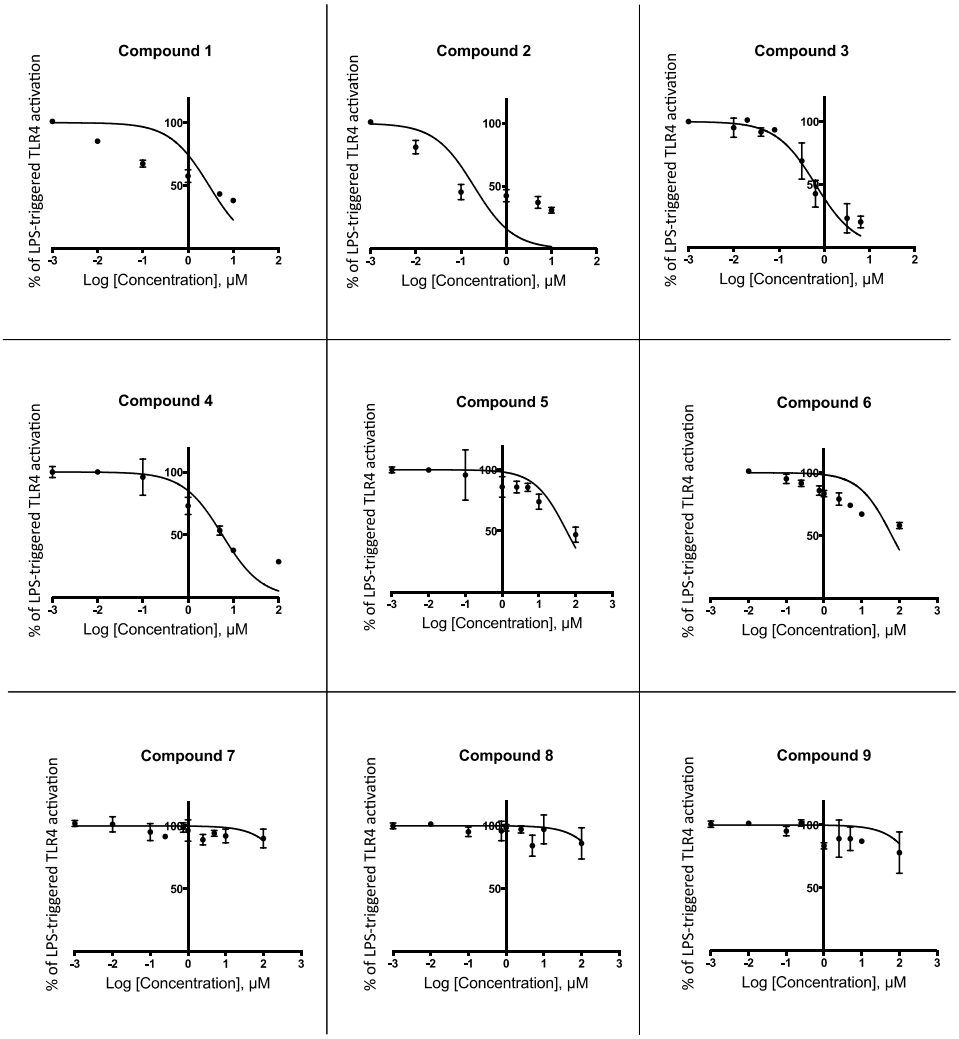


Figure 5. Dose dependent inhibition of LPS-stimulated HEK-Blue cells activation by calixarenes **1–6**. Human TLR4 HEK-Blue was treated with increasing concentrations of compounds and stimulated with LPS (100 ng/mL). The results represent normalized data with positive control (LPS alone) and expressed as the mean of percentage ± SD of at least three independent experiments.

Guanidinocalixarenes **1–6** inhibited TLR4 signal with potencies ranging from 0.2 to 63 µM. Compounds **2**, **3**, and **4** were the most potent antagonists and inhibited LPS-triggered TLR4 signal with IC_{50} of 0.2, 0.7, and 5.7 µM, respectively (Table 2).

Table 2. IC_{50} Values for the Inhibition of LPS-triggered TLR4 Signal in HEK-Blue hTLR4 Cells

Treatment	IC_{50} LPS (µM)
Compound **1**	10
Compound **2**	0.2
Compound **3**	0.7
Compound **4**	5.7
Compound **5**	63
Compound **6**	46
Compound **7**	/
Compound **8**	/
Compound **9**	/

Compounds **1-5** were assessed for their cytotoxicity by MTT viability test, showing no or very low toxicity up to the highest concentration tested (10 µM) (Figure S7, Supp. Info.).

2.3 Inhibition of PHA Lectin-Stimulated TLR4 Signal in HEK-Blue Cells

We were then interested in knowing if the inhibition of TLR4 signal is due to calixarene interaction with LPS or to a direct interaction with the TLR4 receptor system, evidenced as possible by calculations. To investigate this point, we stimulated HEK cells with the plant lectin phytohemagglutinin (PHA from Phaseolus vulgaris) whose property to potently stimulate TLR4 signal acting as agonist has been recently described.[311] We first checked if PHA is able to activate TLR4 signal in HEK-Blue cells, and we found that the lectin was active in stimulating in a dose-dependent way TLR4-dependent SEAP

production (Figure S8, Supp. Info.). To exclude the TLR4 activity could derive from LPS contamination in the PHA, we performed the experiment in the presence of the LPS-neutralizing peptide polymixin-B. We also verified that control HEK-null cells, that is HEK cells transfected with SEAP plasmid and lacking TLR4, MD-2, CD14 genes, were not activated by PHA lectin (Figure S8, Supp. Info.). PHA lectin was then used instead of LPS as a TLR4 agonist to stimulate cells. The highly potent calixarene-based TLR4 antagonists, compounds **3** and **4**, were then investigated for their property to inhibit TLR4 activation by PHA lectin (Figure 6).

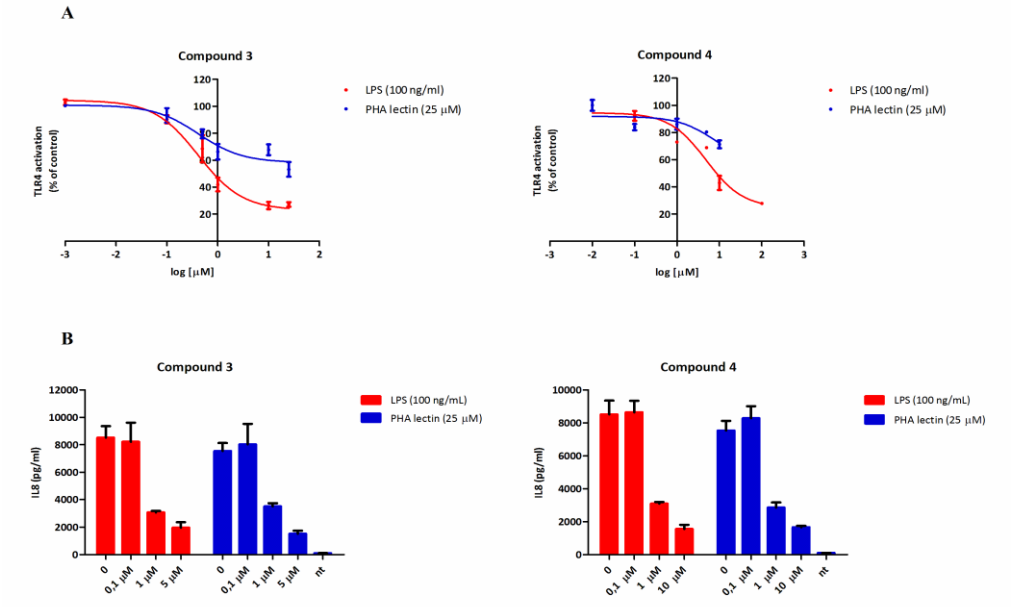


Figure 6. **(A)** Inhibition of TLR4 signaling in HEK-Blue cells stimulated with LPS (100 ng/mL) or PHA lectin (25 µM) and treated with calixarenes 3 and 4. The results represent normalized data with positive control (LPS or PHA lectin alone). **(B)** Quantification of interleukin-8 (IL-8) in HEK- Blue cells stimulated with LPS or PHA and treated with compounds 3 and 4 by performing ELISA assay. Data represent the mean of percentage ± SD of at least three independent experiments.

Guanidinium calixarenes **3** and **4** were indeed active in inhibiting PHA lectin-stimulated TLR4 signal in a concentration-dependent way, with potencies similar to those measured in the inhibition of LPS-stimulated TLR4 signal (Table 2). The fact that the antagonist activity was retained by calixarenes also when TLR4 was stimulated by a non-LPS agonist strongly suggests that the action of calixarenes is mainly based on direct interaction with CD14 and MD-2 receptors.

2.4 Inhibition of LPS-Stimulated TLR4 Signal in Human White Blood Cells

As HEK cells are a non-natural system to study TLR4 activation and to perform preliminary screening, the capacity of lead compounds **3** and **4** to inhibit LPS-triggered TLR4 signalling was further investigated in human white blood cells (h)WBCs that naturally express CD14, MD-2, and TLR4 receptors. We evaluated the production of the main NF-κB-dependent pro-inflammatory cytokines, tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and IL-8 by primary human peripheral blood mononuclear cells (hPBMCs) as readout for TLR4 pathway activation. hPBMCs isolated from the whole blood of healthy volunteers were treated with increasing concentrations (1−10 μM) of compounds **3** and **4** and stimulated with LPS after 30 minutes (100 ng/mL). Compound **3** reduced the production of all the pro-inflammatory cytokines monitored, while compound **4** showed a lower inhibitory activity, reducing only two of the three cytokines evaluated (Figure 7).

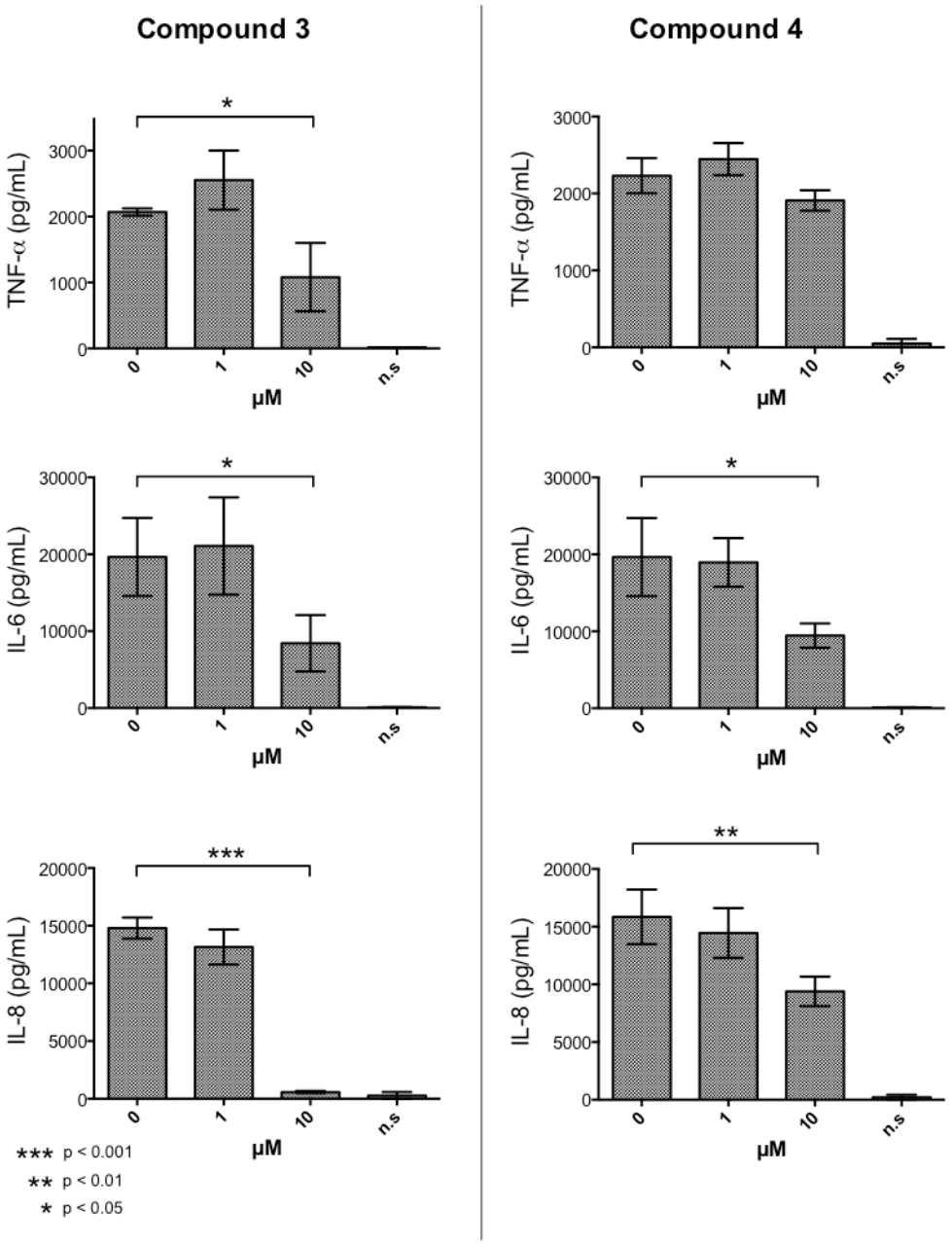


Figure 7. Inhibitory effect of compounds 3 and 4 on LPS-induced pro-inflammatory cytokines production by PBMCs. PBMCs isolated from whole blood were preincubated with synthetic compounds for 30 min and then stimulated with LPS (100 ng/mL). TNFα, IL-6, and IL-8 production was quantified after one night's incubation. Data represent the mean ± SEM of at least three independent experiments.

2.5 Inhibition of LPS-Stimulated TLR4 Signal in Murine White Blood Cells

It is known that human and murine MD-2 have dissimilarities in the LPS binding region, and some ligands have different activity on (h)MD-2 and (m)MD-2, in some cases switching from agonism to antagonism. We therefore aimed to compare the activity of calixarene on human and murine cells. The activity of compounds 3 and 4 was then evaluated in a murine macrophages cell line, RAW-Blue cells. As HEK-Blue cells, RAW-Blue cells are transfected to stably express the SEAP reporter gene in order to monitor the activation of TLR4 signal pathway. Compounds 3 and 4 inhibited in a dose-dependent way the LPS-stimulated TLR4 signal (Figure 8A), revealing that the two calixarenes were also effective on the murine TLR4 system. The abilities of compounds 3 and 4 were further investigated in murine splenocytes. TNF-α relative expression was determined from TLR4−MyD88 pathway activation. Splenocytes from balb/c mice were treated with two concentrations (1 and 10 µM) of compounds 3 and 4 in RPMI and stimulated after 30 min with LPS (100 ng/mL). The LPS-induced TNF-α expression after a 5 h incubation was measured by qPCR. The lower concentration of compounds 3 and 4 (1 µM) was weakly active in reducing LPS-induced TNF-α expression, whereas the higher concentration (10 µM) of both compounds completely inhibited the expression of TNF-α (Figure 8B).

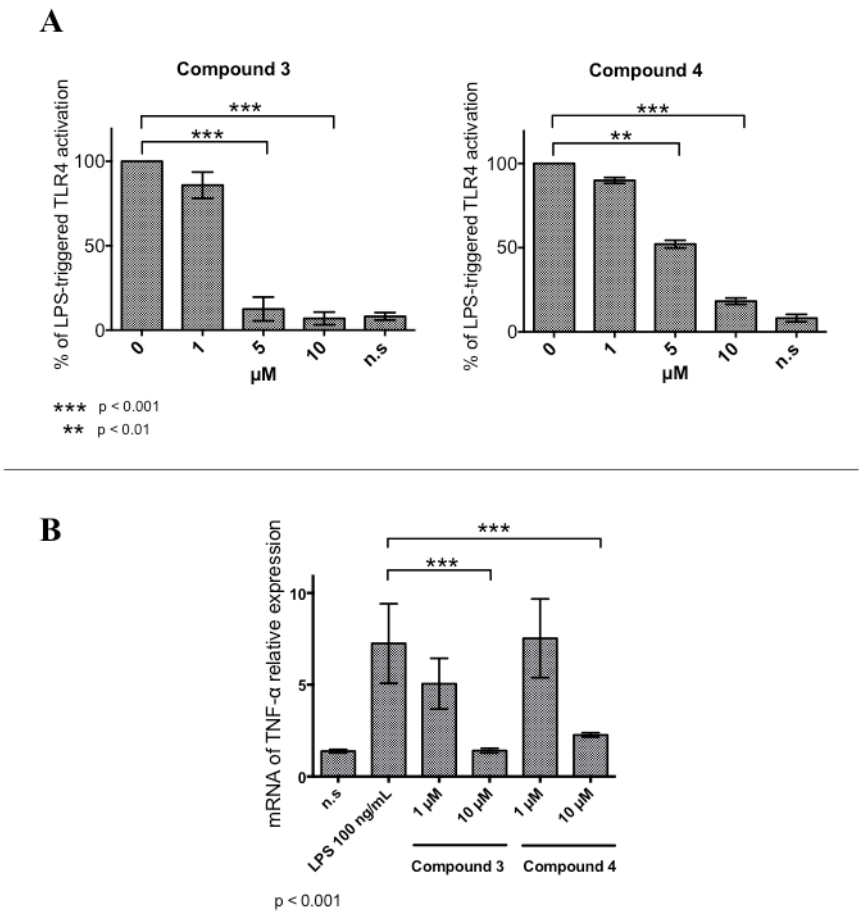


Figure 8. Effects of compounds **3** and **4** on RAW-Blue cells and on murine splenocytes. (**A**) RAW-Blue cells stably transfected with NF-κB-dependent SEAP reporter plasmid were treated with increasing concentrations of compounds 3 and 4 and stimulated with LPS (100 ng/mL) after 30 min. Data represent the mean of percentage of at least three independent experiments. (**B**) Murine splenocytes isolated from murine spleen were preincubated with two concentrations (1 and 10 μM) of compounds 3 and 4 for 30 min and then stimulated with LPS (100 ng/mL). Readout was the TNF-α expression after 5 h of incubation. Normalized data are representative of three independent experiments.

3. EXPERIMETAL SECTION

3.1 Molecular modeling

Structure construction. 3D structures of the ligands were built with PyMOL molecular graphics and modelling package[312] based on the coordinates of the calixarene scaffold retrieved from the PubChem database (CID:562409). 3D Coordinates for the agonist hTLR4/MD-2 complex, the antagonist mTLR4/MD-2 complex, the agonist mTLR4/MD-2 complex and hCD14 were retrieved from the PDB database (www.rcsb.org), under the ID 3FXI, 2Z64, 3VQ2 and 4GLP, respectively. The structures went through a restrained minimization procedure with Maestro using the OPLS3 force field. Gasteiger charges were computed within the AutoDock Tools program and all non-polar hydrogens were merged.
Structure optimization. All compounds (from **1** to **9**) were optimized with *ab initio* calculations, using the density functional theory (DFT) with the hybrid functional B3LYP with the Pople basis set 6-31+g(d,p) using Gaussian g09/e1.[313] Water solvation (with a dielectric constant of ε=78.3553) was simulated with the Gaussian default SCRF method (i.e. using the Polarizable Continuum Model (PCM) with the integral equation formalism variant (IEFPCM)).

Docking procedure. Docking was performed independently with both AutoDock 4.2[314] and AutoDock VINA 1.1.2.[315] In AutoDock 4.2, the Lamarckian evolutionary algorithm was chosen and all parameters were kept default except for the number of genetic algorithm (GA) runs which was set to 200 to enhance the sampling. AutoDockTools 1.5.6 was used to assign the Gasteiger-Marsili empirical atomic partial charges to the atoms of both the ligands and

the receptors. The structure of the receptors was always kept rigid whereas the structure of the ligand was set partially flexible by providing freedom to some appropriately selected dihedral angles. Concerning the boxes, spacing was set to 0.375 Å for AutoDock and is default to 1Å for VINA. In the case of the human and mouse TLR4.MD-2 systems in their agonist and antagonist conformations, the size of the box was set to 33.00 Å in the x-axis, 40.50Å in the y-axis and 35.25 Å in the z-axis. For (h)CD14 the size of the box was set to 33.00 Å in the x-axis, 33.75Å in the y-axis and 33.75 Å in the z-axis. For the (h)TLR4/MD-2 complex the center of the box is located equidistant to the center of mass of residues Arg90 (MD-2), Lys122 (MD-2) and Arg264 (TLR4). For the (m)TLR4/MD-2 complex the center of the box is located equidistant to the center of mass of residues Arg90 (MD-2), Glu122 (MD-2) and Lys263 (TLR4). For (h)CD14 the center of the box is located equidistant to the center of mass of residues Phe69, Tyr82 and Leu89.

Parameters derivation. Parameters for molecular dynamics simulations were set up with the standard Antechamberwang[316] procedure. Briefly, charged were calculated with Gaussian at the Hartree-Fock level (HF/6-31G* Pop=MK iop(6/33=2) iop(6/42=6)) from the solvated DFT B3LYP optimized structure, then derived and formatted for Ambertools15 and Amber14 with Antechamber assigning the general AMBER force field (GAFF) atom types.[317] A new atom type for nitrogen was introduced (nj), within GAFF, to properly describe the guanidine moiety, mirroring the parameters of ff14SB[318] used to describe the guanidine fragment present in arginine. Parameters for this new atom are provided in the supplementary information section.

Molecular Dynamics (MD) simulations. Before being submitted to the production run, the system undergoes a height steps preparation. The first one consists of 1000 steps of steepest descent algorithm followed by 7000 steps of conjugate gradient algorithm; a 100 $kcal.mol^{-1}.A^{-2}$ harmonic potential constraint is applied on both the proteins and the ligand. In the 4 subsequent steps, the harmonic potential is progressively lowered (respectively to 10, 5 and 2.5 $kcal.mol^{-1}.A^{-}$) for 600 steps of conjugate gradient algorithm each time, and then the whole system is minimized uniformly. In the following step the system is heated from 0 K to 100 K using the Langevin thermostat in the canonical ensemble (NVT) while applying a 20 $kcal.mol^{-1}.A^{-2}$ harmonic potential restraint on the proteins and the ligand. The next step heats up the system from 100 K to 300 K in the Isothermal–isobaric ensemble (NPT) under the same restraint condition than the previous step. In the last step the same parameters are used to simulate the system for 100 ps but no harmonic restraint is applied. At this point the system is ready for the production run, which is performed using the Langevin thermostat under NPT ensemble, at a 2 fs time step. All production runs were performed for 90 ns.

LogP calculations. From the optimized 3D structure of compounds **1**-**9**, logP value was calculated with Maestro package (www.schrodinger.com/maestro).

3.2 Biology: Cell Tests

HEK-Blue™ hTLR4 cells activation assay.

HEK-Blue™ hTLR4 cells (*InvivoGen*) are designed for studying the stimulation of human TLR4 (hTLR4) by monitoring the activation of NF-κB and AP-1. HEK-Blue™ hTLR4 cells were obtained by the co-transfection of hTLR4 gene, MD-

2/CD14 co-receptor genes and a secreted embryonic alkaline phosphatase (SEAP) reporter gene into HEK293 cells. The SEAP reporter gene is placed under the control of an IL-12 p40 minimal promoter fused to five NF-κB and AP-1 binding sites. Stimulation with a TLR4 ligand activates NF-κB and AP-1 inducing the production of SEAP. Levels of SEAP can be easily determined with HEK-Blue assay. HEK-Blue hTLR4 cells were cultured in Complete Medium supplemented with 100 µg/mL Normocin and 1X HEK-Blue™ Selection (*InvivoGen*). HEK-Blue™ hTLR4 cells were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, antibiotics and 1× HEK-Blue™ Selection. Cells were detached using a cell scraper, counted and seeded in a 96-well multiwell plate at a density of 4×10^4 cells per well. After overnight incubation (37 °C, 5% CO_2, 95% humidity), supernatants were replaced with new medium supplemented by the compound to be tested dissolved in water or DMSO−Ethanol (1:1). After 30 minutes of pre-incubation, cells were stimulated with 100 ng/mL LPS from *E. coli* O55:B5 (Sigma-Aldrich) or 25 µM lectin from *Phaseolus vulgaris* (PHA-P) and incubated overnight. The SEAP-containing supernatants were collected and incubated with paranitrophenylphosphate (pNPP) for 2−4 h in the dark at room temperature. The wells optical density was determined using a microplate reader set to 405 nm. The results were normalized with positive control (LPS alone) and expressed as the mean of percentage ± SD of at least three independent experiments.

IL-8 quantification

Supernatants from HEK- Blue cells treated with compounds **3** (0.1, 1, 5 µM) and **4** (0.1, 1, 10 µM) and stimulated with LPS (100 ng/mL) or PHA-P (25 µM)

were used to quantify IL-8 concentration by performing ELISA assay (Thermo scientific) according to manufacturer's instructions. The readings were assessed by using a spectrophotometer at 450 nm (LT 4000, Labtech).

Human peripheral blood mononuclear cells (hPBMCs)

PBMCs were isolated by density gradient centrifugation (Lympholyte®-H; Cedarlane Lab) from buffy-coats (Agreement between Ospedale Niguarda Cà Granda - Università degli Studi di Milano-Bicocca for supply of buffy-coats for research use). Briefly, buffy-coats were diluted 1:1 with Phosphate Buffer Saline (PBS), and layered on Lympholyte®-H for density gradient centrifugation according to the manufacturer's instructions. PBMCs were harvested from the interface and washed in PBS. The isolated cells (LPMCs and PBMCs) were counted, checked for viability using 0.1% trypan blue and resuspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 U/mL). Cells were plated in 24 or 96-well U-bottom multiwell culture plates (Falcon Plastic), pre-incubated with two concentrations of compound FP7 (final concentrations 1 and 10 µM) and stimulated with smooth lipopolysaccharide (S-LPS; *Escherichia coli* 055:B5; Sigma; 100 ng/mL) after 30 minutes. The working concentration of LPS was evaluated upon dose-response experiments (data not shown) and it was chosen in order to induce a strong pro-inflammatory response with a massive production of pro-inflammatory cytokines. TNF-α, IL-1β and IL-8 levels were measured in supernatants after 18 hours of LPS stimulation in presence or absence of FP7 (1 and 10 µM) using a sensitive enzyme-linked immunosorbent assay (ELISA) (R&D Systems; #DY206-05, #DY210-05, #DY208-05, Minneapolis, MN). The optical density of each well was determined using a microplate

reader set to 450 nm (wavelength correction 570 nm). All graphs were representative data from at least three independent experiments.

RAW-Blue™ cells activation assay.

RAW-Blue™ Cells (InvivoGen) are derived from RAW 264.7 macrophages. These cells stably express a secreted embryonic alkaline phosphatase (SEAP) gene inducible by NF-κB and AP-1 transcription factors. RAW-Blue™ Cells express all TLRs (with the exception of TLR5). The presence of specific agonists of these receptors induces signaling pathways leading to the activation of NF-κB and AP-1. Upon TLR, stimulation, RAW-Blue™ cells activate NF-κB and/or AP-1 leading to the secretion of SEAP which is easily detectable and measurable with RAW-Blue™ assay. RAW-Blue™ Cells are resistant to Zeocin™ and G418. Cells were cultured in Complete Medium containing supplemented with 100 µg/mL Normocin and 200 µg/mL Zeocin™. Raw-Blue™ cells were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 µg/mL Normocin (InvivoGen), 200 µg/mL Zeocin (InvivoGen). Cells were detached using a cell scraper and the cell concentration was estimated by using Trypan Blue (Sigma-Aldrich). The cells were diluted in DMEM high glucose medium supplemented as described before and seeded in 96-well multiwell plate at a density of 6×10^4 cells per well in 200 µL. After overnight incubation (37 °C, 5% CO_2, 95% humidity), supernatant was removed, cell monolayers were washed with warm PBS and treated with increasing concentrations of compounds dissolved in DMSO−ethanol (1:1) and diluted in DMEM. After 30 minutes, cells were stimulated with 10 ng/mL of LPS from *E. coli* O55:B5 (Sigma- Aldrich) for 16 hours. The supernatants were collected and incubated with

paranitrophenylphosphate (pNPP) for 2–4 hours in the dark at room temperature. The optical density of each well was determined using a microplate reader set to 405 nm. The results were normalized with positive control (LPS alone) and expressed as the mean of percentage ± SEM of at least three independent experiments.

Murine Splenocytes

Murine splenocytes were isolated from murine spleen, counted and resuspended in RPMI supplemented with 10% FBS, 2 mM glutamine and antibiotics. Cells were plated in a 24-well multiwell plate ($1.5x10^6$ cells/well) in presence of different concentrations of the compounds to be tested. After 30 minutes cells were stimulated with 100 ng/mL of LPS and incubated for 4 hours (37°C, 5% CO_2, 95% humidity). Cells were lysed and total RNA was extracted using QIAGEN's RNA Mini Kit (QIAGEN, Milan, Italy) according to the manufacturer's instruction. To remove genomic DNA, on-column digestion using RNase free DNase set (Qiagen) was performed. Reverse transcription was performed with 0.5 - 1 μg of total RNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific) and this was amplified using the following conditions: denaturation for 1 minute at 95°C; annealing for 30 seconds at 58°C for mouse TNF-α and 60°C for mouse β-actin; 30 seconds of extension at 72°C. Primer sequences were as follows: mouse TNF-α (forward 5´-ACCCTCACACTCAGATCATC-3´, reverse 5´-GAGTAGACAAGGTACAACCC-3´); β-actin (forward 5'-AAGATGACCCAGATCATGTTTGAGACC-3', reverse 5'-AGCCAGTCCAGACGCAGGAT-3') was used as a housekeeping gene. TNF-α

expression was calculated using the ΔΔCt algorithm. All graphs were representative data from at least three independent experiments.

MTT Cell Viability Assay

HEK-Blue™ hTLR4 cells were grown in DMEM supplemented with 10% FBS, 2 mM glutamine and antibiotics. Cells were seeded in 100 µL of DMEM without Phenol Red at a density of 4×10^4 cells per well and incubated overnight (37 °C, 5% CO_2, 95% humidity). Cells were treated with the higher dose of compound used in the previous experiments and incubated overnight. MTT solution (5 mg/mL in PBS) was added to each well and after 3 hours incubation, HCl 0.1 N in 2-propanol solution was used to dissolve formazan crystals. Formazan concentration was determined by measuring the absorbance at 570 nm. The results were normalized with untreated control (PBS) and expressed as the mean of percentage ± SEM of three independent experiments.

PAINS

Compounds 1−9 were subjected to the pan assay interference compounds (PAINS) online filter (ZINC PAINS patterns search http://zinc15.docking.org/patterns/home/, accessed Jan 26, 2016) and substructure filters.[319] This analysis showed that none of them were PAINS.

Statistical Analysis

Experimental data were normalized and expressed as means ± standard deviation (SD). The data shown are the average of at least three independent

experiments, each in technical triplicate. Statistical significance was evaluated using Student's *t* test.

4. SUPPORTING INFORMATION

To see Supporting information that regards:

- *Molecular Modeling, Docking Results*
- *Chemistry: General*
- *Synthesis and compounds characterization*

CLICK HERE

or open the following link with a browser:

(http://pubs.acs.org/doi/suppl/10.1021/acs.jmedchem.7b00095)

Supporting information that regards:

- *MTT toxicity test*
- *Biology: activity of PHA plant lectin on HEK cells*

are shown below.

4.1 MTT toxicity test

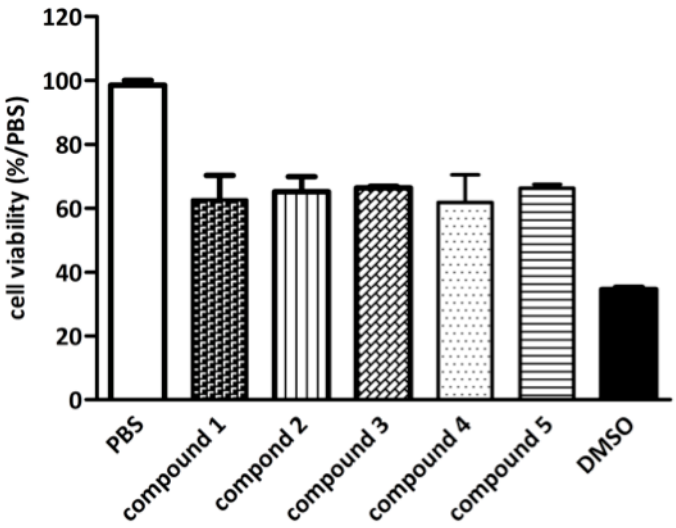


Figure S7. MTT assay of compounds **1**-**5** in HEK Blue cells. Cells were treated with the same concentrations of compounds used in the other assays; the bars represent the cell viability estimated by using 10 µM of compounds, equivalent to the maximum concentration used previously. Data are normalized with PBS and represent the mean of percentage ± SD of at least 3 independent experiments. GO BACK TO THE TEST.

4.2 HEK-Blue cells activation by plant lectin PHA

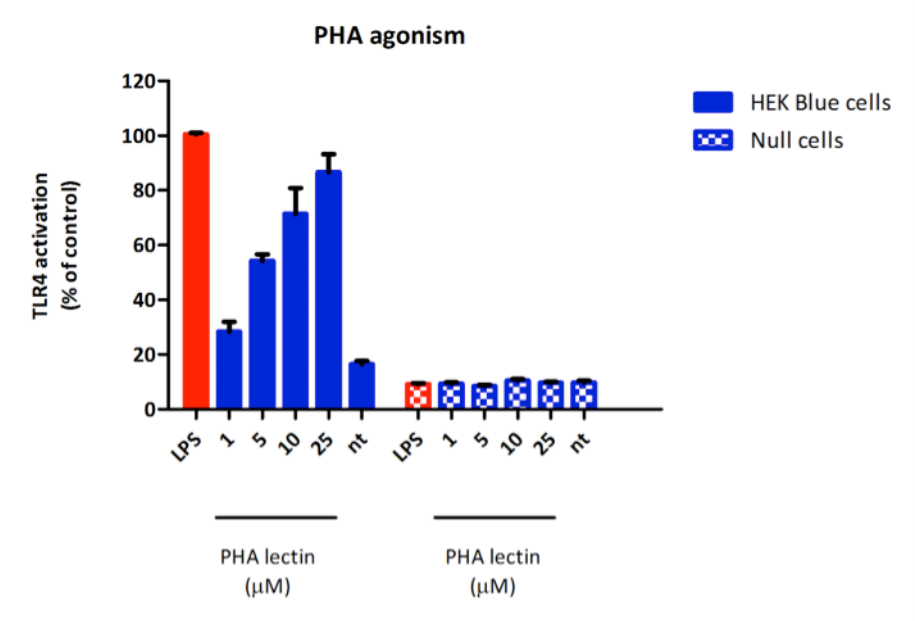


Figure S8. Dose-dependent PHA activation of TLR4 signal in HEK-blue and Null cells. TLR4 HEK Blue and Null cells (control cell line) were stimulated with increasing concentrations of PHA lectin. Data are normalized with LPS (100 ng/mL) and represent the mean of percentage ± SD of at least three independent experiments. nt= not treated. GO BACK TO THE TEST.

CHAPTER II

Co-administration of antimicrobial peptides (AMPs) enhances Toll-like Receptor 4 (TLR4) antagonist activity of a synthetic glycolipid.

Fabio A. Facchini[†], Helena Coelho, [‡][||][⊥] Stefania E. Sestito[†], Sandra Delgado[‡], Alberto Minotti[†], David Andreu[§], Jesús Jiménez-Barbero, [‡][||][▽] Francesco Peri[†]*

[†] Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza, 2, 20126 Milano, Italy

[‡] Molecular Recognition & Host−Pathogen Interactions Programme, CIC bioGUNE, Bizkaia Technology Park, Building 801A, 48170 Derio, Spain

[||] Departament of Organic Chemistry II, Faculty of Science & Technology, University of the Basque Country, 48940 Leioa, Bizkaia Spain.

[⊥] UCIBIO, REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

[§] Department of Experimental and Health Sciences, Pompeu Fabra University, Barcelona Biomedical Research Park, Dr. Aiguader 88, 08003 Barcelona, Spain

[▽] Ikerbasque, Basque Foundation for Science, Maria Diaz de Haro 13, 48009 Bilbao, Spain

Abstract

This study examines the effect of co-administration of antimicrobial peptides and the synthetic glycolipid FP7, which is active in inhibiting inflammatory cytokine production caused by TLR4 activation and signaling. The co-administration of two lipopolysaccharide (LPS)-neutralizing peptides (a cecropin A-melittin hybrid peptide and a human cathelicidin) enhances by an order of magnitude the potency of FP7 in blocking the TLR4 signal. Interestingly, this is not an additional effect of LPS neutralization by peptides, because it also occurs if cells are stimulated by the plant lectin phytohemagglutinin, a non-LPS TLR4 agonist. Our data suggest a dual mechanism of action for the peptides, not exclusively based on LPS binding and neutralization, but also on a direct effect on the LPS-binding proteins of the TLR4 receptor complex. NMR experiments in solution show that peptide addition changes the aggregation state of FP7, promoting the formation of larger micelles. These results suggest a relationship between the aggregation state of lipid A-like ligands and the type and intensity of the TLR4 response.

Manuscript ahead of print on *ChemMedChem.* DOI: 10.1002/cmdc.201700694

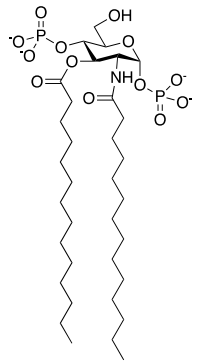
1. INTRODUCTION

Two strategies are usually adopted to interfere in TLR4 activation and signaling with chemicals: i) LPS-binding molecules preventing its interaction with receptors, and ii) inhibition of activated $(TLR4/MD\text{-}2/LPS)_2$ complex formation by molecules directly competing with LPS for the binding to MD-2 and CD14 receptors. While the first strategy is mainly used to block TLR4 stimulation by LPS in sepsis and septic shock, the second one could be, in principle, applied to block a wider array of pathologies deriving from TLR4 activation by DAMPs.

In the first approach, positively charged antimicrobial peptides (AMP) are known to bind to and neutralize LPS and interact with endotoxin.[128] The prototypic AMP is polymyxin B, a cationic, small cyclic lipopeptide, largely investigated for its endotoxin neutralizing property.[139] Further examples are cecropins,[140] magainins,[141] proline-arginine-rich peptides,[142] tachyplesin,[143] defensins,[144] and others.[145, 146] Structures of many of these peptides are known and include turn/loop, helix or □-sheet patterns. Neutralization of LPS by AMPs involves a strong exothermic coulombic interaction between the two species, with ensuing fluidization of LPS acyl chains and a drastic change in LPS aggregate type from cubic into multilamellar and an increase in aggregate sizes, altogether inhibiting the binding of LBP and other mammalian proteins to the endotoxin.[158] The second approach to block LPS/TLR4 signal is based on molecules that directly compete with LPS or other ligands for the binding of MD-2 and CD14 co-receptors. Several small molecules with potent TLR4 antagonist activity are known, such as synthetic phosphorylated disaccharides mimicking lipid A (Eisai's Eritoran being the most famous),[22] synthetic monosaccharides,[198, 199] or natural and synthetic compounds with structures unrelated to lipid A.[320] We recently synthesized FP7 (Figure 1), a di-

phosphorylated glucosamine monosaccharide bearing two myristic (C_{14}) chains linked to C-2 and C-3 positions, that proved to be active as TLR4 antagonist in cells and in animal models.[135, 207, 210] Similarly to disaccharide-based lipid A mimetics that block TLR4, such as Eritoran, FP7 binds to MD-2 by inserting its fatty acid C_{14} linear chains into receptor's binding cavity.[207]

In this section, we were interested in investigating the effect achieved by combining the two strategies: we decided to combine lipid A mimetic FP7 with a small library of AMPs. In particular, we evaluated whether cationic peptides that interact with anionic LPS aggregates could also interact with anionic monosaccharide FP7 thus modulate its antagonist activity on TLR4. To this end, we decided to combine FP7 with cecropin A-melittin hybrids, a class of AMPs where the cationic N-terminus of cecropin A (CA) is fused to the hydrophobic N-terminus of melittin (M) (AMPs **1**-**5**), and with LL-37, a human cathelicidin that possesses a variety of activities including endotoxin neutralization (AMP **6**).[155, 172, 321] Specifically, we screened six synthetic CA-M hybrids (Table 1), namely CA(1-8)M(1-18)[322], **AMP1**; CA(1-7)M(2-9)[175], **AMP2**; [$K_6(Me_3)$] CA(1-7)M(2-9)[323], **AMP3**; N^{α}-Oct-CA(1-7)M(2-9)[324], **AMP4** and CA(1-7)M(5-9)[175], **AMP5**; as well as LL-37[325], **AMP6**, for their effects on TLR4 activation when added alone to cells or in combination with the TLR4 antagonist FP7.



FP7

Figure 1. Molecular formula of **FP7**

Table 1. Primary structures of the AMPs used in this study.

Entry	Common name	Sequence	Ref.
AMP 1	CA(1-8)M(1-8)	KWKLFKKIGIGAVLKVLTTGLPALIS-amide	[322]
AMP 2	CA(1-7)M(2-9)	KWKLFKKIGAVLKVL-amide	[175]
AMP 3	[$K^6(Me_3)$]CA(1-7)M(2-9)	KWKLFK(Me_3)KIGAVLKVL-amide	[323]
AMP 4	N^{α}-Oct-CA(1-7)M(2-9)	Octanoyl-KWKLFKKIGAVLKVL-amide	[324]
AMP 5	CA(1-7)M(5-9)	KWKLFKKVLKVL-amide	[175]
AMP 6	LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	[325]

2. RESULTS

2.1 AMPs potentiation of FP7 antagonist activity on LPS/TLR4 signaling in HEK-Blue hTLR4 cells

The effect of FP7/AMPs co-administration was first investigated in HEK-Blue hTLR4 cells. HEK-Blue hTLR4 cells are HEK293 cells stably transfected with human TLR4, MD-2, and CD14 genes. In addition, these cells possess as reporter gene a secreted embryonic alkaline phosphatase (SEAP) produced upon activation of NF-κB. LPS binding triggers in sequence TLR4 dimerization, myddosome formation and NF-κB activation, leading in the end to SEAP production and secretion. Compound FP7 confirmed its activity as TLR4

antagonist, inhibiting in a dose-dependent way the LPS-stimulated TLR4 activation with a calculated IC_{50} of 2.5 µM (Figure 2, 3 and Table 2).[207] Interestingly, AMPs administered alone did not show any antagonist effect on the same cell line at the concentration range used (Figure 2).

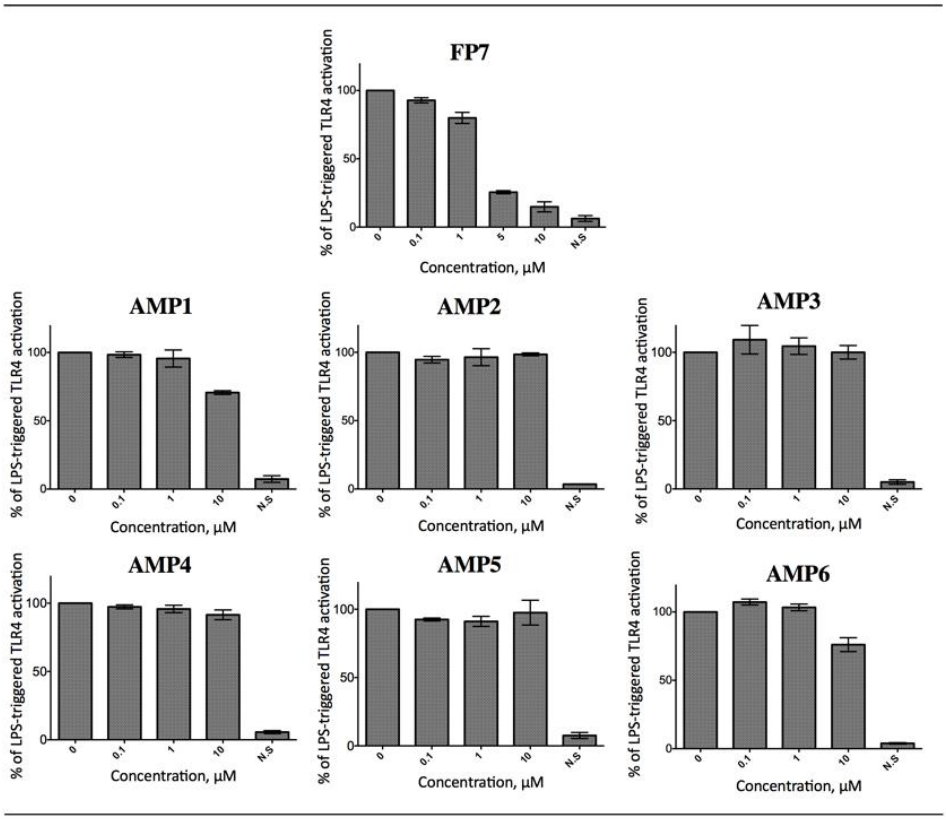


Figure 2. Effects of FP7 and AMPs **1-6** on LPS-stimulated TLR4 signal in HEK-Blue hTLR4 cells. HEK-Blue hTLR4 cells were pre-treated with the indicated concentrations of FP7 and AMPs **1-6** and stimulated with LPS (100 ng/mL) after 30 minutes. Data were normalized to stimulation with LPS alone. Data represent the mean of percentage ± SEM of at least 3 independent experiments.

FP7 was then co-administered (1:1 stoichiometric ratio) with AMPs 1-6. In the concentration range (0 to 10 µM) tested, AMPs 2-5 weakly enhanced FP7 antagonist activity (co-administration IC_{50} around 1.1 - 1.5 µM (Table 2)), whereas AMPs 1 and 6 showed stronger activity (IC_{50} 0.56 µM and 0.18 µM respectively) (Figure 3 and Table 2).

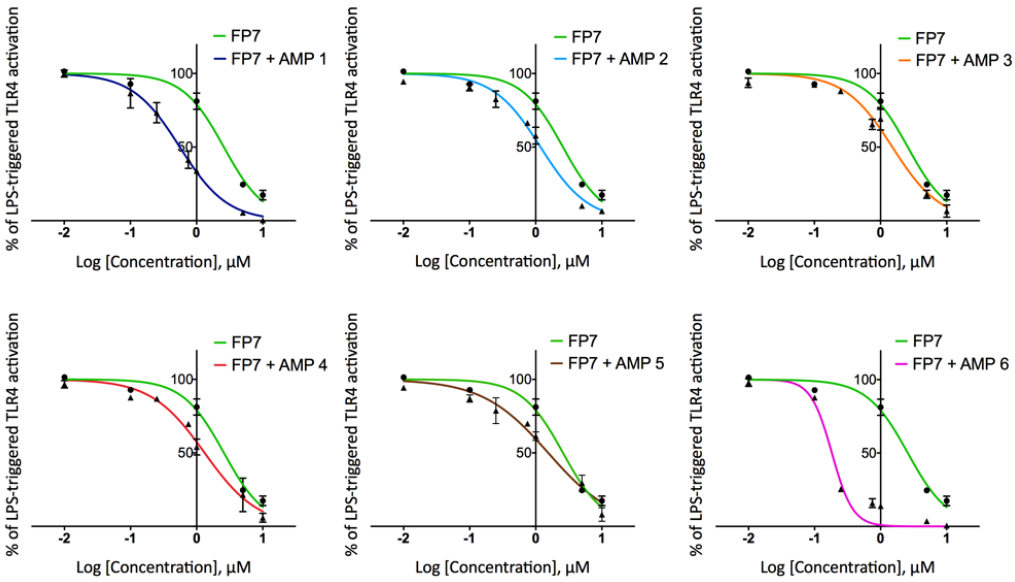


Figure 3. Dose-dependent inhibition of LPS-stimulated TLR4 signal in HEK-Blue hTLR4 cells by FP7/AMPs co-administrations. HEK-Blue hTLR4 cells were pre-treated with increasing concentrations of FP7 and FP7/AMPs mix and stimulated with LPS (100 ng/mL) after 30 m. Data were normalized to stimulation with LPS alone. Concentration-effect data were fitted to a sigmoidal 4 parameter logistic equation to determine IC_{50} values. Data points represent the mean of percentage ± SEM of at least 3 independent experiments. The table below recapitulates the IC_{50} values for the inhibition of LPS- and PHA lectin-stimulated TLR4 signal in HEK-Blue hTLR4 cells.

Table 2. Activities of FP7/AMPs administrations on LPS- and PHA lectin-stimulated TLR4 signal in HEK-Blue hTLR4 cells.

Treatment	**IC_{50} LPS (µM)**	**IC_{50} PHA (µM)**
FP7	2.5	**1.21**
FP7 + AMP 1	0.56	
FP7 + AMP 2	1.18	
FP7 + AMP 3	1.51	
FP7 + AMP 4	1.32	
FP7 + AMP 5	1.54	
FP7 + AMP 6	0.18	**0.14**

To exclude the possibility that the activity increase was due to a cytotoxic effect, all co-administrations were assessed for their toxicity by MTT viability test, showing no or very low toxicity up to the highest concentration tested (10 µM) (Figure 4). CA(1-8)M(1-18) (AMP 1) and LL-37 (AMP 6) most efficiently improve the TLR4 antagonist activity of FP7 in HEK-Blue hTLR4 cells.

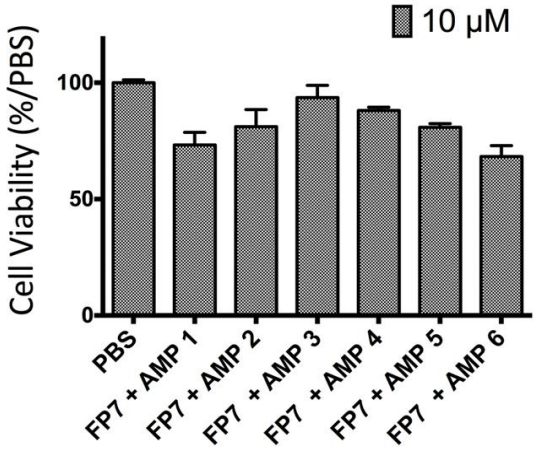


Figure 4. MTT assay of FP7/AMPs co-administrations in HEK-Blue hTLR4 cells. Cells were treated with the six co-administrations used in the other assays; the bars represent the cell viability estimated by using 10 µM of compounds, equivalent to the maximum concentration used previously. Data are normalized with PBS and represent the mean of percentage ± SEM of at least 3 independent experiments.

2.2 AMP6 potentiation of FP7 antagonist activity is maintained in PHA-stimulated cells

Considering that several AMPs are known to interact with high affinity with LPS promoting its neutralization,[155, 321, 326] we next investigated if the potentiation of FP7 antagonist activity by AMPs was due exclusively to a neutralizing effect on endotoxin. If so, the additive effect would be lost by stimulating cells with a TLR4 agonist different from LPS.

For this purpose, we considered phytohemagglutinin (PHA) plant lectins (i.e., PHA-L and PHA-P), which are described to induce TLR4-dependent NF-κB activation in a dose-dependent way, with a lower potency than LPS.[311] We first confirmed the activity of PHA-P as TLR4 agonist (Figure 5A) and, in order to excluded this effect could be due to LPS contamination, we treated cells with PHA-P in the presence of the LPS sequestrant polymyxin B (PMB), obtaining similar NF-κB activation values (Figure 5B). Next we used PHA-P-stimulated HEK-Blue hTLR4 cells to evaluate the antagonist activity of FP7 in the presence of AMP6. As expected, treatment with FP7 inhibited TLR4 activation in a dose-dependent way, confirming that the compound interferes with receptor-ligand recognition (Figure 5C). Interestingly, on the same PHA-P-activated cells the potentiation of FP7 antagonism by AMP6 co-administration was maintained (Figure 5C), suggesting that the enhancement of FP7 activity is at least in part independent from a LPS neutralizing effect.

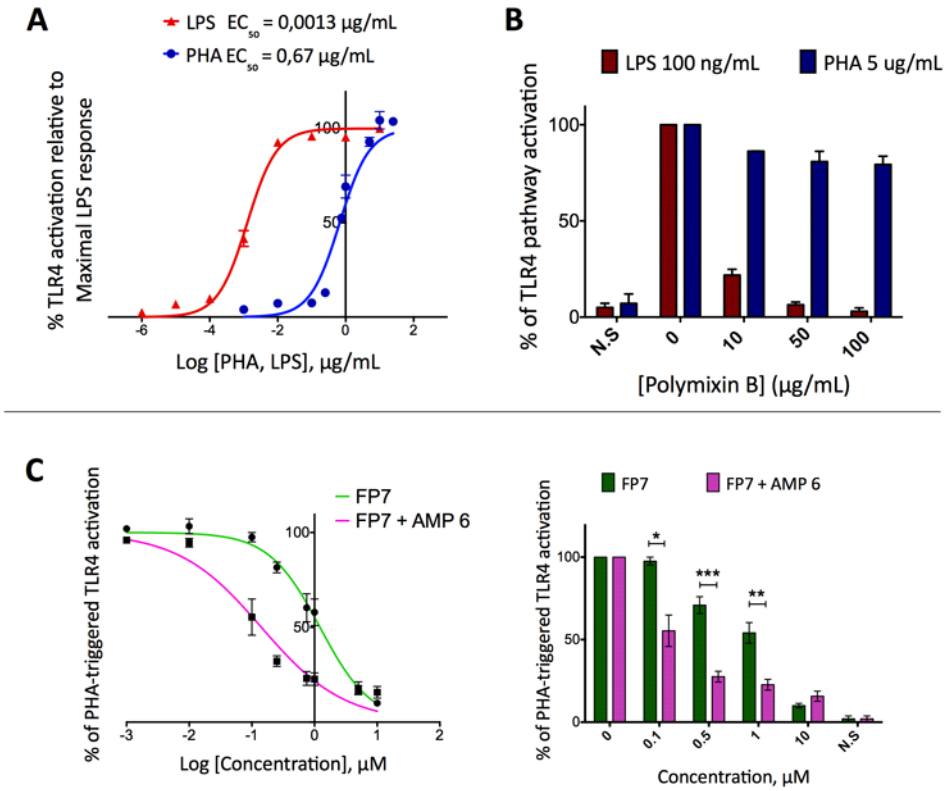


Figure 5. A) Dose-dependent PHA- and LPS-stimulated TLR4 activation in HEK-Blue hTLR4 cells. HEK-Blue hTLR4 cells were stimulated with increasing concentrations of LPS and PHA lectin and SEAP levels in media were quantified after 16 h. The percentages of TLR4 activation are relative to maximal LPS response. **B)** HEK-Blue hTLR4 cells were stimulated with LPS (100 ng/mL) and PHA lectin (5 µg/mL) in the absence or presence of increasing concentrations of polymixin B. **C)** Dose-dependent inhibition of PHA-stimulated TLR4 activation by FP7 and FP7/AMP6. Cells were treated with increasing concentrations of compounds and stimulated with PHA-P (5 µg/mL). The results represent normalized data with positive control (PHA-P alone). Concentration-effect data were fitted to a sigmoidal 4 parameter logistic equation to determine IC_{50} values and represent the mean of percentage ± SEM of at least 3 independent experiments. The IC_{50} values are shown in table 1

2.3 AMP6 potentiation of FP7 antagonist activity in human peripheral blood mononuclear cells (h)PBMCs

We investigated whether the capacity of the most potent peptide, LL-37 (AMP6), to enhance FP7 antagonist activity also occurred in human monocytes. For this purpose, human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats, pre-incubated with increasing concentrations (0.1-10 µM) of FP7 or FP7/AMP6 mix and stimulated with LPS (100 ng/mL) after 30 minutes. We evaluated the production of the NF-κB-dependent pro-inflammatory cytokine interleukin-1β (IL-1β) as readout for LPS-triggered TLR4 pathway activation. As expected, FP7 was able to reduce the production of IL-1β in a dose-dependent way, halving the amount of cytokine released when administered at a concentration of 5 µM. The addition of AMP6 to FP7 produces a much more powerful inhibitory response, inhibiting the production of interleukin already at the lower dose of 1 µM (Figure 6).

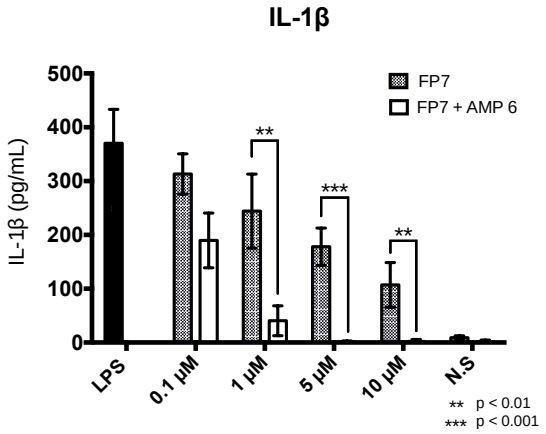


Figure 6. LL-37 (AMP6) potentiation of FP7 antagonist activity in human PBMCs. PBMCs isolated from buffy coats were preincubated with FP7 or FP7/AMP6 mix for 30 m and then stimulated with LPS (100 ng/mL). IL-1β production was quantified after one night's incubation. Data represent the mean ± SEM of at least three independent experiments**.**

2.4 NMR analysis of glycolipid/peptide interaction

The additive effect of AMP 6 on FP7 activity in lectin-stimulated cells suggests that a direct interaction between peptides and glycolipid could have an effect on TLR4 antagonism. The peptide/glycolipid interaction was investigated by NMR for AMP1 and AMP6, by analyzing the perturbations observed on characteristic NMR parameters (e.g. chemical shifts, line widths, and signal intensities) of either binding partner. The titration of AMP 1 and AMP 6 with FP7 (see Figure S3 and S11 in Supp. Info.) permitted to observe the broadening of the peptides NMR resonance signal upon addition of FP7. In particular, a clear perturbation of the signals of hydrophobic amino acids lateral chain was observed. The experimentally observed reduction in intensity (see Figure S4 and S12 in Supp. Info.), due to specific line broadening of these signals, probably arise from the changes in the transverse relaxation time of these signals; a clear indication of binding events between AMPs (1 and 6) and FP7. The DOSY spectra of AMP1 and AMP6 showed a strikingly low diffusion coefficient, far from its small/medium molecular weight indicating peptide aggregation (see Figure S5 and S13 in Supp. Info.). This experimental evidence was also confirmed by using TEM negative staining analysis (see Figure S6 and S14 in Supp. Info.), where filament-like shapes were observed for the peptides alone. Interestingly, the diffusion coefficient of both AMPs remained unaltered in the presence of FP7 (see Figure S5 and S13 in Supp. Info.). Thus, the interaction of FP7 with the two AMPs (in excess) does not show a large effect in the average size of the AMPs aggregates. The process was also monitored by looking at the NMR signals of FP7 protons upon addition of AMP 1 (Figure 7 A-I) and AMP6 (Figure 7 B-I). In both cases, the dramatic reduction of the intensity of aliphatic moieties NMR signal suggested that the interaction with

AMP1 and 6 involves the lipid chains (Figure 7 A-I for AMP 1 and B-I for AMP 6). Three alternative hypothesis can explain these data. The peptide could act as linker between different FP7 aggregates (see Figure S7 in Supp. Info.) and/or deform the FP7 micelles (see Figure S8 in Supp. Info.). Alternatively, the peptide could participate in the formation of large aggregates (see Figure S9 in Supp. Info.), behaving as a large molecule, as earlier described for MD-2.[207] This model is in agreement with the DOSY observations (see Figure S5 and S13 in Supp. Info.) described above.

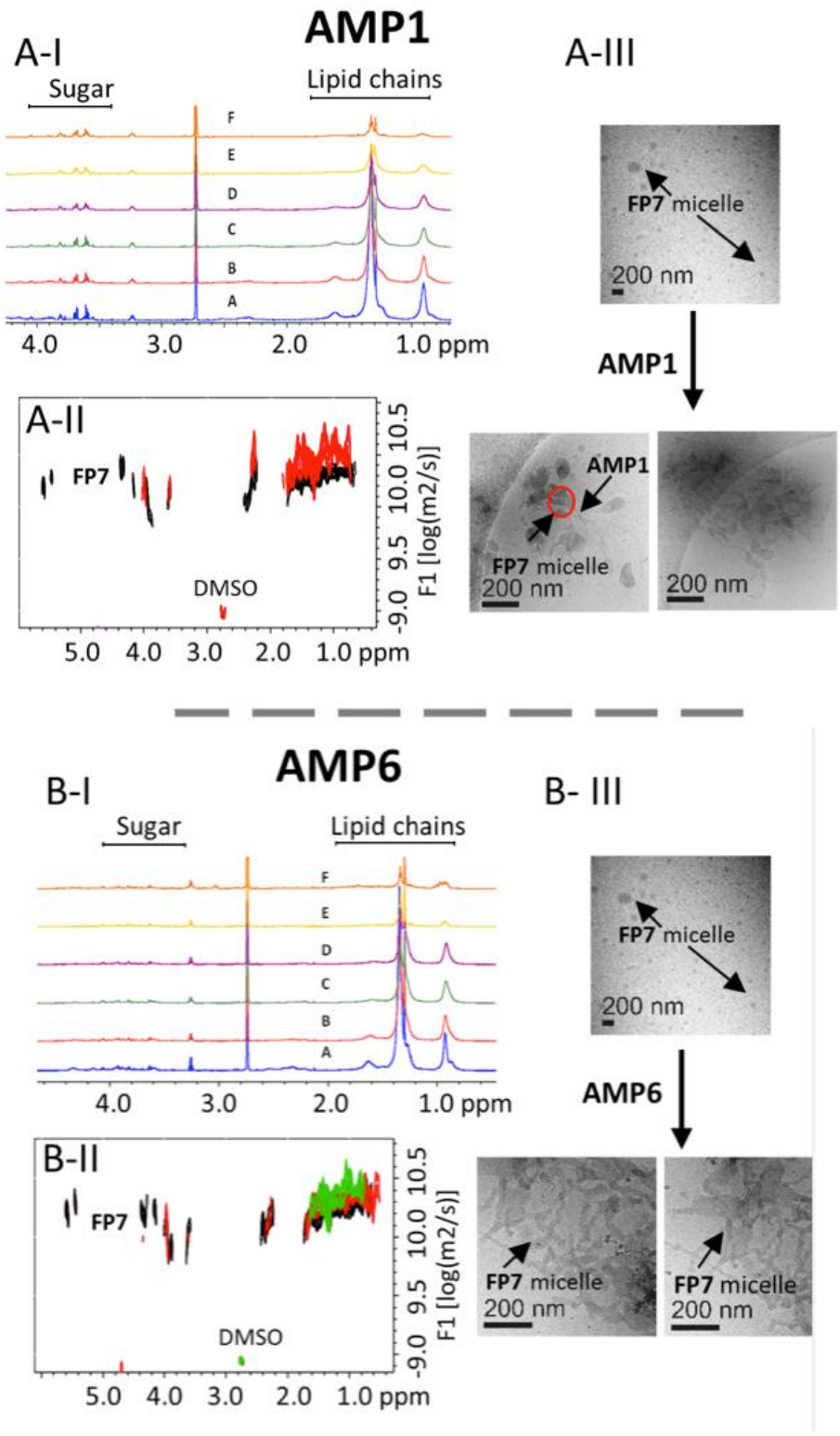

AMP1
A-I
Sugar
Lipid chains
F
E
D
C
B
A
4.0
3.0
2.0
1.0 ppm
A-II
FP7
DMSO
5.0 4.0 3.0 2.0 1.0 ppm
-9.0 9.5 10.0 10.5
F1 [log(m2/s)]
A-III
FP7 micelle
200 nm
AMP1
AMP1
FP7 micelle
200 nm
200 nm
AMP6
B-I
Sugar
Lipid chains
F
E
D
C
B
A
4.0
3.0
2.0
1.0 ppm
B-II
FP7
DMSO
5.0 4.0 3.0 2.0 1.0 ppm
-9.0 9.5 10.0 10.5
F1 [log(m2/s)]
B- III
FP7 micelle
200 nm
AMP6
FP7 micelle
200 nm
FP7 micelle
200 nm

Figure 7. A-I) ^{1}H-NMR titration of FP7 with AMP1. A: FP7 alone (500 μM); B: + 10 μM AMP1; C: + 30 μM AMP1; D: + 50 μM AMP1; E: + 90 μM AMP1; F: + 170 μM AMP1. **A-II)** DOSY spectrum. Black: FP7 (500 μM) Red: FP7 (500 μM) with AMP1 (30 μM). **A-III)** Transmission Electron Microscopy - Cryo-TEM of FP7 alone (2.5 mg/mL) and after addition of AMP1 (80 μM), with a nominal magnification of 30000X (0.36 nm/pixel). **B-I)** ^{1}H-NMR titration of FP7 with AMP6. A: FP7 alone (500 μM); B: + 10 μM AMP6 ; C: + 20 μM AMP6; D: + 30 μM AMP6; E: + 50 μM AMP6; F: + 90 μM AMP6. **B-II)** DOSY spectrum. Black: FP7 (500 μM) alone; Red: FP7 (500 μM) with AMP6 (10 μM) Green: FP7 (500 μM) with AMP6 (50 μM). **B-III)** Transmission Electron Microscopy - Cryo-TEM of FP7 (2.5 mg/mL) and after addition of AMP6 (400 μM), nominal magnification of 30000 X (0.36 nm/pixel). The samples have 10% DMSO in PBS 100 mM pH=5.5. Cryo-TEM after addition of AMPs shown in two figures to demonstrate that the same structures are present in different grid locations.

Indeed, the DOSY experiment of FP7 in the presence of AMPs (Figure 7 A-II for AMP1 and B-II for AMP6) showed clear perturbations of the diffusion coefficient measured for FP7 alone. In case of AMP1, for substoichiometric ratios of the peptide ([AMP1]/[FP7]=0.06), the decrease in the diffusion coefficient of FP7 is evident (Table 3). Also AMP6 causes a decrease in the diffusion coefficient of FP7, although the observed perturbation is smaller compared to that in the presence of AMP1 peptide (Table 3).

Table 3 Diffusion coefficient values estimated for FP7 (500 μM) from DOSY NMR experiments.

Compounds	**D/m^{2}s^{-1}**
FP7	6.31 x 10^{-11}
[**AMP 1**]/[FP7]=0.06	3.16 x 10^{-11}
[**AMP 6**]/[FP7]=0.02	5.01 x 10^{-11}
[**AMP 6**]/[FP7]=0.1	3.98 x 10^{-11}

This effect could be, in principle, due to changes either in the size or shape (see Figures S7-S8 in Supp. Info.) of the lipid. Thus, we used TEM with negative staining analysis (see Figure S10 and S14 in Supp. Info.) and cryo-microscopy (Figure 7 A-III and B-III) to obtain the required morphological information. It was possible to observe that the presence of AMP1 induced formation of aggregates between different FP7 micelles, thus supporting the change in size. The peptide is linking various FP7 micelles, displaying peanut-shaped structures. This suggests the presence of fusion events (indicated with the red circle in Figure 4 A-III and Figure S10 in Supporting Information). In contrast, the TEM analysis of FP7 in the presence of AMP6 showed a dramatic change in the shape, from spheres to cylinders. Long entangled cylindrical micelles are now displayed in the cryo-TEM image (Figure 7 B-III) and in the negative staining analysis (see Figure S14-Right in Supporting Information).

3. EXPERIMENTAL SECTION

3.1 Peptides and biochemical

The six AMPs in Table 1 were produced in high purity (>95% by analytical HPLC) by Fmoc solid phase synthesis methods, as reported (Table 1). The HPLC purified materials had the expected composition as determined by electrospray or MALDI-TOF mass spectrometry. E. coli LPS (O55:B5) and PHA-P Lectin (Phaseolus vulgaris) were supplied from Sigma-Aldrich. Polymyxin B was purchased from InvivoGen.

3.2 Cell Tests

HEK-Blue™ hTLR4 cells activation assay.

HEK-Blue™ hTLR4 cells (*InvivoGen*) are designed for studying the stimulation of human TLR4 (hTLR4) by monitoring the activation of NF-κB and AP-1. HEK-Blue™ hTLR4 cells were obtained by the co-transfection of hTLR4 gene, MD-2/CD14 co-receptor genes and a secreted embryonic alkaline phosphatase (SEAP) reporter gene into HEK293 cells. The SEAP reporter gene is placed under the control of an IL-12 p40 minimal promoter fused to five NF-κB and AP-1 binding sites. Stimulation with a TLR4 ligand activates NF-κB and AP-1 inducing the production of SEAP. Levels of SEAP can be easily determined with HEK-Blue assay. HEK-Blue hTLR4 cells were cultured in Complete Medium supplemented with 100 µg/mL Normocin and 1X HEK-Blue™ Selection (*InvivoGen*). HEK-Blue™ hTLR4 cells were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, antibiotics and 1× HEK-Blue™ Selection. Cells were detached using a cell scraper, counted and seeded in a 96-well multiwell plate at a density of 4×10^4 cells per well. After overnight incubation (37 °C, 5% CO_2, 95% humidity), supernatants were replaced with new medium supplemented by the compound to be tested dissolved in water or DMSO−Ethanol (1:1). After 30 minutes of pre-incubation, cells were stimulated with 100 ng/mL LPS from *E. coli* O55:B5 (Sigma-Aldrich) or 5 µg/mL PHA-P and incubated overnight. The SEAP-containing supernatants were collected and incubated with paranitrophenylphosphate (pNPP) for 2−4 h in the dark at room temperature. The wells optical density was determined using a microplate reader set to 405 nm. The results were normalized with positive control (LPS alone) and expressed as the mean of percentage ± SEM of at least three independent experiments.

Human peripheral blood mononuclear cells (hPBMCs)

PBMCs were isolated by density gradient centrifugation (Lympholyte®-H; Cedarlane Lab) from buffy-coats (Agreement between Ospedale Niguarda Cà Granda - Università degli Studi di Milano-Bicocca for supply of buffy-coats for research use). Briefly, buffy-coats were diluted 1:1 with Phosphate Buffer Saline (PBS), and layered on Lympholyte®-H for density gradient centrifugation according to the manufacturer's instructions. PBMCs were harvested from the interface and washed in PBS. The isolated cells were counted, checked for viability using 0.1% trypan blue and resuspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 U/mL). Cells were plated in 96-well U-bottom multiwell culture plates (Euroclone), pre-incubated with increasing concentrations of FP7 and FP7/AMP6 mix (0.1, 1, 5, 10 µM) and stimulated with smooth LPS (100 ng/mL) after 30 minutes. Each patient who took part in the study gave written informed consent, and the study protocol was approved by the local Ethics Committees (Agreement between Ospedale Niguarda Cà Granda - Università degli Studi di Milano-Bicocca for supply of buffy-coats for research use). IL-1β levels were measured as read-out of LPS-triggered TLR4 activation. Supernatants were collected after 18 hours upon LPS stimulation in presence or absence of the treatment and IL-1β levels were quantified using a sensitive enzyme-linked immunosorbent assay (ELISA) (R&D Systems; #DY210-05, Minneapolis, MN). The optical density of each well was determined using a microplate reader set to 450 nm (wavelength correction 570 nm). All graphs were representative data from at least three independent experiments.

MTT Cell Viability Assay

HEK-Blue™ hTLR4 cells were grown in DMEM supplemented with 10% FBS, 2 mM glutamine and antibiotics. Cells were seeded in 100 µL of DMEM without Phenol Red at a density of 4×10^4 cells per well and incubated overnight (37 °C, 5% CO_2, 95% humidity). Cells were treated with the higher dose of compound used in the previous experiments and incubated overnight. MTT solution (5 mg/mL in PBS) was added to each well and after 3 hours incubation, HCl 0.1 N in 2-propanol solution was used to dissolve formazan crystals. Formazan concentration was determined by measuring the absorbance at 570 nm. The results were normalized with untreated control (PBS) and expressed as the mean of percentage ± SEM of three independent experiments.

Statistical Analysis

Experimental data were normalized and expressed as means ± standard error of measurement (SEM). The data shown are the average of at least three independent experiments, each in technical triplicate. Statistical significance was evaluated using Student's *t* test.

3.3 NMR experiments

All NMR experiments were recorded on a Bruker Avance III 800 MHz spectrometer equipped with a TCI cryoprobe and Bruker Avance III 600 MHz spectrometer equipped with a TBI probe. The ^{1}H NMR resonances of the peptides (AMP 1 and AMP 6) were characterized through 2D-TOCSY (75 ms mixing time) and 2D-NOESY experiments (300 ms mixing time). The concentration of the compounds was set to 500 µM (AMP 6) and 300 µM (AMP 1) in perdeuterated PBS 100 µM in H_2O/D_2O 90:10 with 10% DMSO,

uncorrected pH meter reading 5.5. The peptide characterization was accomplished either at 293 K. The resonance of 2,2,3,3-tetradeutero-3-trimethylsilylpropionic acid (TSP) was used as a chemical shift reference in the ^{1}H NMR experiments (δ TSP = 0 ppm). Peak lists for the 2D-TOCSY and 2D-NOESY spectra were generated by interactive peak picking using the CARA software (Keller, R. Computer-aided Resonance Assignment Tutorial CARA; Cantina Verlag: Goldau, Switzerland, 2004).

The DOSY spectra of FP7 were recorded at 310 K with the tdDOSYccbp.2D pulse sequence by acquisition of 256 scans, with a diffusion time of 300 ms, a gradient length of 2 ms, and a gradient ramp from 5% to 95 % in 16 linear steps. Additions of the peptides to the solution were then performed and new DOSY spectra recorded up to a molar ratio of [AMP 1]/[FP7]=0.06 and [AMP 6]/[FP7]=0.1.

The DOSY spectra of the isolated AMP 1 and AMP 6 peptides as blank were recorded at 310 K with the tdDOSYccbp.2D pulse sequence by acquisition of 128 scans, with a diffusion time of 250 ms, a gradient length of 1.5 ms, and a gradient ramp from 5 % to 95 % in 16 linear steps. Additions of the FP7 to the solution were then performed and new DOSY spectra recorded up to a molar ratio of [FP7]/[AMP 1]=0.667 and [FP7]/[AMP 6]=0.667. FP7 samples were prepared by diluting the stock solution of FP7 (50 mM in DMSO) with the PBS buffer 100 mM pH=5.5, with a final 10 % DMSO ratio. Peptide samples were prepared by dissolving the solid molecules in DMSO (20 mM stock solution).

3.4 Transmission Electron Microscopy

All samples were prepared with 90 % of H_2O and 10 % of DMSO. Negative staining samples were applied to glow-discharged carbon-coated copper grids

and stained with 2 % (w/v) NANOVAN. Digital micrographs were taken at room temperature in low dose mode radiation on a Jeol transmission electron microscope operated at 100 kV and equipped with an orius camera. For cryo-microscopy study the samples were vitrified on quantifoil 2/2 grids, using vitrobot (FEI) and were analyzed at nitrogen liquid temperature with a TEM operated at 200 kV in low dose conditions. The samples were applied to glow-discharged carbon-coated copper grids and stained with 2 % (w/v) NANOVAN. Micrographs were taken at low radiation dose on a Jeol JEM-2200 FS transmission electron microscope operated at 200 kV and equipped with an UltraScan4000 CCD camera.

4. SUPPORTING INFORMATION

To see Supporting information that regards:

- NMR and TEM experiments

CLICK HERE

or open the following link with a browser:

(https://drive.google.com/open?id=1SjVNUChWDzOkjje82KCeWkTXgyKXZeNP)

CHAPTER III

TLR4 synthetic antagonist FP7 inhibits LPS-induce production of pro-inflammatory cytokines in mononuclear cells of IBD patients and shows anti-Inflammatory effects in dextran-sulfate-sodium (DSS)-induced colitis model.

Fabio Alessandro Facchini [a], Davide Di Fusco [b], Simona Barresi [a], Lenny Zaffaroni [a], Alberto Minotti [a], Roman Jerala[c], Francesca Granucci [a], Giovanni Monteleone [b], Francesco Peri [a] and Ivan Monteleone [b]

[a] Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza, 2; 20126 Milano (Italy).

[b] Department of Systems Medicine, University of Rome "Tor Vergata", Rome, Italy.

[c] Department of Biotechnology, National Institute of Chemistry and EN-FIST Center of Excellence, Hajdrihova 19 SI-1001 Ljubljana, Slovenia

Paper in Preparation

Journal: *Pharmacological Research or Mucosal Immunology*

1. INTRODUCTION

Inflammatory bowel diseases (IBDs), include ulcerative colitis (UC), Crohn's disease (CD), and indeterminate colitis, and indicates a group of intestinal debilitating disorders that cause prolonged inflammation of the digestive tract.[213] Although the causes of these disorders are still unknown, there is evidence that IBD pathogenesis is closely associated with multiple factors including environmental changes, genetic background, gut dysbiosis and host immunity.[213] Furthermore studies conducted in mouse models of colitis revealed that the persistent inflammatory condition in IBD results from an abnormal and out of control immune response against gut bacterial flora,[211, 212] which is not offset by counter-regulatory mechanisms.[218]

Human gastrointestinal tract is inhabited by a large community of bacteria, commonly named "gut microbiota".[2] Between the microbiota and the host exists a symbiotic mutualism in which both partners have benefits, that is the result of at least half a billion years of co-evolution.[238] In order to maintain this beneficial homeostasis, the host adopted multiple mechanisms to be tolerant towards commensals and to minimize potentially dangerous immune responses.[244, 245] Immune cells that inhabit the gut mucosa perform host immune-surveillance without inducing any inflammatory response, which could be fatal in an environment with myriads of bacteria.[124, 245, 270, 271] Mucosal macrophages, for instance, turn out to be inflammation anergic compared to circulating monocytes, showing a certain tolerance towards many bacterial antigens, also called pathogen-associated molecular patterns (PAMPs), including the lipopolysaccharide (LPS) of Gram-negative bacteria. The anergy of mucosal macrophages seems to be related to the potent down-regulation of several innate immunity molecules, including the receptors

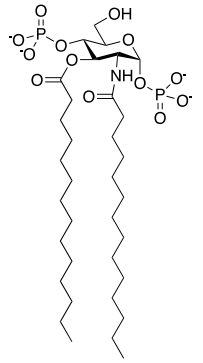
responsible for PAMPs detection, named PAMPs-recognition receptors (PRRs), and multiple signalling effectors activated by PRRs to induce the pro-inflammatory response.[124, 245, 270, 272-277] When circulating monocytes enter the healthy intestinal mucosa to replace senescent and apoptotic macrophages, the gut environment induces them to change their gene expression profile in order to become tolerant to the microbiota.[245, 275, 278] In IBD the delicate symbiosis between microbiota and host is compromised leading to a prolonged and persistent inflammatory condition that cause intestinal epithelium injury and reduced healing.[244] On one hand immune cells of IBD patients are less tolerant towards the microbiota, triggering excessive immune responses towards commensals. Indeed in IBD patients the expression and activity of many PRRs, including toll-like receptors (TLRs) is dysregulated,[94, 248, 249] leading to the activation of downstream signals that cause inflammation.[244] Moreover, inflammation promotes the rapid replacement of inflammation anergic gut resident macrophages with new pro-inflammatory monocytes coming from the circulating blood, further aggravating the situation.[126, 279] On the other hand the alteration of commensals-host symbiosis can set up optimal conditions for the spreading of harmful bacterial populations, normally present at low levels in the healthy gut, which cause an extensive host immune response.[238]

In this scenario one possible strategy to ameliorate IBD inflammation could be to block the abnormal pro-inflammatory activity of mucosal immune cells by reducing their ability to excessively respond to microbial antigens. Considering the key role played by TLRs in PAMPs recognition and in the initiation of the inflammatory response, the modulation of TLRs activity may represents a promising approach to reduce IBD inflammation. Among TLRs, TLR4 is the host

sensor of LPS (or endotoxin), one of the most immunogenic PAMPs contained in the outer membrane of Gram-negative bacteria.[18] LPS is detected by TLR4 via a complex mechanism that involves different LPS-binding proteins, which sequentially bind the endotoxin to activate the signalling. LPS binding protein (LBP) extracts monomeric LPS from endotoxin aggregates and transferred it to cluster of differentiation 14 (CD14) co-receptor;[39-41] CD14 presents monomeric LPS to TLR4 ectodomain, which binds endotoxin through an adaptor protein named myeloid differentiation factor 2 (MD-2).[38, 39, 41, 42] LPS recognition triggers TLR4/MD-2 dimerization leading to the initiation of the intracellular pro-inflammatory signaling cascade (MyD88-dependent pathway). Intracellular signalling promotes the activation of mitogen-activated protein kinases (MAPKs) and of I kappa kinases (IKKs), which induce the activation of transcription factors AP1 and of NF-κB, respectively.[49, 50] The nuclear translocation of these transcription factors leads to the production and secretion of multiple pro-inflammatory mediators of the innate immunity, including cytokines TNFα, IL-1β and IL-6. Concomitant with TLR4 signalling from the plasma membrane, LPS binding to CD14 also promotes TLR4 endocytosis,[15] leading to the recruitment of signaling adaptor TRIF (TRIF-dependent pathway), and to the subsequent expression of type I IFNs and IFN-stimulated genes (ISGs).[52]

Considering that in IBD-susceptible hosts aberrant TLR4 signalling may contribute to destructive host immune responses and to maintain inflammation,[294] the inhibition of TLR4 signalling may be beneficial. Our research group developed FP7, a synthetic TLR4 ligand able to potently[207] and selectively[210] inhibit TLR4 activation. FP7 is a diacylated, di-phosphorylated monosaccharidic small-molecule (Figure 1) that mimic a part of the active

portion of LPS, the lipid A. We present here a pre-clinical study in which FP7 is evaluated for its anti-inflammatory property *in vitro* and in an experimental model of inflammatory bowel disease (IBD) *in vivo*.



FP7

Figure 1. Molecular formula of **FP7**

2. RESULTS

2.1 FP7 reduces LPS-induced pro-inflammatory cytokines production in PBMCs

As stated in the introductory section, circulating monocytes are normally recruited to replace senescent and apoptotic macrophages in the gastrointestinal tract mucosa. When they localize in the lamina propria to become resident macrophages, the expression of CD14 is greatly decreased.[124] This mechanism reduces LPS sensitivity allowing intestinal macrophages to be more tolerant to endotoxin.[126] In inflamed intestine, however, the rapid and continuous influx of blood monocytes results in an abundant accumulation of $CD14^+$ LPS-sensitive macrophages in the lamina propria.[126] This can led to the persisting production of pro-inflammatory cytokines and other inflammatory

factors produced in increased amounts in IBD tissue.[126] Consistent with this idea, we investigated the activity of FP7 on LPS-stimulated peripheral blood mononuclear cells (PBMCs), evaluating whether FP7 is able to reduce the production of the main NF-κB-dependent pro-inflammatory cytokines. PBMCs were pre-incubated with two concentrations (1 and 10 µM) of FP7 and stimulated with LPS after 30 minutes. TNFα, IL-1β and IL-6 expression and production were evaluated via real-time qPCR and ELISA assay respectively. We found that the higher concentration of FP7 (10 µM) was able to strongly reduce the expression and production of all the pro-inflammatory cytokines monitored, confirming the anti-inflammatory property of the compound (Fig. 2A and B). Therefore we decide to perform the next experiments using FP7 higher concentration of 10 µM.

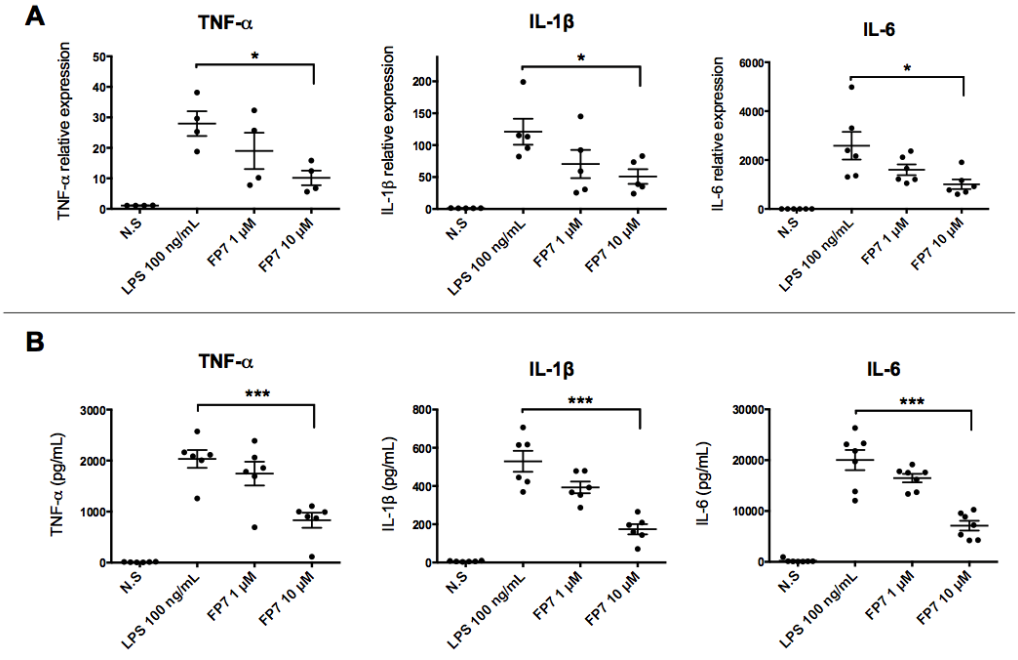


Figure 2. Inhibitory effect of FP7 on the expression and production of pro-inflammatory cytokines in LPS-stimulated PBMCs. PBMCs isolated from whole blood were preincubated with two concentration of FP7 (1 and 10 µM) for 10 min and then

stimulated with LPS (100 ng/mL). **A)** TNFα, IL-1β and IL-6 mRNa relative expression was measured by real-time PCR after 4 hours upon LPS exposure. **B)** TNFα, IL-1β and IL-6 cytokine levels were quantified after one night's incubation by ELISA assay. Each point in the graph represents the value of a single experiment and horizontal bars indicate the median values ± SEM (*P<0.05; ***P<0.001).

2.2 FP7 decreases TLR4 signaling reducing NF-κB and MAPKs activation

In order to investigate whether the reduced pro-inflammatory cytokines production was due to a minor TLR4 signaling, we evaluated the activation of the main MyD88-dependent pathway effectors: NF-κB and MAPKs. We first investigated the activation of transcription factors NF-κB. In the absence of stimulus NF-κB is kept inactivated in the cytosol through the interaction with the inhibitory protein IκBα. Upon LPS stimulation, IKK protein kinases phosphorylate IκBα causing its rapid degradation by the 26S proteasome. This event allows NF-κB to be also phosphorylated and to translocate into the nucleus and activate gene transcription. Therefore we treated PBMCs with LPS (10 ng/mL), FP7 alone (10 μM) and FP7+LPS and we monitored over time IκBα degradation and NF-κB phosphorylation. As expected, stimulation with LPS caused the rapid degradation of IκBα inhibitor leading to NF-κB phosphorylation, which is maintain up to 30 minutes after the stimulus (Figure 3A). Interestingly, the presence of FP7 reduced the temporal window in which NF-κB is active, delaying IκBα degradation after LPS exposure and favoring the reappearing of the inhibitor after 30 minutes (Figure 3A). Next, we evaluated the activation of AP-1 transcription factor, monitoring over time phosphorylation of the MAPKs p38, ERK1/2 and JNK. As well as for NF-κB, LPS addition strongly caused the phosphorylation of all three MAPKs monitored. In particular p38 and JNK phosphorylation occurred after only 5 minutes upon

stimulation. The presence of FP7 is able to significantly reduce the phosphorylation, thus the activation, of all MAPKs analyzed (Figure 3B). These collective data reveal that FP7 dampened LPS-induced pro-inflammatory cytokines production reducing the activation of the Myd88-dependent pathway.

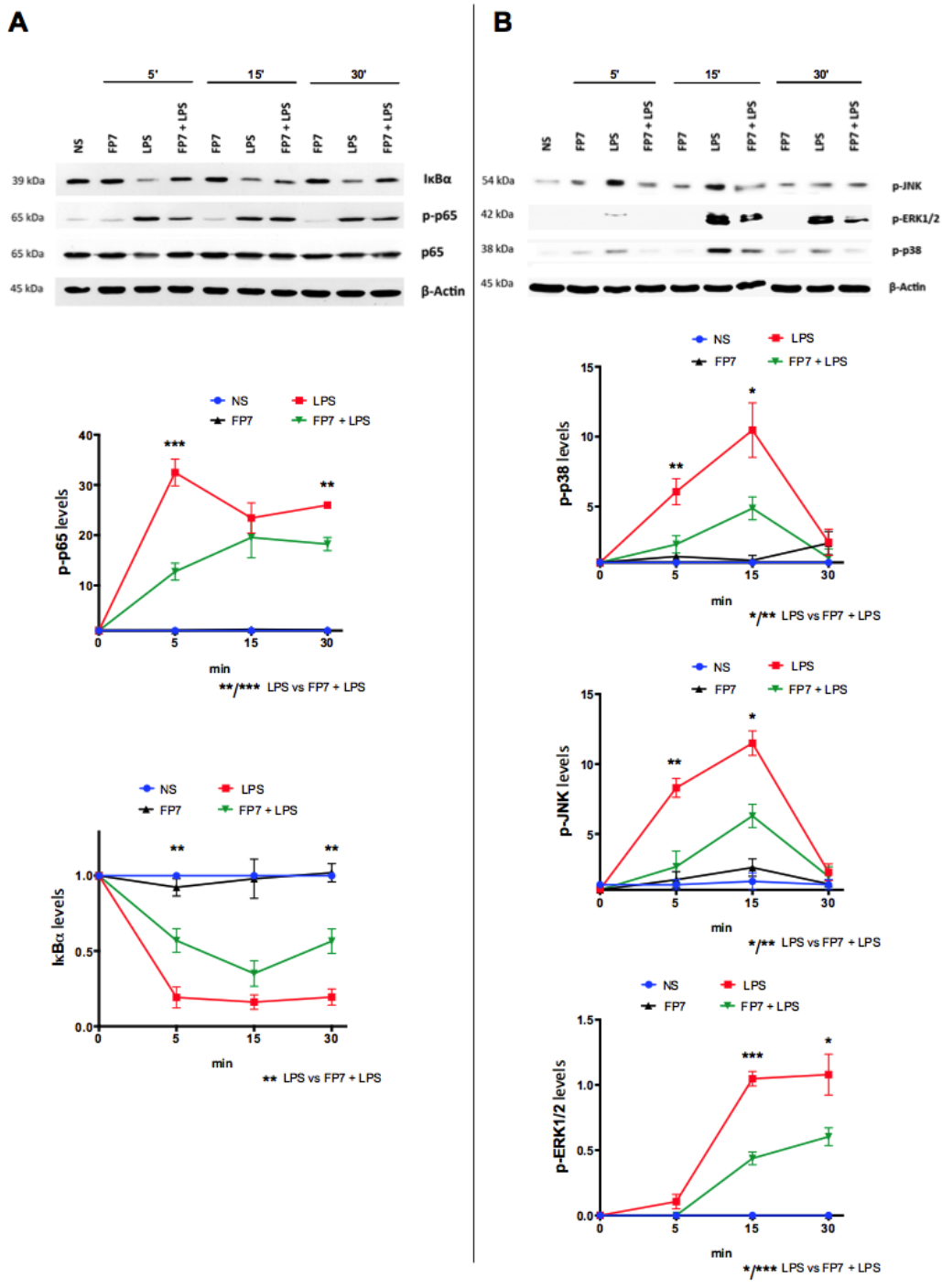


Figure 3. Effect of FP7 on LPS-induced NF-κB and MAPKs activation. PBMCs isolated from whole blood were treated with LPS (100 ng/mL), FP7 (10 µM) and LPS + FP7 and

collected after the indicated times. Cells were lysed and crude lysate were separated by SDS-page. The levels of IκBα, phospho-NF-κB and actin **(A)** and phospho-p38, phospho-ERK1/2, phospho-JNK and actin **(B)** were detected by immunoblot. Data are normalized to β-actin and represent the mean ± SEM of at least three independent experiments (*P<0.05; **P<0.01; ***P<0.001).

2.3 FP7 competes with LPS for the binding to MD-2 receptor

The observation that FP7 reduces Myd88-dependent pathway activation made us speculate that the compound really acts in the extracellular compartment interfering with the ligand-receptor recognition. In order to clarify whether compound FP7 interacts with the endotoxin receptor complex, competing with LPS for MD-2 binding, we performed an optimized ELISA-like cell-free binding assay.[327] MD-2 binding was assessed with the 9B4 antibody, which detects the co-receptor only when the hydrophobic cavity of MD-2 that accommodates LPS is not occupied. Because this antibody recognizes the "empty" MD-2, loss of antibody-linked enzyme activity means that MD-2 cavity has been occupied. Consistent with this idea, recombinant human (rh)MD-2 was immobilized on a microtiter plate and increasing concentrations of LPS or FP7 were added. Similar to what previously reported,[327] the presence of LPS decreased 9B4 antibody binding to rhMD-2 (Fig. 4.A). Interestingly, the decreased 9B4 binding was also monitored when mimetic FP7 was incubated with rhMD-2 (90% and 95% of binding decreased using FP7 at 10 and 20 μM respectively), suggesting that the compound really interact with MD-2 lipophilic cavity (Fig. 4.A). Furthermore, to verify whether FP7 competes with LPS for MD-2 binding, we performed a biotin-LPS displacement assay. Immobilized rhMD-2 was first pre-incubated with biotin-LPS and then incubated with increasing concentration of FP7. Although with higher

concentrations than those used on cells, FP7 was able to displace biotinylated LPS from the MD-2 hydrophobic pocket in a dose-dependent manner (highest displacement of 60-65% using FP7 at 160 µM) (Fig 4.B). Collectively, these data reveal that compound FP7 is able to bind MD-2 co-receptor and that its inhibitory activity is probably due to the capacity to compete with LPS for MD-2 binding.

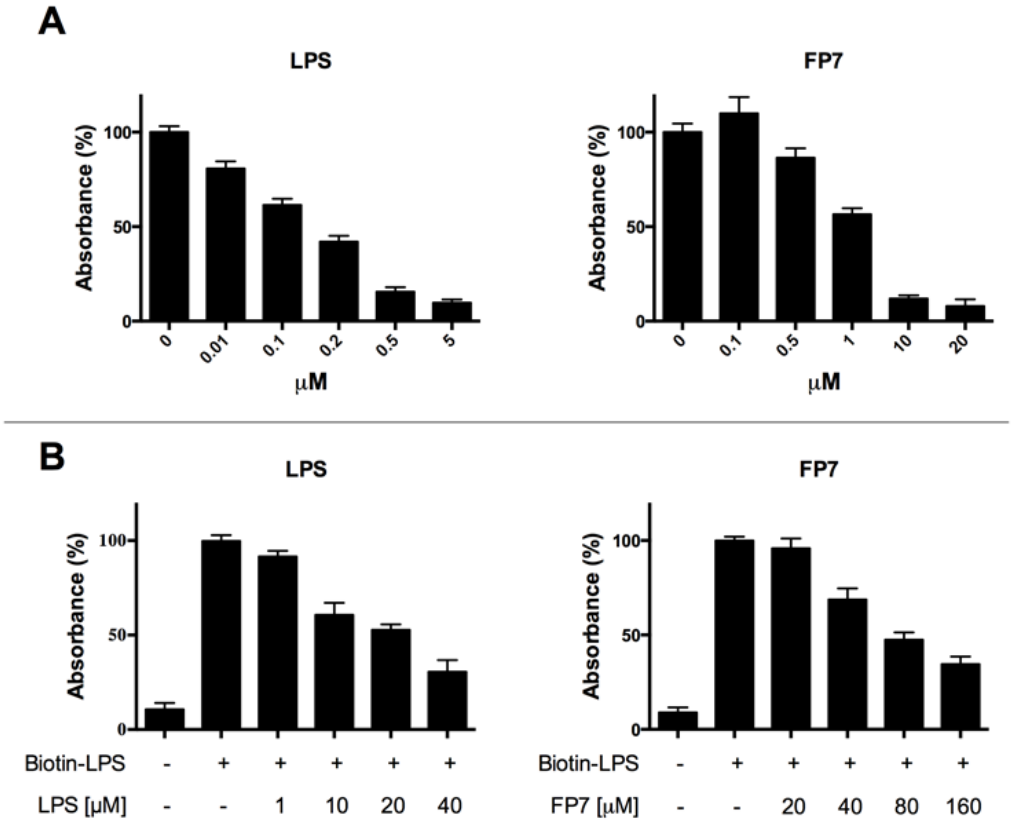


Figure 3. FP7 competes with LPS for the binding to MD-2 receptor. A) rhMD-2 was immobilized on a microtiter plate and incubated with the indicated concentrations of LPS (left) and FP7 (right). Binding was determined by the ELISA test in competition with 9B4 anti-human MD-2 monoclonal antibody as described in experimental procedures. **B)** Immobilized rhMD-2 was first incubated with biotin-LPS and then the indicated concentrations of LPS (left)

and FP7 (right) were added. FP7 capacity to displace biotin-LPS was determined by ELISA test as described in experimental procedures.

2.4 FP7 reduces LPS-induced CD14 and TLR4 internalization in PBMCs

The results obtained with ELISA-like binding assays strongly suggest that the mechanism of action of FP7 is based on the capacity of the molecule to interact with MD-2, hindering the LPS recognition process. To verify whether FP7 acts in the same way also on human cells, we monitored a cell surface event that occurs when LPS binds first CD14 and then TLR/MD-2 receptors. As described in paragraph 1.2.2, concomitant with the initiation of Myd88-dependent pathway from the plasma membrane, LPS also triggers CD14 and TLR4/MD-2 endocytosis.[52] Receptors internalization only occurs when LPS is bound by CD14 co-receptor and subsequently transferred to TLR4/MD-2 complex. We reasoned that whether FP7 interferes with LPS detection process, then receptors internalization would be reduced in the presence of the molecule. Consistent with this idea, we monitor via flow cytometry the over time amount of surface TLR4 and CD14 receptors upon LPS exposure in the presence or absence of FP7. LPS stimulation causes a decrease of surface CD14 confirming that the co-receptor undergoes endocytosis, however the presence of FP7 prevents this process to occur (Figure 5, left panel). We observed a similar trend when we monitored the amount of TLR4 on cell surface. LPS promotes TLR4 endocytosis within 30 minutes and the receptor re-appears at the plasma membrane at late time point (90 minutes) (Figure 5, right panel). Also in this case the presence of FP7 is able to abolish the process (Figure 5, right panel). The results obtained reveal that the anti-inflammatory effect of FP7 is due to the ability of the molecule to interfere with the LPS-detection process, by competing with endotoxin for receptor binding.

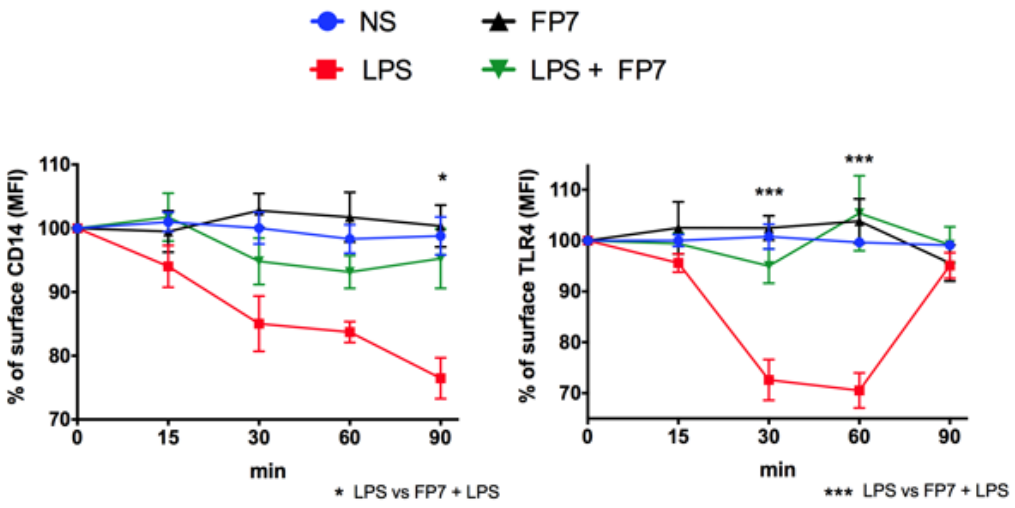


Figure 5. Effect of FP7 on LPS-induced CD14 and TLR4 internalization. PBMCs isolated from whole blood were treated with LPS (1 µg/mL), FP7 (10 µM) and LPS + FP7 and collected after the indicated times. Surface level of CD14 (left) and TLR4 (right) was determined by flow cytometry. Data represent the mean ± SEM of at least three independent experiments (*P<0.05; ***P<0.001).

2.5 FP7 reduces hyper-responsiveness to LPS in LPMCs of patients with IBD

As describe in the introduction, in patients with IBD a lower tolerance toward bacterial antigens, including LPS, is observed. Indeed, lamina propria mononuclear cells (LPMCs) isolated from the inflamed gut of IBD patients, respond to LPS exposure triggering the production and release of pro-inflammatory cytokines. Therefore, in order to investigate whether FP7 is able to reduce the capacity of the inflamed mucosa to respond to LPS challenge, we started to test the compound on LPMCs isolated from patients with UC (two patients). IBD LPMCs were stimulated with LPS in presence of two concentrations of FP7 (1 and 10 µM) and the expression and production of the main pro-inflammatory mediators was monitored. As expected,[126] LPS is able

to induce the expression and production of TNFα, IL-1β and IL-6 in IBD LPMCs (Figure 6 A and B). Interestingly, the presence of the higher dose of FP7 is able to reduce the expression and production of the same cytokines (Figure 6 A and B). Although a higher number of experiments on biopsy of UC patients is needed, this preliminary result suggests that the compound can carry out its anti-inflammatory activity on the inflamed mucosa.

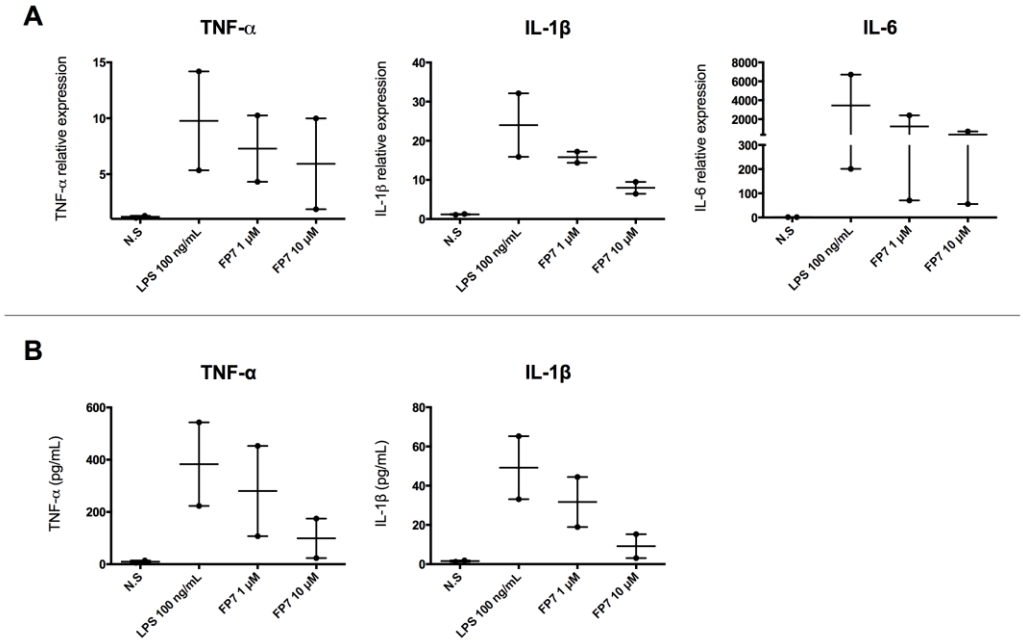


Figure 6. Inhibitory effect of FP7 on the expression and production of pro-inflammatory cytokines in LPS-stimulated LPMCs. LPMCs isolated from biopsy of patients with UC were preincubated with two concentration of FP7 (1 and 10 µM) for 10 min and then stimulated with LPS (100 ng/mL). **A)** TNFα, IL-1β and IL-6 mRNa relative expression was measured by real-time PCR after 4 hours upon LPS exposure. **B)** TNFα, IL-1β and IL-6 cytokine levels were quantified after one night's incubation by ELISA assay.

2.6 FP7 reduced inflammation in a mouse model of colitis

We sought to evaluate whether FP7 anti-inflammatory effect is also observed in vivo. For these experiments, we planned to use a dextran-sulfate-sodium (DSS)-induced model, in which colitis is induced by administering DSS (3.25%) to the mice. After four days of DSS administration, we started to treat mice with FP7 (250 μg/kg/day) or vehicle (Control). The compound was intraperitoneally injected to the mice for four days, following normal administration procedures and the mice body weight was recorded every day. The dose per day of FP7 was chosen upon a preliminary DSS experiment performed with two doses of the compound: 250 and 2500 μg/kg/day. Considering that the higher dose showed some adverse effects, we selected 250 μg/kg/day for the subsequent experiments.

On day nine the two groups of animals were sacrificed and the distal portion of the colon was analyzed for the histology and the level of pro-inflammatory cytokines (Figure 7A). Histological analysis showed that DSS caused extensive bowel damage with the epithelial layer architecture greatly compromised and the presence of inflammatory infiltrate in the lamina propria (Figure 7B). However the treatment with compound FP7 reduces inflammation leading to significant amelioration of the histological framework (Figure 7B). Amelioration is also indicated by mice body weight monitoring, indeed, only animals treated with FP7 have shown a lower body weight decrease (Figure 7A). The anti-inflammatory effect of FP7 was also confirmed when the levels of pro-inflammatory cytokines were measured by real-time PCR and ELISA assay. In particular mice treated with FP7 showed lower amounts of TNFα, IL-1β and IL-6 compared with mice that did not receive any treatment (Figure 7C).

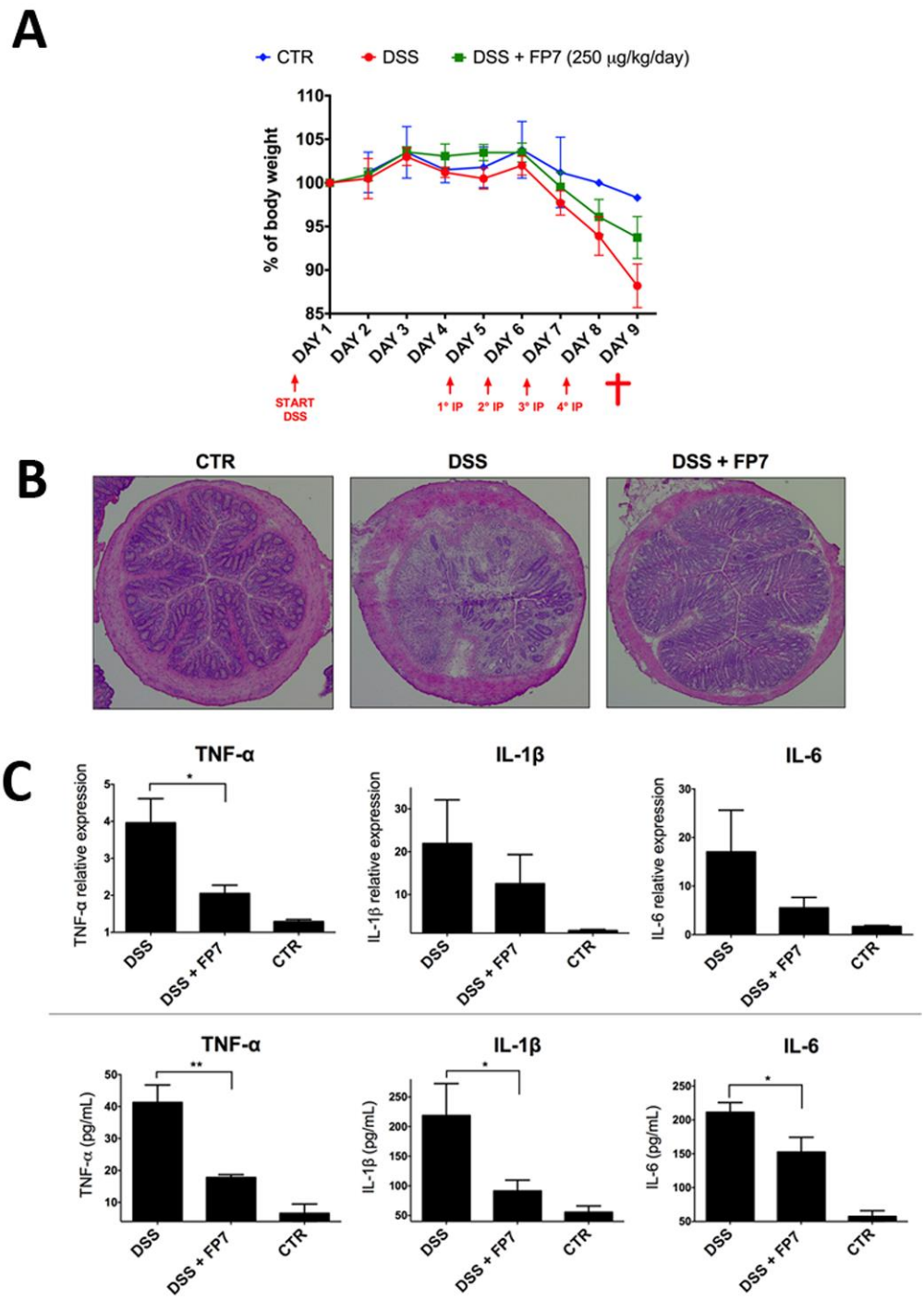


Figure 7. FP7 is therapeutic in mice with DSS-induced colitis. A) Mice received either regular drinking water (CTR) or dextran sulfate sodium (DSS) for 8 days. After 4 days

of DSS treatment, mice were treated with FP7 (250 μg/kg/day) (DSS + FP7) by intraperitoneal injection. Body weight was recorded daily and each point on the graph indicates cumulative mean ± SEM of 3 separate experiments. In each experiment, at least 4 mice per group were included. **B)** Mice were treated as above and killed at day 9. Representative H&E-stained colonic sections of CTR, DSS and DSS + FP7 treated mice. **C)** FP7 reduces TNFα, IL-1β and IL-6 mRNA expression and cytokine levels in the colon of DSS-treated mice. Cytokine RNA transcripts were determined by real-time PCR and normalized to β-actin, while protein levels were quantified by ELISA assay. (*P<0.05; **P<0.01) Data represent the mean ± SEM of at least three independent experiments.

3. EXPERIMENTAL SECTION

LPMCs and PBMCs Isolation and Culture

Lamina propria mononuclear cells (LPMCs) were isolated from biopsy specimens of patients with irritable bowel syndrome. Mucosal biopsy specimens were taken from involved areas of patients with active UC (median age, 35 y, range, 25–55 y). LPMCs were isolated by the dithiothreitol (DTT)–ethylenediaminetetraacetic acid (EDTA)–collagenase sequence as previously described in detail.[328] Briefly, the dissected intestinal mucosa was incubated at 37 °C with DTT-EDTA for 30 minutes to remove mucus and epithelial cells and then digested using Liberase™ and collagenase (2 mg/mL) (Sigma Chemical Co., St. Louis, MO). The medium containing the mononuclear cells was collected and centrifuged at 400g for 10 minutes. Cell suspension was filtered and washed twice in Hank's balanced salt solution, calcium and magnesium free (HBSS – CMF, Sigma). PBMCs were isolated by density gradient centrifugation (Lympholyte®-H; Cedarlane Lab) from patients with UC (informed consent) or from buffy-coats (Agreement between Ospedale Niguarda Cà Granda - Università degli Studi di Milano-Bicocca for supply of

buffy-coats for research use). Briefly, buffy-coats were diluted 1:1 with Phosphate Buffer Saline (PBS), and layered on Lympholyte®-H for density gradient centrifugation according to the manufacturer's instructions. PBMCs were harvested from the interface and washed in PBS. The isolated cells (LPMCs and PBMCs) were counted, checked for viability using 0.1% trypan blue and resuspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 U/mL). Cells were plated in 24 or 96-well U-bottom multiwell culture plates (Falcon Plastic), pre-incubated with two concentrations of compound FP7 (final concentrations 1 and 10 μM) and stimulated with smooth lipopolysaccharide (S-LPS; *Escherichia coli* 055:B5; Sigma; 100 ng/mL) after 30 minutes. Each patient who took part in the study gave written informed consent, and the study protocol was approved by the local Ethics Committees (Tor Vergata University Hospital, Rome, Italy).

RNA extraction, cDNA synthesis and Real-Time polymerase chain reaction.

Total RNA was extracted using QIAGEN's RNA Mini Kit (QIAGEN, Milan, Italy) according to the manufacturer's instruction. To remove genomic DNA, on-column digestion using RNase free DNase set (Qiagen) was performed. Reverse transcription was performed with 0.5 - 1 μg of total RNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific) and this was amplified using the following conditions: denaturation for 1 minute at 95°C; annealing for 30 seconds at 62°C for human TNF-α, 58°C for human interleukin (IL)-1β, 61°C for human interleukin (IL)-6 and 60°C for human β-actin; 30 seconds of extension at 72°C. Primer sequences were as follows: human TNF-α forward 5'-AGGCGGTGCTTGTTCCTCAG-3' reverse 5'-GGCTACAGGCTTGTCACTCG-3'; human IL-1β forward 5'-

AGAATGACCTGAGCACCTTC-3', reverse 5'-GCACATAAGCCTCGTTATCC-3'; human IL-6 forward 5'-CCACTCACCTCTTCAGAACG-3', reverse 5'-GCCTCTTTGCTGCTTTCACAC-3'; β-actin (forward 5'-AAGATGACCCAGATCATGTTTGAGACC-3', reverse 5'-AGCCAGTCCAGACGCAGGAT-3') was used as a housekeeping gene. Gene expression was calculated using the ΔΔCt algorithm. All graphs were representative data from at least three independent experiments.

Enzyme-Linked Immunosorbent Assay (ELISA)

TNF-α, IL-1β and IL-6 levels were measured in LPMC and PBMC supernatants after 18 hours of LPS stimulation in presence or absence of FP7 (1 and 10 μM) using a sensitive enzyme-linked immunosorbent assay (ELISA) (R&D Systems; #DY206-05, #DY210-05, #DY208-05, Minneapolis, MN). The optical density of each well was determined using a microplate reader set to 450 nm (wavelength correction 570 nm). All graphs were representative data from at least three independent experiments.

Western blot analysis

The effect of FP7 on LPS-dependent IκBα degradation, and on NF-κB and MAPKs phosphorylation was evaluated over time in PBMCs. PBMCs were seeded in 15 mL tubes at a concentration of $1x10^6$ cells/mL in presence or absence of FP7 (10 μM). Cells were then stimulated with LPS and collected after 5, 15 and 30 minutes. Cells were washed twice in ice-cold PBS and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 % NP-40, 0.1 % SDS, 0.5 % sodium deoxycholate) supplemented with Protease and Phosphatase Inhibitors (ROCHE). After centrifugation at 13.000 rpm for 30 min at 4 °C, the

supernatants were collected as whole cell protein samples. An equal amount of protein was separated on 10 % polyacrylamide gels and transferred on nitrocellulose membrane filters (VWR). Proteins were revealed by chemioluminescence (ECL, Amersham Biosciences AB) and detected on an X-ray film (Thermo Scientific, CL-XPosure™ Film, #34090). The following primary antibodies were used: anti-phospho NF-κB (Ser536) (93H1) Rabbit mAb (Cell Signaling #3033; diluted 1:1000), anti-NF-κB p65 (D14E12) XP® Rabbit mAb (Cell Signaling #8242; diluted 1:1000); anti-IκB-α (L35A5) Mouse mAb (Amino-terminal Antigen) (Cell Signaling #4814; diluted 1:1000); anti-phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb (Cell Signaling #4511; diluted 1:1000); anti-p38 MAPK Rabbit (Cell Signaling #9212; diluted 1:1000); anti-phospho-JNK (14.Thr 183/Tyr 185) Mouse mAb (Santa Cruz, sc-293136; diluted 1:500); anti-JNK (D-2) Mouse mAb(Santa Cruz, sc-7345; diluted 1:500); anti–phospho-p44/42 MAPK (Thr202/Tyr204) Rabbit Ab (Cell Signaling #9101), Anti-p44/42 MAPK (Erk1/2) Rabbit Ab (Cell Signaling #9102, diluted 1:1000), Anti-β-Actin (D6A8) Rabbit mAb (Cell Signaling Tech, Euroclone #BK8457; diluted 1:1000); Secondary antibodies used: anti-rabbit or anti-mouse IgG, HRP-linked secondary antibody (Cell Signaling #7074 and #7076, diluted 1:5000). Densitometric analysis were carried out using Image J.

Preparation and purification of recombinant hMD-2 from Pichia pastoris

hMD-2 was produced in *Pichia pastoris*, analyzed by SDS–PAGE and its biological activity tested on 293/hTLR4a cells. The coding sequence of mature hMD-2 was amplified by PCR (primers F-hMD2-Q19 CAGAAGCAGTATTGGGTCTGC and R-Spe-hMD2 TTTACTAGTATTTGAATTAGGTTGGT GTAGG) from a plasmid template and

ligated into the SnaBI/SpeI opened pPpT4AlphaS-His expression vector (under the control of AOX1 promoter), in frame with the N-terminal *S. cerevisiae* α-MF pre-pro leader sequence and the C-terminal 6xHis tag. The resulting recombinant plasmid pPpT4AlphaS-His was transformed into *E. coli* DH5α competent cells, and the positive recombinant plasmid which was confirmed by DNA sequencing, was linearized and transformed into *Pichia pastoris* GS115 by electroporation. MD-2 expressing transformant was selected and cultured in a 250 mL shake flask containing 10 mL of YPD liquid media at 28 °C for 24 h. 2L Flasks containing 250 mL of BMGY (1% glycerol) medium at 28 °C were inoculated with 1 mL of overnight inoculum. After being cultured for 24 h, cells were aseptically collected by centrifugation at room temperature 10 minutes at 5,000 rpm. BMGY medium was replaced with 250 mL of methanol-complex medium BMMY (1% methanol) to induce protein expression at 28 °C (250 rpm), adding 1% of methanol every 12 h. After 2 days of fermentation in BMMY, cells were removed by centrifugation 10 minutes at 5,000 rpm. Supernatant was supplemented with 2 mM $MgCl_2$ (Sigma), 100 mg/L of reduced glutathione (Sigma), and pH was adjusted to 7.5 with NaOH (Sigma). Precipitate was removed by centrifugation for 20 minutes at 1,900 g, followed by filtration using Stericup-GP 0.22 µm (Sigma). A 0.5 M solution of TRIS HCl pH 7.5, 1.5 M NaCl (Sigma) was added to the medium to a final concentration of 50 mM TRIS HCl, 150 mM NaCl. High Density Nickel resin (ABT) was added to the medium (30 mL every liter of medium) and incubated in batch at room temperature for 4 hours. High Density Nickel resin was washed several times with 50 mM TRIS HCl pH 7.5, 150 mM NaCl solution. hMD-2 was eluted with 0.5 M Imidazole (Sigma) in 2 mL fractions, which were analysed for protein concentration and by SDS-PAGE. Pooled fractions containing hMD-2 were

extensively dialysed against 50 mM TRIS, 150 mM NaCl, 0.5% Tween 20, pH 7.5 at 4°C and purified hMD-2 biological activity was tested on 293/hTLR4a cells.
For measuring the activity of recombinant expressed hMD-2, HEK 293 cells stably transfected with the human TLR4 gene (293/hTLR4a (Invivogen)) were used. Various dilutions of hMD-2 (stock concentration was 10 µM) were incubated with 100 ng/mL of LPS (Sigma) and then added to 293/hTLR4a cells. Supernatants were collected and IL-8 levels were evaluated by ELISA assay (IL-8 Cytosets™, Invitrogen).

Antibody-sandwich ELISA for the detection of compound-rhMD-2 binding

The method of antibody-sandwich ELISA for the detection of the binding of compounds to MD-2 was modified from a previous study.[327] A microtiter plate was coated overnight at 4 °C with 100 µL/well of 5 µg/mL of chicken polyclonal anti-hMD-2 antibodies, diluted in 50 mM Na_2CO_3 buffer, pH 9.6 and blocked with 1% BSA in PBS. After washing, 1 µM hMD-2 with tested compounds was added and incubated for 2 hours. 0.1 µg/mL mouse anti-hMD-2 MAb (9B4) and 0.1 µg/mL goat anti-mouse IgG conjugated with HRP in PBS were added, followed by detection at 420 nm after the addition of 100 µL of ABTS (Sigma). Chicken anti-hMD-2 polyclonal antibodies were prepared against recombinant hMD-2 by GenTel (Madison, WI, USA), monoclonal mouse anti-hMD-2 9B4 antibodies were from eBioscience (San Diego, CA, USA), and secondary goat anti-mouse IgG conjugated with horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

LPS displacement assay

The ability of the compounds to displace LPS from hMD-2 hydrophobic pocket

was determined by ELISA. A microtiter plate was coated overnight at 4 °C with 100 µL/well of 5 µg/mL of chicken polyclonal anti-hMD-2 antibodies, diluted in 50 mM Na_2CO_3 buffer, pH 9.6 and blocked with 1% BSA in PBS. After washing, 1 µM of hMD-2 with biotin-labeled LPS was added and incubated for 2 hours. After washing, the compounds were added at different concentration and incubated for 1.5 hours. After washing, 0.5 µg/mL HRP-conjugated streptavidin (Sigma) in PBS was added, followed by detection at 420 nm after the addition of 100 µL ABTS (Sigma). Chicken anti-hMD-2 polyclonal antibodies were prepared against recombinant hMD-2 by GenTel (Madison, WI, USA).

Flow Cytometry

Human PBMCs were resuspended in RPMI 1640 medium, supplemented with 10 % inactivated FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL), seeded in 96-well U-bottom multiwell plates in presence or absence of FP7 (10 µM) and stimulated with LPS (1 µg/mL). Cells were collected after 15, 30, 60 and 90 minutes, washed in 5% BSA FACS buffer and stained with the following antibodies: anti-CD45-APC-H7, anti-human CD14-Pacific Blue™ and anti-human CD284 (TLR4)-APC (BioLegend, San Diego, CA). Cells were washed onece and analyzed using a FACSVerse flow cytometer and FACSSuite software (BD Biosciences).

DSS-Induced Colitis

Eight-week-old female Balb/c mice received either regular drinking water (control) or 2.5-3.25% of dextran sulfate sodium (DSS) in drinking water for 7 days. After four days of DSS treatment, mice were intraperitoneal (IP) injected with 5 µg of compound FP7 (250 µg/kg/day) or the vehicle solution (PBS 0.25%

DMSO) for four days (one IP per day). Weight changes were recorded daily. At day 8 DSS administration was discontinued and at day 9 the mice were sacrificed. Tissues were collected for histology and cytokines quantification. The colitis histologic score was assigned as described elsewhere [329].

Statistical Analysis

All experimental results represent the means ± standard errors of the means (SEM) of at least three independent experiments. In real-time PCR and western blot experiments gene expression and protein amount were evaluated in relation to the housekeeping gene β-actin. H&E-stained colonic sections shown were representative data from at least three independent experiments. Means were compared by t tests (two groups) or one-way ANOVA (three or more groups). Tukey multiple comparisons testing following one-way ANOVA was performed to obtain adjusted P value.

4. DISCUSSION AND CONCLUSIONS

In the introduction the role of aberrant TLR4 signaling in several acute and chronic inflammatory pathologies was described. Considering that most of these pathologies still lack a specific and efficient pharmacological treatment, the development of new small molecules able to selectively modulate the activation of TLR4 has attracted increasing interest in a wide range of possible clinical settings. Two main strategies are used to modulate the TLR4 signalling. The first is based on molecules that bind endotoxin with high affinity thus neutralizing its toxic effect,[128-130] while the second involves the use of small-molecules that directly interact with the extracellular endotoxin receptor system, competing with LPS for CD14 and TLR4-MD-2 binding.[45, 131-134] The first approach aims to overcome the limitations of the standard clinical therapies used to counteract Gram-negative bacterial infection and their systemic complications. The bactericidal effect of antibiotics treatment may cause bacterial cell wall disruption leading to the release of pro-inflammatory LPS. Although therapeutic agents able to directly bind and neutralize LPS would be highly useful in the clinic, this therapeutic approach is limited to the treatment of endotoxin-related pathologies. On the contrary, the use of compounds that target TLR4/MD-2 and CD14 receptors can be extended to the treatment of TLR4-related inflammatory disorders caused by DAMPs. The majority of TLR4/MD-2 and CD14-targeting molecules are anionic amphiphiles that mimic lipid A (disaccharide) or lipid X (monosaccharide). However, some experimental results showed that also cationic amphiphiles are active as TLR4 modulators. In Chapter I of this thesis guanidine-calixarene cationic

amphiphiles were synthesized and evaluated for their capacity to inhibit TLR4 signal. Calixarenes with guanidinium groups on one rim and fatty acid chains on the other are defined “facial” amphiphiles in which the charged polar face and the hydrophobic apolar are spatially organized in two different regions or faces. Thanks to this structural feature, they can be potential modulators of the TLR4 activation through direct binding to MD-2, CD14 and LBP receptors. We designed the series of calixarenes **1**-**9** aiming to explore the possible direct binding to CD14 and MD-2 co-receptors. Docking studies demonstrated that compounds **2**-**4** and **1**-**9** are in principle able to form complexes with CD14 and TLR4/MD2 heterodimer (human and murine), respectively, independently from the relative disposition of the polar and apolar residues and from the nature of the charged groups. The lipophilic chains, linked at the lower (**1** and **2**) or at the upper (**3**-**5**) rim, resulted in all cases to bind to the CD14 or MD-2 hydrophobic pocket, while the charged heads established contacts with polar residues located in proximity of the entry of the pockets. Therefore, our computational studies provide plausible binding poses into the TLR4 co-receptors for the investigated compounds and this supports our hypothesis of a direct binding of calixarene derivatives to these proteins in competition with LPS. These findings thus open the possibility to explore calixarenes as a platform for the design of TLR4.MD-2 modulators. The activity of positively charged calixarenes was investigated on HEK cells expressing (h)TLR4/MD-2 and human and murine leukocytes. Cationic calixarenes **1**-**4** inhibited in a dose-dependent way LPS-stimulated TLR4 activation in both human and murine cells with potency in the low micromolar range. Paradoxically, negatively charged amphiphilic calixarenes, that should mimic better the anionic nature of lipid A (the natural MD-2 and CD14 ligand) were not active in

inhibiting the TLR4 signal. Compound **6**, with more polar ether chains on the lower rim instead of hydrocarbon chains turned out to be substantially inactive as TLR4 inhibitor in both cell types. Additionally, calculations of the logP values predicted high values for compounds **7**, **8** and **9** (calculated logP values above 15), pointing to a high lipophilicity, while compound **6** was predicted to be extremely hydrophilic (calculated logP value below zero, Figure S6, Supp. Info.). These unfavourable values for logP could be correlated with poor physical-chemical properties thus explaining the lack of activity in the cells assays. The very close IC_{50} values (Table 3.2) found for guanidinocalixarenes **2** and **3** suggest that the relative disposition of polar and hydrophobic residues with respect to the macrocyclic cavity does not have a significant impact on the inhibition activity of these ligands. Furthermore, by comparing compounds **3**-**5** it seems that an increasing lipophilicity results detrimental for the inhibition potency, even if the less amphiphilic derivative **6** is very poorly active with a IC_{50} two order of magnitude higher than that of **3**. It is worth noting that lead compounds **3** and **4** show antagonist activity on both human and murine TLR4. Indeed as described in paragraph 1.2.3, several TLR4 modulators that mimic lipid A have species-specific activity. The species-specific activity is generally attributed, among other factors, to structural dissimilarities in the shape of the hydrophobic binding pocket of (h) and (m)MD-2[330] and to the variations in the electrostatic potentials at the rim of the binding cavity of MD-2 and at the dimerization interface. The most significant example of this different behavior is the natural compound tetraacylated lipid IVa that, as described in the introduction, acts as an antagonist on human but as an agonist on murine TLR4.[330] However, several synthetic phospho-glycolipids with a monosaccharide scaffold also showed

agonist activity on murine and antagonist activity on human TLR4.[199] In order to understand whether the antagonist activity of calixarenes was due to their interaction with receptors or with LPS, we performed studies of the TLR4 activation with non-LPS ligands. We reasoned that if the inhibitory effect of calixarenes on TLR4 activation is due to a neutralizing effect on endotoxin, the antagonist effect would be lost by stimulating cells with a TLR4 agonist structurally different from LPS. Besides the natural agonists LPS, lipid A, lipid A mimetics as monophosphoryl lipid A (MPL),[331] and aminoalkyl glucosaminide 4-phosphates[137] (AGPs), TLR4 can also be activated by DAMPs such as HMGB1[332] and fibronectin[65] and by other small molecules like the natural compound taxol,[327] neoseptins[333] and protein lectins. Lectins constitute a very large class of carbohydrate-binding proteins, and plant lectins have immunostimulating activity, that recently has been related to TLR agonism. In fact, the activity as potent TLR4 agonists of plant lectins KML-C (Korean mistletoe lectin)[334] and PHA (phytohaemagglutinin from *Phaseolus vulgaris*)[311] has been described. Although the experimental data indicate a strong TLR4 agonist activity by lectins, the mechanism of action of these proteins should still be clarified. Because lectins recognize and bind sugars, it is possible that lectins promote the formation of the $(TLR4/MD\text{-}2/LPS)_2$ heterodimer by binding sugars attached to the surface of glycosylated MD-2 and TLR4 proteins thus bringing together two TLR4/MD-2 complexes. According to these literature data, we first validated plant PHA lectin as agonist in HEK-Blue cells. A dose-dependent activation of TLR4 signal was observed when cells were treated with PHA lectin in the presence of polymixin-B to neutralize the agonist effect of any possible LPS contamination. The addition of calixarenes **1**-**4** followed by lectins inhibited in a dose-dependent way the TLR4 signal,

showing that cationic calixarenes antagonize TLR4 signal also in the case of non-LPS stimulation. This would suggest a direct interaction of calixarenes with CD14 and MD-2 receptors, according to the predicted binding poses by docking calculations and molecular dynamic simulations. Calixarenes **3** and **4** showed a potent TLR4 antagonist activity in cells inhibiting the production of the main pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 in LPS-stimulated human PBMCs and murine splenocytes. Although solubility and distribution properties of calixarenes **3** and **4** should be optimized for *in vivo* studies and preclinical development, the lack of toxicity (Figure S8, Supp Info) and the potent TLR4 blocking activity point to these compounds as plausible drug hits targeting TLR4. The flexibility of calixarene scaffold will allow to modulate the hydrophilicity profile of cationic amphiphiles and optimize their pharmacokinetic. The possibility of the calix cavity to complex metal ions or small organic fluorophores could be exploited to generate labeled compounds for diagnostic and therapeutic applications.

In Chapter II, we were interested in investigating the effect achieved by combining the two strategies mentioned above, in order to develop an innovative therapeutic approach to inhibit TLR4 signalling. With a view to studying the synergistic combination of TLRs modulators with antibacterial drugs for inflammatory and autoimmune pathologies, we have examined the combination of a potent TLR4/MD-2 targeting molecule with various AMPs. Compound FP7 has been rationally designed by F. Peri research group to be a MD-2 ligand and shown to inhibit TLR4 signal in cells in the low μM range.[207] FP7 is designed to directly compete with LPS for interacting with MD-2 binding pocket.[207] We recently observed that FP7 targets selectively TLR4 and not TLR2, and that it is able to block TLR4 signal activated by microbial PAMPS but

also by endogenous DAMPs such as HMGB1 protein.[210] The potent and selective antagonist activity on TLR4 plus the lack of cytotoxicity make FP7 a good hit for therapeutic development against pathologies generated by PAMP- and DAMPs-mediated TLR4 signaling. Out of the six AMPs studied, AMP1 and AMP6 present the most pronounced effect in boosting FP7 antagonist activity. A solution structure for CA(1-8)M(1-18) (AMP 1) is not available. Nevertheless, a 6 residue-shorter version, CA(1-8)M(1-12), in aqueous solution (in the presence of structure-inducing trifluoroethanol)[174] has been shown to adopt a major helical structure, with three and one helix turns in the melittin and cecropin A moieties, respectively, separated by a rather flexible hinge (residues Gly-Ile-Gly). These features have been postulated to be required for membrane disruption against both prokaryotic and eukaryotic cells. On the other hand, a high quality structure of human cathelicidin LL-37 (AMP 6) in SDS micelles has been determined by NMR using a $^{13}C,^{15}N$-labeled version.[173] In the SDS micelles, the peptide adopted a curved amphipathic helix-bend-helix motif spanning most of its length (residues 2-31), a pattern not unlike that of CA(1-8)M(1-12) discussed above. Several mechanisms can underlie the potentiating action of AMPs. A direct neutralizing effect on LPS by AMP interaction cannot completely explain the result, which is still observed when cells are stimulated by the plant lectin PHA instead of LPS. Two alternative mechanisms could be based on: 1) direct binding of AMPs to CD14 and/or MD-2 receptors, with concomitant LPS displacement, or 2) the AMPs affecting the aggregation state of FP7. The amphiphilic glycolipid FP7 has a CMC of about 9 μM,[207] in the same order of magnitude than that calculated for *E. coli* LPS (between 1.3 and 1.6 μM).[335] In the concentration range of our cell experiments (0.1-10 μM), FP7 is therefore in equilibrium between aggregate and monomeric species. Both

NMR and TEM experiments clearly show an effect of either AMP1 or AMP6 on FP7 aggregation state. NMR shows addition of either AMP to FP7 to cause the formation of larger aggregates, as revealed by the reduction in the intensities of FP7 aliphatic chain proton signals and the decrease of FP7 diffusion coefficient in DOSY. Cryo-TEM images confirm these data and clearly show that, upon peptide addition, FP7 micellar aggregates (at 500 μM concentration) undergo a change in size and 3D shape from spherical to rod-like cylindrical. NMR experiments provide a valuable indication on the ability of these AMPs to affect FP7 aggregation state in aqueous environment. However, since they have been performed at a concentration two orders of magnitude higher than that at which FP7 displays biological activity, they may suggest a similar behavior of the peptides on FP7 at the biological conditions but do not allow a definitive conclusion on this. More detailed physico-chemical characterization of FP7/AMPs co-aggregates is in progress. In pathologies where inflammation is exacerbated by bacterial infection, combination of anti-TLRs (anti-inflammatory) small molecules with AMPs, as discussed here for the TLR4 antagonist FP7 and peptides CA(1-8)M(1-18) and LL-37, may become a valuable and innovative therapeutical approach.

In chapter III a preclinical study was carried out, in which the anti-inflammatory activity of FP7 has been studied in a model of chronic inflammation (IBD). As described above FP7 is a lipid X mimetic rationally designed to interact with MD-2 and compete with LPS.[207] The observation that FP7 selectively targets TLR4 and not other TLRs,[210] suggested to use this small molecule as a tool to selectively target TLR4 signalling to investigate the involvement of this receptor in different PAMPs and/or DAMPs-driven inflammatory conditions in IBD. Results obtained in this study reveal that FP7

is able to strongly inhibit TLR4 activation both *in vitro* and *in vivo*. Considering that IBD inflammation is characterized by a persistent influx of $CD14^+$ circulating blood monocytes in the inflamed gut mucosa,[126] we performed all the *in vitro* studies on PBMCs collected from patients with UC. We have shown that the higher concentration of FP7 (10 μM) is able to strongly reduce the production of the main pro-inflammatory cytokines released upon LPS stimulation and we demonstrated that this anti-inflammatory effect was due to a reduced LPS-induced TLR4 signalling. In particular we have shown that the presence of FP7 reduces the state of activation of the two main effectors of the MyD88-dependent signalling, NF-κB and MAPKs, normally induced by TLR4 upon LPS exposure. FP7 strongly delays LPS-induced NF-κB phosphorylation and promotes the reappearance of the NF-κB inhibitor, IκBα after 30 minutes. Furthermore FP7 reduces the activation of the three MAPKs monitored, p38, ERK1/2 and JNK suggesting a reduced induction of the pro-inflammatory transcription factor AP1. A possible explanation of these results is that FP7 exerts its effect by acting upstream of these effectors in the TLR4 signalling pathway. In order to clarify the molecular target of FP7 we performed an *in vitro* binding assay using recombinant human MD-2 (hMD-2) isolated from *P. pastoris.* The results obtained performing ELISA-like cell-free binding assays revealed that FP7 binds to MD-2 with high affinity and that it is able to displace LPS from MD-2 binding. This result is in agreement with the mechanism of action of FP7 predicted by previous docking studies,[207] which demonstrated that FP7 is in principle able to form complexes with TLR4/MD2 heterodimer. Furthermore this result strengthens the preliminary NMR result that showed a physical FP7-MD-2 interaction using recombinant hMD-2 purified from *E. coli*.[207]

Recent studies demonstrated that concomitant with TLR4 signaling cascade initiated from the plasma membrane, LPS recognition triggers the internalization of both CD14 and TLR4/MD-2 complex promoting the initiation of the TRIF-dependent pathway.[15] This event is strictly dependent on the physical interaction of LPS with CD14 and on the transfer of LPS to the TLR4/MD-2 complex. Indeed mutations of the LPS-binding site of both CD14 and MD-2 that compromise interaction with monomeric LPS abolished the endocytosis process.[15] Considering that CD14/TLR4 internalization is entirely triggered by extracellular events,[15] we used this process as an indirect indicator to monitor LPS-CD14 and LPS-TLR4 binding. The results obtained clearly showed that the presence of FP7 strongly reduce the LPS-promoted internalization of both receptors, suggesting that the molecule interfere with the LPS detection process by competing with endotoxin for receptor binding. Although this result is extremely interesting, a direct proof of FP7-CD14 and/or FP7-MD-2 interaction in living cells is still lacking. In this respect different approaches are in progress to label our small-molecule without altering its biological activity. For instance, we are considering to conjugate FP7 with different fluorophores including cyanins.

The first two experiments conducted on LPMCs isolated from the inflamed gut of UC patients, suggest that FP7 is able to reduce the expression and production of the same cytokines monitored in LPS-stimulated PBMCs. Although a higher number of experiments on biopsy of UC patients is needed, this preliminary result suggests that the compound can carry out its anti-inflammatory activity on the inflamed mucosa of IBD patients. The *in vivo* experiments conducted on the mouse model of colitis (DSS-induced colitis model) showed that FP7 is able to reduce the development of severe colitis

reducing inflammation. In this model, DSS causes a serious damage to the epithelial barrier of mice large intestine, allowing luminal antigens and bacterial products (including LPS) to penetrate in the lamina propria. Although not all the bacteria present in the gut lumen are Gram negative, the result obtained using a small molecule that selectively targets TLR4 suggests that this receptor, and consequently LPS, plays a crucial role in the development and worsening of gut colitis. Indeed several studies demonstrated that inflammation in IBD patients could set up optimal conditions for the bloom of pathogens like *Enterobacteriaceae* normally present at low levels in the healthy colon.[238] Many studies demonstrated the overgrowth of invasive strains of *E. coli* in patients with both CD and UC, the two major forms of IBD.[252-254] This observation was also confirmed by many studies conducted on experimental models of colitis, were inflammation-induced *E. coli* bloom caused first bacteremia and then mouse mortality.[263] On the other hand, however, other studies demonstrated that TLR4 signalling is essential to promote mucosal healing and epithelial cells regeneration in mice treated with DSS, indicating a protective role of the receptor after inflammation. Indeed TLR4 KO and MyD88 KO mice showed inability to heal the damage caused by DSS, which increase intestinal bleeding and leading to mortality.[336] These observations suggest that TLR4 signalling plays a crucial role in the initiation of intestinal inflammation in IBD mice models, and that it is involved in epithelial healing at a later time. Therefore a therapeutic treatment based on a molecule as FP7 that selectively and temporarily modulate TLR4 activity, may prove to be an effective strategy to ameliorate IBD and other inflammatory pathologies in which TLR4 signalling plays a dual role as initiator of the inflammation and healing promoter. In summary, chapters 1-3 encompass three important steps

regarding the research of TLR4 modulators for therapeutic applications: the early phases of drug development, the characterization of drug interactions and mechanism of action, and the selection of promising candidates to perform pre-clinical studies. Indeed, in chapter I, performing all the early stages of the drug development process, we discovered a new class of TLR4 signalling modulators and we partially characterized their mechanism of action.

In chapter II, with the aim to investigate the effects of co-administered LPS- and TLR4/MD-2-targeting molecules on TLR4 signalling, we described an interesting synergic interaction between the glycolipid FP7 and AMPs. Although the results achieved do not allow a definitive explanation of this synergy, NMR and TEM experiments suggest that the aggregation state of lipid A analogs in aqueous environment plays an important role for TLR4 modulation, and that some molecules like AMPs can affect this state. In the final chapter III we carried out a pre-clinical study in which our best characterized compound FP7 showed interesting anti-inflammatory properties suggesting a possible clinical setting for IBD.

5. PAPERS

Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Amphiphilic Guanidinocalixarenes Inhibit Lipopolysaccharide (LPS)- and Lectin-Stimulated Toll-like Receptor 4 (TLR4) Signaling

Stefania E. Sestito,[†] Fabio A. Facchini,[†] Ilaria Morbioli,[‡] Jean-Marc Billod,[§] Sonsoles Martin-Santamaria,[§] Alessandro Casnati,[‡] Francesco Sansone,[*,‡] and Francesco Peri[*,†]

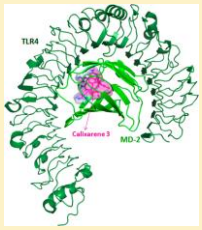
[†]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza, 2, 20126 Milano, Italy

[‡]Dipartimento di Scienze Chimiche, della Vita e della Sostenibilità Ambientale, Università di Parma, Parco Area delle Scienze 17/a, 43124 Parma, Italy

[§]Department of Chemical and Physical Biology, Centro de Investigaciones Biologicas, CIB-CSIC, C/Ramiro de Maeztu, 9, 28040 Madrid, Spain

Supporting Information

ABSTRACT: We recently reported on the activity of cationic amphiphiles in inhibiting TLR4 activation and subsequent production of inflammatory cytokines in cells and in animal models. Starting from the assumption that opportunely designed cationic amphiphiles can behave as CD14/MD-2 ligands and therefore modulate the TLR4 signaling, we present here a panel of amphiphilic guanidinocalixarenes whose structure was computationally optimized to dock into MD-2 and CD14 binding sites. Some of these calixarenes were active in inhibiting, in a dose-dependent way, the LPS-stimulated TLR4 activation and TLR4-dependent cytokine production in human and mouse cells. Moreover, guanidinocalixarenes also inhibited TLR4 signaling when TLR4 was activated by a non-LPS stimulus, the plant lectin PHA. While the activity of guanidinocalixarenes in inhibiting LPS toxic action has previously been related to their capacity to bind LPS, we suggest a direct antagonist effect of calixarenes on TLR4/MD-2 dimerization, pointing at the calixarene moiety as a potential scaffold for the development of new TLR4-directed therapeutics.



INTRODUCTION

The members of the Toll-like receptor (TLR) family are among the first receptors to be activated during many host−pathogen interactions. They are responsible for detecting microbial products and inducing innate and adaptive immune responses.[1] TLRs are pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). Among TLRs, TLR4 is the sensor of Gram-negative bacteria endotoxins lipopolysaccharide (LPS) and lipooligosaccharide (LOS).[2] TLR4 is mainly expressed on monocytes, dendritic cells (DCs), and macrophages (MΦs). LPS binds sequentially to lipid binding protein (LBP), to cluster of differentiation 14 (CD14, GPI-linked or soluble), and finally to myeloid differentiation factor 2 (MD-2)[3] that noncovalently associates with TLR4 promoting the formation of the activated receptor multimer $(TLR4/MD\text{-}2\text{-}LPS)_2$ on the plasma membrane.[4] While the role of TLR4 as LPS sensor is fundamental for initiating inflammatory and immune responses, excessive and deregulated TLR4 activation leads to acute sepsis and septic shock, syndromes associated with high lethality for which no specific pharmacological treatment is available.[5,6] TLR4 can also be activated by endogenous factors called damage-associated molecular patterns (DAMPs), derived from damaged, necrotic, or infected tissues. DAMPs-activated TLR4 signaling has been implicated in a large array of pathologies including atherosclerosis,[7] rheumathoid arthritis,[8] neuroinflammations, neuropathic pain,[9] and neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS).[10]

To block abnormal TLR4 signaling in bacterial sepsis, two different strategies have been developed. The first one is based on LPS neutralization by the formation of noncovalent adducts with cationic compounds: positively charged antimicrobial peptides (AMPs)[11] including polymixin B,[12] and synthetic dendrimeric polyamines[13,14] contain positively charged groups (most frequently amino and guanidinium groups) and form noncovalent complexes with negatively charged LPS, thus preventing LPS from interacting with the receptors.

The second strategy is based on the use of molecules that compete with endotoxic LPS in binding to the same site on CD14 and MD-2, thereby inhibiting the induction of signal transduction by impairing LPS-initiated receptor dimerization. To date, several lipid A variants, which specifically block the LPS-binding site on human (h)MD-2, have been identified: natural compounds such as lipid IVa (a biosynthetic precursor of *E. coli* lipid A)[15] and a nonpathogenic lipid A from *R. sphaeroides*,[16] and synthetic molecules such as the tetraacylated disaccharide eritoran (E5564),[17,18] the aminoalkyl glucosaminide 4-phosphates (AGPs),[19,20] and some phosphorylated monosaccharide glycolipids.[21] These compounds inhibit

Received: January 28, 2017
Published: May 4, 2017

DOI: 10.1021/acs.jmedchem.7b00095
J. Med. Chem. 2017, 60, 4882−4892

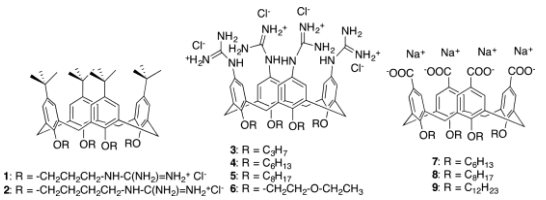


Figure 1. Positively charged guanidinocalixarenes **1**–**6** and negatively charged carboxy calixarenes **7**–**9**.

TLR4 signaling by accommodating into the deep hydrophobic pocket of the co-receptor, MD-2, and blocking ligand-induced dimerization.[22] Eritoran[23] and other small molecules with TLR4 antagonist activity[24] also potently inhibit LPS binding to CD14. While the use of LPS neutralizing agents is limited to sepsis and septic shock, TLR4 antagonists that directly bind CD14 and MD-2 have potential also as therapeutics to treat neuroinflammations[25] and viral syndromes[26] caused by DAMP-TLR4 signaling. We recently observed that glycoamphiphiles with a sugar core (trehalose or glucose) functionalized with lipid chains and positively charged ammonium groups are able to inhibit LPS-stimulated TLR4 signal in vitro with IC_{50} values ranging from about 5 to 0.2 μM and to reduce TLR4-dependent production of inflammatory cytokines in vivo.[27] The main structural feature of these molecules is their "facial" arrangement with positive charges and lipophilic chains disposed in spatially well-defined regions.

Therefore, we hypothesized that calixarene-based facial amphiphiles could also be suitable as scaffolds to obtain TLR4 ligands with antagonist activity. Actually, in a biological context, amphiphilic calixarenes showed remarkable properties significantly related to their amphiphilicity.[28] The calixarene scaffold represents a very versatile structure to build amphiphilic compounds due to the possibility of variably and selectively functionalizing both its upper (aromatic para positions) and lower (phenolic oxygens) rim. Moreover, the possibility of linking to the macrocyclic platform several binding moieties, resulting in preorganized arrays, gives rise to systems that, exploiting a multivalent effect, frequently show improved biological activity with respect to corresponding monovalent models.[28,29] From this point of view, also the tight compaction of hydrophobic chains located at one of the rims can result in the enhancement of some properties such as (self)assembling capabilities in an aqueous environment.[28−31]

We present here a study on the inhibition of TLR4/MD-2 signaling by a series of positively and negatively charged calixarene-based amphiphiles (compounds **1**–**6** and **7**–**9** in Figure 1, respectively) and the investigation of their mechanism of action. In the series we included calixarene **2** as reference compound whose activity in this biological context has been previously reported[32] and associated with its capacity to bind and neutralize LPS as topomimetic of LPS-binding peptides. Since we hypothesized that calixarene derivatives could directly bind to human and murine MD-2 and CD14 in a similar fashion as LPS, we preliminarily performed docking calculations to support this mode of interaction. Moreover, we aimed here to verify if the TLR4 antagonist activity is a rather general property of positively charged amphiphilic calixarenes and if the antagonist effect also derives from the direct interaction of calixarenes with the receptors and not exclusively from LPS neutralizing action, as suggested for calixarene **2**.

RESULTS

Rational Design of Amphiphilic Calixarenes as CD14/MD-2 Ligands. We were inspired by the hypothesis that the calixarenes could be TLR4 modulators similar to lipid A variants and to trehalose or glucose-based glycoamphiphiles previously developed by one of the groups involved in the present study.[27] Positively charged guanidinocalix[4]arenes **1** and **3**–**6** and negatively charged carboxylate calixarenes **7**–**9** were designed in order to investigate the suitability of this macrocyclic scaffold to build CD14 and TLR4/MD-2 ligands (Figure 2). These calixarene derivatives have an amphiphilic

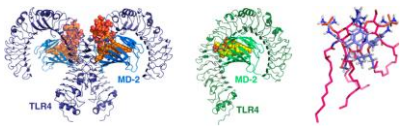


Figure 2. Left: 3D structure of human TLR4/MD-2/LPS dimer from PDB code 3FXI. Middle: 3D structure of TLR4/MD-2/lipid-IVa from PDB code 2E56. Right: Superimposition of lipid IVa (from PDB code 2E56, magenta) and calixarene **3** (purple).

character due to the presence of lipophilic tails on one rim and charged polar groups on the other. Only compound **6**, having ethoxyethyl chains at the lower rim, has a reduced amphiphilicity and was included in the library precisely to verify the possible relevance of this property in the biological activity.

Calixarenes **1** and **2**[32] present lipophilic upper rims bearing four *tert*-butyl groups and polar lower rims with positively charged guanidinium groups linked through, respectively, propyl and butyl chains. Calixarenes **3**–**6** present a reversed arrangement of lipophilic and charged groups: guanidinium groups are directly linked to the scaffold on the upper rim, and hydrocarbon chains of different length (C_3, C_6, and C_8 for compounds **3**, **4**, and **5**, respectively), or an ethoxy ethyl chain in the case of compound **6**, are linked at the lower rim. Finally, anionic calixarenes **7**–**9** were designed with the purpose of studying the influence of negatively charged groups. Thus, these anionic calixarenes present carboxylate groups at the upper rim, aiming to mimic the phosphate groups of LPS, and hydrocarbon chains of variable length (C_6, C_8, and C_{12}) at the lower rim.

DOI: 10.1021/acs.jmedchem.7b00095
J. Med. Chem. 2017, 60, 4882−4892

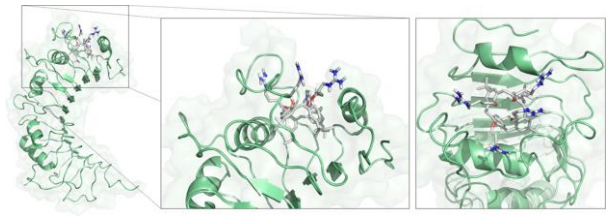


Figure 3. Docked pose for compound **3** inside CD14 (PDB code 4GLP). Left: full perspective. Middle: side view. Right: top view.

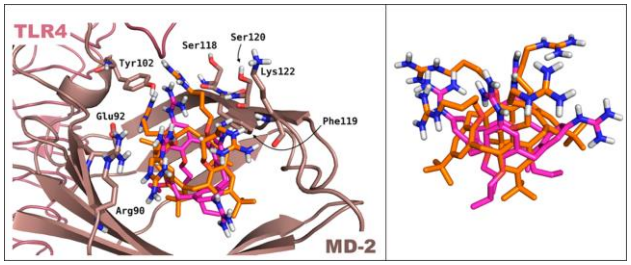


Figure 4. Superimposition of the best docked poses for compounds **2** (orange) and **3** (magenta) in TLR4/MD-2 heterodimer (PDB code 2Z65). A 90° rotated view is shown on the right (TLR4/MD-2 has been hidden for the sake of clarity).

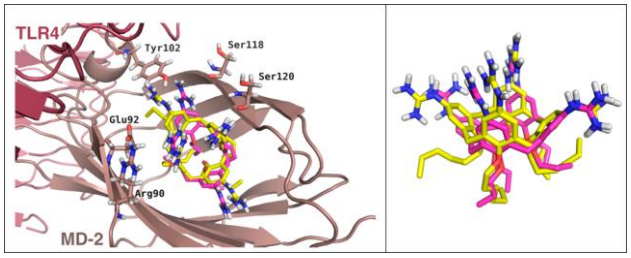


Figure 5. Superimposition of the best docked poses for compounds **3** (magenta) and **4** (yellow) in (h)TLR4/MD-2 heterodimer (PDB code 2Z65). A 90° rotated view is shown on the right (TLR4/MD-2 has been hidden for the sake of clarity).

Three-dimensional (3D) structures of compounds **1**–**9** were built and optimized by means of computational techniques (see Experimental Section). We superimposed the 3D structures of the calixarenes **2** and **3** with that of lipid IVa, a natural underacylated MD-2 ligand with activity as (h)TLR4 antagonist. From a comparison of lipid IVa (3D structure from the X-ray crystallography structure) with compound **3** (Figure 2, right), the oppositely charged groups (phosphate vs guanidinium) aligned perfectly, and also did the disaccharide over the aromatic calix backbones, and the acyl over the alkoxy chains. This preliminary result regarding the geometrical similarity prompted us to further study calixarenes **1**–**9** as putative TLR4/MD-2 and CD14 ligands.

First, compounds **2**, **3**, and **4**, as representative derivatives, were docked into the binding site of the human CD14 protein (PDB code 4GLP). For all these three compounds, docking calculations predicted favorable binding poses inside the human CD14 protein (Figure 3), where the guanidinium moieties are placed at the rim of CD14 and the hydrophobic chains are inserted into the hydrophobic pocket.

Docking calculations were also performed with compounds **1**–**9** into four different structures of the TLR4/MD-2 system: human and mouse, in agonist and antagonist conformations of MD-2 (see Figures 4 and 5 and Supporting Information Figures S1, S2, and S3). Overall, all the ligands were predicted to bind inside the different TLR4/MD-2 structures, with the

DOI: 10.1021/acs.jmedchem.7b00095
J. Med. Chem. 2017, 60, 4882−4892

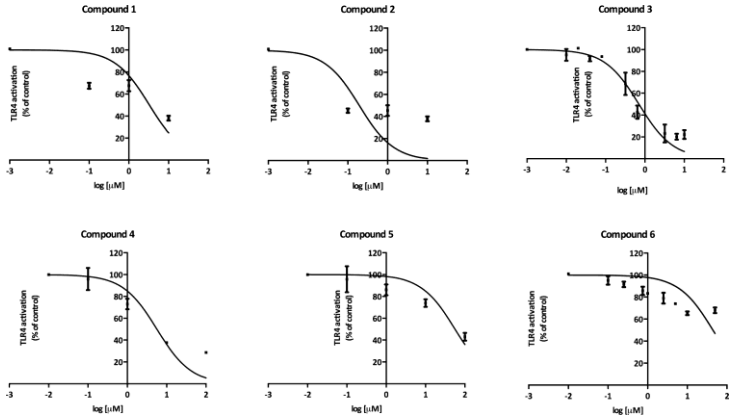


Figure 6. Dose dependent inhibition of LPS-stimulated HEK-Blue cells activation by calixarenes **1**–**5**. Human TLR4 HEK-Blue was treated with increasing concentrations of compounds and stimulated with LPS (100 ng/mL). The results represent normalized data with positive control (LPS alone) and expressed as the mean of percentage ± SD of at least three independent experiments.

guanidinium/carboxylate moieties placed at the rim of MD-2, where polar interactions predominate, and the lipophilic groups (alkoxy or *tert*-butyl chains) inside the MD-2 pocket. These docked poses are in agreement with calculations reported by us of compounds binding both CD14 and MD-2 proteins. Although MD-2 is more specific in the ligand recognition, both MD-2 and CD14 binding pockets share some similarities regarding volume and accessible surface area.[33,34]

Regarding reported compound **2**, in the docked poses in both agonist and antagonist conformations of human MD-2, the guanidinium groups establish H-bonds with the side chains of Glu92, Tyr102, and Ser118 and the backbone of Lys122 (Figure 4 and Supporting Information Figures S1and S2), while one of the aromatic rings of the macrocycle is engaged in a π–π-stacking interaction with the Phe119 side chain.

In detail, the guanidinium groups at the upper rim of compounds **3**–**5** establish H-bonds with the backbone of Ser120 and with the side chains of Glu92 and Tyr102 (Figure 5). The longer alkyl chains of compounds **4** and **5** occupy deeper regions of the MD-2 pocket. Interestingly, when comparing the best predicted docked poses for compounds **2** and **3**, it was observed that they are half turn rotated one from another in regard to the calixarene moiety (Figure 4 and Supporting Information Figure S2). In both cases, the guanidinium moieties are accommodated at the entrance of the pocket while the hydrophobic groups (*tert*-butyl and propyl for compounds **2** and **3**, respectively) are buried inside the MD-2 hydrophobic pocket.

Regarding compounds **7**–**9**, they presented similar docked poses where the alkyl chains were also buried inside the hydrophobic MD-2 pocket and the carboxylate moieties were establishing polar interactions with the residues at the MD-2 rim. Compounds **8** and **9** presented docked poses protruding slightly more than compound **7**, probably due to the longer alkyl chains, although the difference was very subtle (Figure S3, Supporting Information).

To ensure the stability of the docked poses of compound **3** with TLR4/MD-2 and to gain insights on the interactions that take place, we performed 90 ns molecular dynamic simulations of the (h)TLR4/MD-2/**3** complex starting from the docked geometries for both the antagonist and the agonist conformations of (h)TLR4/MD-2. In the simulation starting from the agonist conformation of MD-2 we could observe that compound **3** rotates of almost 90° around its plan of symmetry (a partial rotation happens at 5 ns of simulation and the full rotation at approximately 38 ns) to find a more stable bound conformation that was maintained stable for the rest of the simulation (Figure S4, Supporting Information). This rotation forced the MD-2 pocket to adopt an antagonist-like conformation (characterized by, inter alia, great motion of residue Phe126). In this new binding mode, two guanidinium groups of compound **3** continued to interact through hydrogen bonds with the side chains of Glu92 and Ser120, a third guanidinium group formed a new hydrogen bond with the CO group of Pro88, and the fourth guanidinium group was involved in polar interactions with the solvent. Moreover, later in the simulation (starting at 42 ns), the loop made by residues 80–90 undergoes a considerable deformation (Figure S5, Supporting Information). In contrast, in the simulation of the TLR4/MD-2/**3** complex starting from the antagonist conformation, the geometries of both compound **3** and MD-2 were stable during the 90 ns run (Figure S5, Supporting Information), not experiencing important conformational changes. These results clearly indicated that the complex of calixarene **3** with MD-2 in agonist conformation is less stable than the complex with the antagonist one, therefore providing explanations for the antagonist activity later observed (see below). Taken together, our computational studies provided plausible binding poses for compounds **2**–**4** into CD14 and for compounds **1**–**9** into

DOI: 10.1021/acs.jmedchem.7b00095
J. Med. Chem. 2017, 60, 4882–4892

TLR4/MD-2, supporting a putative direct binding to these proteins.

Synthesis. The already known guanidinocalixarenes **1**[35] and **3**–**6**[36,37] were synthesized according to the procedures we already reported in the literature. Derivative **2**, previously reported,[32] was prepared from tetra-*tert*-butyl-25,26,27,28-tetrabutylaminocalix[4]arene[38] through condensation with bis-BOC-*N*-triflylguanidine to obtain the protected precursor[32] and subsequent removal of the Boc protecting groups by treatment with 1% HCl in dioxane in the presence of triethylsilane as scavenger. The anionic ligands **8** and **9** were obtained from the corresponding tetraacid derivatives[39] by titration with NaOH, while the tetracarboxylate **7**, bearing hexyl chains at the lower rim, was obtained from the tetraformyl-tetrahexylcalix[4]arene precursor[40] by oxidation with $NaClO_2$ in the presence of sulfamic acid and subsequent titration with NaOH.

Inhibition of LPS-Stimulated TLR4 Signal in HEK-Blue Cells. Calixarenes **1**–**9** were first screened for their capacity to interfere with LPS-stimulated TLR4 activation and signaling on HEK-Blue cells. HEK-Blue cells are stably transfected with TLR4, MD-2, and CD14 genes. In addition, these cells stably express an optimized alkaline phosphatase gene engineered to be secreted (sAP), placed under the control of a promoter inducible by several transcription factors such as NF-κB and AP-1.[36] This reporter gene allows monitoring of the activation of TLR4 signal pathway by endotoxin. All calixarenes were inactive in stimulating TLR4 signal when provided alone, thus indicating the absence of agonist activity, in agreement with the molecular modeling studies. On the other hand, compounds **1**–**5** inhibited in a dose-dependent way the LPS-stimulated TLR4 signal (Figure 6), while calixarene **6** with oxygenated ethylene glycol chains instead of hydrocarbon chains showed weak antagonistic activity.

Guanidinocalixarenes **1**–**5** inhibited TLR4 signal with potencies ranging from 0.2 to 63 μM (Table 1). Compounds

Table 1. IC_{50} Values for the Inhibition of LPS-Stimulated TLR4 Signal in HEK Cells

compd	IC_{50} LPS (μM)
1	10
2	0.2
3	0.7
4	5.7
5	63
6	45

2, **3**, and **4** were the most potent antagonists and inhibited LPS-stimulated TLR4 signal with IC_{50} of 0.2, 0.7, and 5.7 μM, respectively. In contrast, negatively charged amphiphilic calixarenes **7**–**9** with carboxylic groups on the upper rim showed no or very weak inhibition of LPS-TLR4 signal (Figure S9, Supporting Information).

Inhibition of PHA Lectin-Stimulated TLR4 Signal in HEK-Blue Cells. We were then interested in knowing if the inhibition of TLR4 signal is due to calixarene interaction with LPS or to a direct interaction with the TLR4 receptor system, evidenced as possible by calculations. To investigate this point, we stimulated HEK cells with the plant lectin phytohemagglutinin (PHA from *Phaseolus vulgaris*) whose property to potently stimulate TLR4 signal acting as agonist has been recently described.[41] We first checked if PHA is able to activate TLR4 signal in HEK-Blue cells, and we found that the lectin was active in stimulating in a dose-dependent way TLR4-dependent SEAP production (Figure S7, Supporting Information). To exclude the TLR4 activity that could be derived from LPS contamination in the PHA, we performed the experiment in the presence of the LPS-neutralizing peptide polymixin-B. We also verified that control HEK-null cells, that is, HEK cells transfected with SEAP plasmid and lacking TLR4, MD-2, CD14 genes, were not activated by PHA lectin (Figure S7, Supporting Information). PHA lectin was then used instead of LPS as a TLR4 agonist to stimulate cells. The highly potent calixarene-based TLR4 antagonists, compounds **3** and **4**, were then investigated for their property to inhibit TLR4 activation by PHA lectin (Figure 7).

Guanidinocalixarenes **3** and **4** were indeed active in inhibiting PHA lectin-stimulated TLR4 signal in a concentration-dependent way, with potencies similar to those measured in the inhibition of LPS-stimulated TLR4 signal (Table 1). The fact that the antagonist activity was retained by calixarenes also when TLR4 was stimulated by a non-LPS agonist strongly suggests that the action of calixarenes is mainly based on direct interaction with CD14 and MD-2 receptors.

Inhibition of LPS-Stimulated TLR4 Signal in Human White Blood Cells. As HEK cells are a non-natural system to study TLR4 activation and to perform preliminary screening, the capacity of lead compounds **3** and **4** to inhibit LPS-stimulated TLR4 signaling was further investigated in human white blood cells (h)WBCs that naturally express CD14, MD-2, and TLR4 receptors. We evaluated the production of the main NF-κB-dependent proinflammatory cytokines tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and IL-8 by primary human peripheral blood mononuclear cells (hPBMCs) as readout for TLR4 pathway activation. hPBMCs isolated from the whole blood of healthy volunteers were treated with increasing concentrations (1–10 μM) of compounds **3** and **4** and stimulated after 30 min with LPS (100 ng/mL). Compound **3** reduced the production of all the proinflammatory cytokines monitored, while compound **4** showed a lower inhibitory activity, reducing only two of the three cytokines evaluated (Figure 8).

Inhibition of LPS-Stimulated TLR4 Signal in Murine White Blood Cells (m)WBCs. It is known that human and murine MD-2 have dissimilarities in the LPS binding region, and some ligands have different activity on (h)MD-2 and (m)MD-2, in some cases switching from agonism to antagonism. We therefore aimed to compare the activity of calixarene on human and murine cells. The activity of compounds **3** and **4** was then evaluated in a murine macrophages cell line, RAW-Blue cells. As HEK-Blue cells, RAW-Blue cells are transfected to stably express the SEAP reporter gene in order to monitor the activation of TLR4 signal pathway. Compounds **3** and **4** inhibited in a dose-dependent way the LPS-stimulated TLR4 signal (Figure 9A), revealing that the two calixarenes were also effective on the murine TLR4 system.

The abilities of compounds **3** and **4** were further investigated in murine splenocytes. TNF-α relative expression was determined from TLR4–MyD88 pathway activation. Splenocytes from balb/c mice were treated with two concentrations (1 and 10 μM) of compounds **3** and **4** in RPMI and stimulated after 30 min with LPS (100 ng/mL). The LPS-induced TNF-α expression after a 5 h incubation was measured by qPCR. The lower concentration of compounds **3** and **4** (1 μM) was weakly active in reducing LPS-induced TNF-α expression, whereas the

DOI: 10.1021/acs.jmedchem.7b00095
J. Med. Chem. 2017, 60, 4882–4892

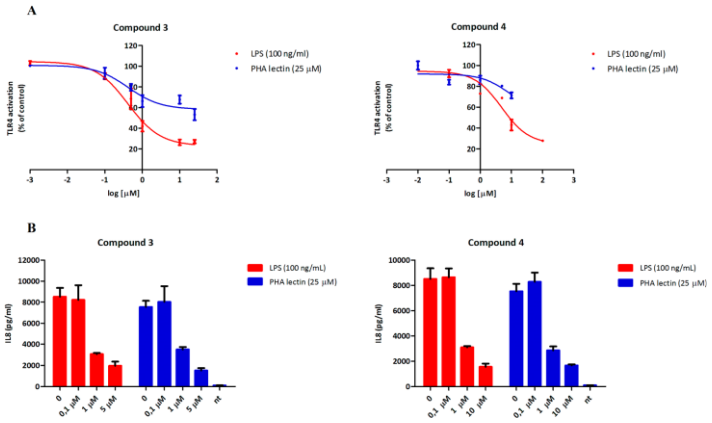


Figure 7. (A) Inhibition of TLR4 signaling in HEK-Blue cells stimulated with LPS (100 ng/mL) or PHA lectin (25 μM) and treated with calixarenes **3** and **4**. The results represent normalized data with positive control (LPS or PHA lectin alone). (B) Quantification of interleukin-8 (IL-8) in HEK-Blue cells stimulated with LPS or PHA and treated with compounds **3** and **4** by performing ELISA assay. Data represent the mean of percentage ± SD of at least three independent experiments.

higher concentration (10 μM) of both compounds completely inhibited the expression of TNF-α (Figure 9B).

■ DISCUSSION AND CONCLUSIONS

Calixarenes with guanidinium groups on one rim and hydrocarbon chains on the other are facial amphiphiles in which the charged polar face and the hydrophobic apolar one are spatially organized. Because of this structural feature, they can be potential modulators of the TLR4 activation through direct binding to the receptor or one of the co-receptors involved in the signaling process. We designed the series of calixarenes **1**–**9** aiming to explore the plausible direct binding to CD14 and MD-2 co-receptors. Docking studies demonstrated that compounds **2**–**4** and **1**–**9** are in principle able to form complexes with CD14 and TLR4/MD-2 heterodimer (human and murine), respectively, independently from the relative disposition of the polar and apolar residues and from the nature of the charged groups. The lipophilic chains, linked at the upper (**1** and **2**) or at the lower (**3**–**5**) rim, were in all cases buried into the CD14 or MD-2 hydrophobic pocket, while the charged heads established contacts with polar residues located in proximity of the entry of the pockets. Therefore, our computational studies provide plausible binding poses into the TLR4 co-receptors for the investigated compounds, and this supports our hypothesis of a direct binding of calixarene derivatives to these proteins in competition with LPS. These findings thus open the possibility of exploring calixarenes as a platform for the design of TLR4/MD-2 modulators. Calculations of the stability of the complexes between the guanidinocalixarene **3** and TLR4/MD-2 suggested that this derivative, and for analogy at least all the other positively charged analogues, could act as antagonist.

The activity of positively charged calixarenes was tested on HEK cells expressing (h)TLR4/MD-2 and human and murine leukocytes. Cationic calixarenes **1**–**4** inhibited in a dose-dependent way LPS-stimulated TLR4 activation in both human and murine cells. Cells were first stimulated by LPS and then treated with synthetic molecules. In agreement with the theoretical studies, compounds **1**–**4** showed an antagonist activity in the low micromolar range on human and murine TLR4. Paradoxically, negatively charged amphiphilic calixarenes, which should mimic better the anionic nature of lipid A, the natural MD-2 and CD14 ligand, were not active both in inhibiting and stimulating TLR4. Compound **6**, with more polar ether chains on the lower rim instead of hydrocarbon chains turned out to be substantially inactive as TLR4 inhibitor in both cell types.

Additionally, calculations of the log *P* values predicted high values for compounds **7**, **8**, and **9** (calculated log *P* values above 15), pointing to a high lipophilicity, while compound **6** was predicted to be extremely hydrophilic (calculated log *P* value below zero, Figure S6, Supporting Information). These unfavorable values for log *P* could be correlated with poor physical–chemical properties, thus explaining the lack of activity in the cells assays.

The very close IC_{50} values (Table 1) found for guanidinocalixarenes **2** and **3** indicate that the relative disposition of polar and hydrophobic residues with respect to the macrocyclic cavity does not have a significant impact on the inhibition activity of these ligands. Furthermore, by comparing compounds **3**–**5**, it seems that an increasing lipophilicity is detrimental for the inhibition potency, even if the less amphiphilic derivative **6** is very poorly active with an IC_{50} 2 orders of magnitude higher than that of **3**. A subtle balance between lipophilic and hydrophilic portions in the ligand structure seems then to be the key to determine the activity.

It is worth noting that lead compounds **3** and **4** show antagonist activity on both human and murine TLR4. Several

DOI: 10.1021/acs.jmedchem.7b00095
J. Med. Chem. 2017, 60, 4882–4892

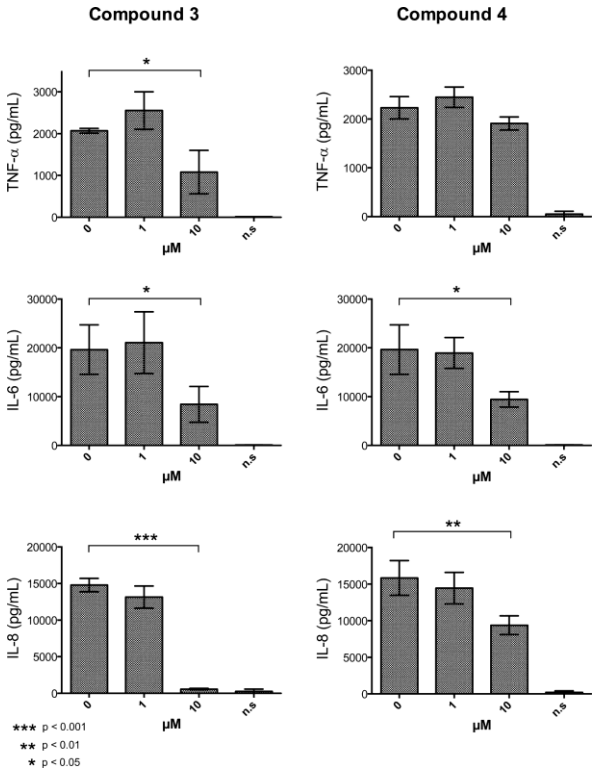


Figure 8. Inhibitory effect of compounds **3** and **4** on LPS-induced proinflammatory cytokines production by PBMCs. PBMCs isolated from whole blood were preincubated with synthetic compounds for 30 min and then stimulated with LPS (100 ng/mL). TNFα, IL-6, and IL-8 production was quantified after one night's incubation. Data represent the mean ± SEM of at least three independent experiments.

TLR4 modulators resembling lipid A have species-specific activity that is generally attributed, among other factors, to the dissimilarities in the shape of the hydrophobic binding pocket of (h)- and (m)MD-2[42] and to the variations in the electrostatic potentials at the rim of the binding cavity of MD-2 and at the dimerization interface. The most significant example of this is the natural compound tetraacylated lipid IVa that acts as an antagonist on human but as an agonist on murine TLR4.[42] However, several synthetic phosphoglycolipids with a monosaccharide scaffold also showed agonist activity on murine and antagonist activity on human TLR4.[43]

Trying to understand whether the antagonist activity of calixarenes was due to their interaction with receptors or with LPSs, we also undertook studies of the TLR4 activation with non-LPS ligands. We reasoned that if the contribution of calixarenes in inhibiting TLR4 activation is due to a neutralizing effect on endotoxin, the antagonist effect would be lost by stimulating cells with a TLR4 agonist structurally different from LPS. Besides the natural agonists LPS, lipid A, lipid A mimetics as monophosphoryl lipid A (MPL),[44] and aminoalkyl glucosaminide 4-phosphates[19] (AGPs), TLR4 can also be activated by small molecules, such as the natural compound paclitaxel,[45] oxidized phospholipids, and synthetic pyrimidoindoles and neoseptins,[46,47] and by protein DAMPs such as high mobility group box 1 (HMGB1)[48] and lectins. Lectins constitute a very large class of carbohydrate-binding proteins, and plant lectins have immunostimulating activity that recently has been related to TLR agonism. In fact, the activity as potent TLR4 agonists of plant lectins KML-C (Korean mistletoe lectin)[49] and PHA (phytohemagglutinin from *Phaseolus vulgaris*)[41] has been described. Although the experimental data indicate a strong TLR4 agonist activity by lectins, the

DOI: 10.1021/acs.jmedchem.7b00095
J. Med. Chem. 2017, 60, 4882−4892

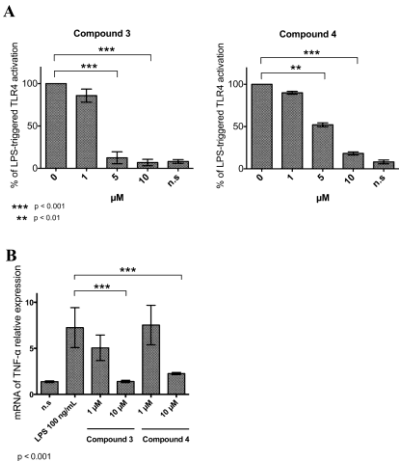


Figure 9. Effects of compounds **3** and **4** on RAW-Blue cells and on murine splenocytes. (A) RAW-Blue cells stably transfected with NF-κB-dependent SEAP reporter plasmid were treated with increasing concentrations of compounds **3** and **4** and stimulated with LPS (100 ng/mL) after 30 min. Data represent the mean of percentage of at least three independent experiments. (B) Murine splenocytes isolated from murine spleen were preincubated with two concentrations (1 and 10 μM) of compounds **3** and **4** for 30 min and then stimulated with LPS (100 ng/mL). Readout was the TNF-α expression after 5 h of incubation. Normalized data are representative of three independent experiments.

mechanism of action of these proteins should still be clarified. Because lectins recognize and bind sugars, it is possible that lectins promote the formation of the $(TLR4/MD\text{-}2/LPS)_2$ heterodimer by binding to the sugars attached to the surface of glycosylated MD-2 and TLR4 proteins, thus bringing together two TLR4/MD-2 complexes.

According to these literature data, we first validated plant PHA lectin as agonist in HEK-Blue cells. A dose-dependent activation of TLR4 signal was observed when cells were treated with PHA lectin in the presence of polymixin-B to neutralize the agonist effect of any possible LPS contamination. The addition of calixarenes **1**–**4** followed by lectins inhibited in a dose-dependent way the TLR4 signal, showing that cationic calixarenes antagonize TLR4 signal also in the case of non-LPS stimulation. This would suggest a direct interaction of calixarenes with CD14 and MD-2 receptors, according to the predicted binding poses by docking calculations and molecular dynamic simulations.

Calixarenes **3** and **4** showed a potent TLR4 antagonist activity in cells and inhibited the production of the inflammatory TNF-α production in LPS-stimulated murine splenocytes and in mice. Although solubility and distribution properties of calixarenes **3** and **4** should be optimized for in vivo studies and preclinical development, the lack of toxicity (Figure S8, Supporting Information) and the potent TLR4 blocking activity point to these compounds as plausible drug hits targeting TLR4. The flexibility of calixarene scaffold will allow modulation of the hydrophilicity profile of cationic amphiphiles and optimization of their pharmacokinetics. The possibility of the calix cavity to complex metal ions or small organic fluorophores could also be exploited to generate labeled compounds for diagnostic and therapeutic applications.

EXPERIMENTAL SECTION

Molecular Modeling. *Structure Construction.* 3D structures of the ligands were built with PyMOL molecular graphics and modeling package[50] based on the coordinates of the calixarene scaffold retrieved from the PubChem database (CID: 562409). 3D coordinates for the agonist hTLR4/MD-2 complex, the antagonist mTLR4/MD-2 complex, the agonist mTLR4/MD-2 complex, and hCD14 were retrieved from the PDB database (www.rcsb.org) under codes 3FXI, 2Z64, 3VQ2, and 4GLP, respectively. The structures went through a restrained minimization procedure with Maestro using the OPLS3 force field. Gasteiger charges were computed within the AutoDockTools program, and all nonpolar hydrogens were merged.

Structure Optimization. All compounds (from **1** to **9**) were optimized with ab initio calculations, using the density functional theory (DFT) with the hybrid functional B3LYP with the Pople basis set 6-31+g(d,p) using Gaussian g09/e1.[51] Water solvation (with a dielectric constant of ε = 78.3553) was simulated with the Gaussian default SCRF method (i.e., using the polarizable continuum model (PCM) with the integral equation formalism variant (IEFPCM)).

Docking Procedure. Docking was performed independently with both AutoDock 4.2[52] and AutoDock VINA 1.1.2.[53] In AutoDock 4.2, the Lamarckian evolutionary algorithm was chosen and all parameters were kept default except for the number of genetic algorithm (GA) runs which was set to 200 to enhance the sampling. AutoDockTools 1.5.6 was used to assign the Gasteiger–Marsili empirical atomic partial charges to the atoms of both the ligands and the receptors. The structure of the receptors was always kept rigid, whereas the structure of the ligand was set partially flexible by providing freedom to some appropriately selected dihedral angles.

Concerning the boxes, spacing was set to 0.375 Å for AutoDock and is default to 1 Å for VINA. In the case of the human and mouse TLR4/MD-2 systems in their agonist and antagonist conformations, the size of the box was set to 33.00 Å in the *x*-axis, 40.50 Å in the *y*-axis, and 35.25 Å in the *z*-axis. For (h)CD14 the size of the box was set to 33.00 Å in the *x*-axis, 33.75 Å in the *y*-axis, and 33.75 Å in the *z*-axis. For the (h)TLR4/MD-2 complex the center of the box is located equidistant to the center of mass of residues Arg90 (MD-2), Lys122 (MD-2), and Arg264 (TLR4). For the (m)TLR4/MD-2 complex the center of the box is located equidistant to the center of mass of residues Arg90 (MD-2), Glu122 (MD-2), and Lys263 (TLR4). For (h)CD14 the center of the box is located equidistant to the center of mass of residues Phe69, Tyr82, and Leu89.

Parameters Derivation. Parameters for molecular dynamics simulations were set up with the standard Antechamber[54] procedure. Briefly, charges were calculated with Gaussian at the Hartree–Fock level (HF/6-31G* Pop=MK iop(6/33=2) iop(6/42=6)) from the solvated DFT B3LYP optimized structure, then derived and formatted for Ambertools15 and AMBER 14[55] with Antechamber assigning the general AMBER force field (GAFF) atom types.[56] A new atom type for nitrogen was introduced (nj), within GAFF, to properly describe the guanidine moiety, mirroring the parameters of ff14SB[57] used to describe the guanidine fragment present in arginine. Parameters for this new atom are provided in the Supporting Information.

Molecular Dynamics (MD) Simulations. Before being submitted to the production run, the system undergoes a height steps preparation. The first one consists of 1000 steps of steepest descent algorithm followed by 7000 steps of conjugate gradient algorithm; a 100 $kcal \cdot mol^{-1} \cdot A^{-2}$ harmonic potential constraint is applied on both the proteins and the ligand. In the four subsequent steps, the harmonic potential is progressively lowered (respectively to 10, 5, and 2.5 $kcal \cdot mol^{-1} \cdot A^{-2}$) for 600 steps of conjugate gradient algorithm each time, and then the whole system is minimized uniformly. In the following

DOI: 10.1021/acs.jmedchem.7b00095
J. Med. Chem. 2017, 60, 4882–4892

step the system is heated from 0 to 100 K using the Langevin thermostat in the canonical ensemble (*NVT*) while applying a 20 kcal·mol^{-1}·A^{-2} harmonic potential restraint on the proteins and the ligand. The next step heats up the system from 100 to 300 K in the isothermal−isobaric ensemble (*NPT*) under the same restraint condition as in the previous step. In the last step the same parameters are used to simulate the system for 100 ps but no harmonic restraint is applied. At this point the system is ready for the production run, which is performed using the Langevin thermostat under *NPT* ensemble, at a 2 fs time step. All production runs were performed for 90 ns.

Calculations of log P. From the optimized 3D structure of compounds **1**−**9**, log *P* value was calculated with the Maestro package (www.schrodinger.com/maestro).

Biology: Cell Tests. *HEK-Blue Activation Assay.* HEK-Blue-TLR4 cells (InvivoGen) were cultured according to the manufacturer's instructions. Briefly, cells were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1× Penstrep, 1× Normocin (InvivoGen), 1× HEK-Blue Selection (InvivoGen). Cells were detached by trypsin, and the cell concentration was estimated by using Trypan Blue (Sigma-Aldrich). The cells were diluted in DMEM high glucose medium supplemented as described before and seeded in multiwell plate at a density of 2 × 10^4 cells per well in 200 μL. After overnight incubation (37 °C, 5% CO_2, 95% humidity), supernatant was removed and cell monolayers were washed with warm PBS and treated with increasing concentrations of compounds dissolved in water or DMSO−ethanol (1:1) and diluted in DMEM. After 30 min, the cells were stimulated with 100 ng/mL LPS from *E. coli* O55:B5 (Sigma- Aldrich) or 25 μM lectin from *Phaseolus vulgaris* (PHA-P) and incubated overnight. As control, the cells were treated with or without LPS (100 ng/mL) or PHA-P (25 μM) alone. Then the supernatants were collected and an amount of 50 μL of each sample was added to 100 μL of PBS, pH 8, 0.84 mM para-nitrophenyl phosphate (pNPP) for a final concentration of 0.8 mM pNPP. Plates were incubated for 2−4 h in the dark at room temperature, and then the plate reading was assessed by using a spectrophotometer at 405 nm (LT 4000, Labtech). The results were normalized with positive control (LPS or PHA-P alone) and expressed as the mean of percentage ± SD of at least three independent experiments. As control, the same procedure was performed in HEK-Blue Null cells, the parental cell line of TLR4 HEK-Blue.

Human Peripheral Blood Mononuclear Cells (hPBMCs). Whole blood was collected from healthy volunteers. Informed consent was obtained from all volunteers. Research using biological samples from mice was performed in accordance with institutional guidelines defined by EU Directive 2010/63/EU for Europe.

Whole blood was diluted 1:1 with PBS and layered on Lymphoprep (STEMCELL Technologies) for density gradient centrifugation according to the manufacturer's instructions. PBMCs were harvested from the interface, washed in PBS, and resuspended in complete RPMI with 10% FBS, 2 mM glutamine, and antibiotics. Informed consent was obtained from all volunteers. Cells were then plated in a 96 multiwell plate (10^5 cells/well) in the presence of different concentrations of the two compounds to be tested. After 30 min, cells were stimulated with 100 ng/mL of LPS and incubated for 18 h (37 °C, 5% CO_2, 95% humidity). Cell supernatants were harvested, and TNF-α, IL-6, and IL-8 cytokines were quantified by ELISA assay (R&D Systems; no. DY206-05, no. DY210-05, no. DY208-05) according to the manufacturer's instructions. The optical density of wells were determined using a microplate reader set to 450 nm (LT 4000, Labtech). All graphs were representative data from at least three independent experiments.

RAW-Blue Cells. RAW-Blue cells (InvivoGen) were cultured according to the manufacturer's instructions. Briefly, cells were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 μg/mL Normocin (InvivoGen), 200 μg/mL Zeocin (InvivoGen). Cells were detached using a cell scraper, and the cell concentration was estimated by using Trypan Blue (Sigma-Aldrich). The cells were diluted in DMEM high glucose medium supplemented as described before and seeded in multiwell plate at a density of 6 × 10^4 cells cells per well in 200 μL. After overnight incubation (37 °C, 5% CO_2, 95% humidity), supernatant was removed and cell monolayers were washed with warm PBS and treated with increasing concentrations of compounds dissolved in DMSO−ethanol (1:1) and diluted in DMEM. After 30 min, the cells were stimulated with 100 ng/mL LPS from *E. coli* O55:B5 (Sigma-Aldrich). Then the supernatants were collected and an amount of 50 μL of each sample was added to 100 μL of PBS, pH 8, 0.84 mM para-nitrophenyl phosphate (pNPP) for a final concentration of 0.8 mM pNPP. Plates were incubated for 2−4 h in the dark at room temperature, and then the plate reading was assessed by using a spectrophotometer at 405 nm (LT 4000, Labtech). The results were normalized with positive control (LPS alone) and expressed as the mean of percentage ± SD of at least three independent experiments.

Murine Splenocytes. Murine splenocytes were isolated from the spleen of balb/c mice (11−13 weeks old), counted, and resuspended in complete RPMI with 10% FBS, 2 mM glutamine, and antibiotics. Cells were then plated in a 24 multiwell plate (1.5 × 10^6 cells/well) in the presence of different concentrations of the two compounds to be tested. After 30 min, cells were stimulated with 100 ng/mL of LPS and incubated for 5 h (37 °C, 5% CO_2, 95% humidity). Cells were lysed, and total RNA was isolated by means of the Quick-RNAMiniPrep purification kit (Zymo Research, R1054). TNF-α expression analyses were performed by real-time qPCR. Gene induction fold changes were normalized to β-actin, shown as mean and SEM of two technical replicates. All graphs were representative data from at least three independent experiments.

MTT Cell Viability Assay. Human embryonic kidney (HEK) 293 cells were grown in DMEM supplemented with 10% FBS, 2 mM glutamine and Penstrep 1×. The cells were seeded in 100 μL of DMEM without phenol red at a density of 2 × 10^4 cells per well 100 μL and incubated overnight (37 °C, 5% CO_2, 95% humidity). Then, the cells were treated with 10 μL of compounds, dissolved in DMSO−ethanol, diluted in DMEM, and incubated again. DMSO 5% and PBS were included as controls. The day after, an amount of 10 μL of MTT solution (5 mg/mL in PBS) was added to each well, and after a 3 h incubation, HCl 0.1 N in 2-propanol was added (100 μL per well) to dissolve formazan crystals. Formazan concentration in the wells was determined by measuring the absorbance at 570 nm (LT 4000, Labtech). The results were normalized with untreated control (PBS) and expressed as the mean of percentage ± SD of three independent experiments.

IL-8 Quantification. Supernatants from HEK-Blue cells treated with compounds **3** (0.1, 1, 5 μM) and **4** (0.1, 1, 10 μM) and stimulated with LPS (100 ng/mL) or PHA-P (25 μM) were used to quantify IL-8 concentration by performing ELISA assay (Thermo scientific) according to the manufacturer's instructions. The readings were assessed by using a spectrophotometer at 450 nm (LT 4000, Labtech).

PAINS. Compounds **1**−**9** were subjected to the pan assay interference compounds (PAINS) online filter (ZINC PAINS patterns search http://zinc15.docking.org/patterns/home/, accessed Jan 26, 2016) and substructure filters.[38] This analysis showed that none of them were PAINS.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b00095.

Molecular modeling, docking results, synthesis and compounds characterization, activity of PHA plant lectin and of compounds **7**−**9** on HEK cells, and MTT toxicity test (PDF)

Molecular formula strings and some data (CSV)

DOI: 10.1021/acs.jmedchem.7b00095
J. Med. Chem. 2017, 60, 4882−4892

AUTHOR INFORMATION

Corresponding Authors

*F.S.: phone, +39.0521.905458; e-mail, francesco.sansone@unipr.it.

*F.P.: phone, +39.02.64483453; e-mail, francesco.peri@unimib.it.

ORCID

Francesco Peri: 0000-0002-3417-8224

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This study was financially supported by the H2020-MSC-ETN-642157 Project TOLLerant. The Italian Ministry for Foreign Affairs and International Cooperation (MAECI) "Progetti di Grande Rilevanza", the Italian Ministry of Instruction, University and Research (MIUR, PRIN2010JMAZML Multi-NanoIta), the COST Action CM1102 "MultiGlycoNano", and the Spanish Ministry for Economy and Competitiveness (MINECO) Grant CTQ2014-57141-R are also acknowledged.

ABBREVIATIONS USED

TLR, Toll-like receptor; PRR, pattern recognition receptor; PAMP, pathogen-associated molecular pattern; MD-2, myeloid differentiation 2; PHA, phytohemagglutinin; LOS, lipooligosaccharide; DC, dendritic cell; DAMP, damage-associated molecular pattern; RA, rheumatoid arthritis; ALS, amyotrophic lateral sclerosis; LBP, lipid binding protein; CD14, cluster of differentiation 14; AMP, antimicrobial peptide; hPBMC, human peripheral blood mononuclear cell; IL, interleukin

REFERENCES

(1) Akira, S.; Takeda, K. Toll-like Receptor Signalling. *Nat. Rev. Immunol.* **2004**, *4*, 499−511.

(2) Poltorak, A.; He, X.; Smirnova, I.; Liu, M.; Van Huffel, C.; Du, X.; Birdwell, D.; Alejos, E.; Silva, M.; Galanos, C.; Freudenberg, M.; Ricciardi-Castagnoli, P.; Layton, B.; Beutler, B. Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in Tlr4 Gene. *Science* **1998**, *282*, 2085−2088.

(3) Gioannini, T.; Teghanemt, A.; Zhang, D.; Coussens, N.; Dockstader, W.; Ramaswamy, S.; Weiss, J. Isolation of an Endotoxin-MD-2 Complex that Produces Toll-like Receptor 4-dependent Cell Activation at Picomolar Concentrations. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 4186−4191.

(4) Park, B.; Song, D.; Kim, H.; Choi, B.; Lee, H.; Lee, J. The Structural Basis of Lipopolysaccharide Recognition by the TLR4-MD-2 Complex. *Nature* **2009**, *458*, 1191−1195.

(5) Gogos, C. A.; Drosou, E.; Bassaris, H. P.; Skoutelis, A. Pro- versus Anti-inflammatory Cytokine Profile in Patients with Severe Sepsis: a Marker for Prognosis and Future Therapeutic Options. *J. Infect. Dis.* **2000**, *181*, 176−180.

(6) Brunialti, M.; Martins, P.; Barbosa de Carvalho, H.; Machado, F.; Barbosa, L.; Salomao, R. TLR2, TLR4, CD14, CD11B, and CD11C Expressions on Monocytes Surface and Cytokine Production in Patients with Sepsis, Severe Sepsis, and Septic Shock. *Shock* **2006**, *25*, 351−357.

(7) Erridge, C. The Roles of Toll-like Receptors in Atherosclerosis. *J. Innate Immun.* **2009**, *1*, 340−349.

(8) Abdollahi-Roodsaz, S.; Joosten, L. A.; Roelofs, M. F.; Radstake, T. R.; Matera, G.; Popa, C.; van der Meer, J. W.; Netea, M. G.; van den Berg, W. B. Inhibition of Toll-like Receptor 4 Breaks The Inflammatory Loop in Autoimmune Destructive Arthritis. *Arthritis Rheum.* **2007**, *56*, 2957−2967.

(9) Cao, L.; Tanga, F. Y.; Deleo, J. A. The Contributing Role of CD14 in Toll-like Receptor 4 Dependent Neuropathic Pain. *Neuroscience* **2009**, *158*, 896−903.

(10) Casula, M.; Iyer, A. M.; Spliet, W. G.; Anink, J. J.; Steentjes, K.; Sta, M.; Troost, D.; Aronica, E. Toll-like Receptor Signaling in Amyotrophic Lateral Sclerosis Spinal Cord Tissue. *Neuroscience* **2011**, *179*, 233−243.

(11) Andreu, D.; Rivas, L. Animal Antimicrobial Peptides: an Overview. *Biopolymers* **1998**, *47*, 415−433.

(12) Rifkind, D. Studies on the Interaction Between Endotoxin and Polymyxin B. *J. Infect. Dis.* **1967**, *117*, 433−438.

(13) David, S. Towards a Rational Development of Anti-endotoxin Agents: Novel Approaches to Sequestration of Bacterial Endotoxins with Small Molecules. *J. Mol. Recognit.* **2001**, *14*, 370−387.

(14) Burns, M.; Jenkins, S.; Kimbrell, M.; Balakrishna, R.; Nguyen, T.; Abbo, B.; David, S. Polycationic Sulfonamides for the Sequestration of Endotoxin. *J. Med. Chem.* **2007**, *50*, 877−888.

(15) Ohto, U.; Fukase, K.; Miyake, K.; Satow, Y. Crystal Structures of Human MD-2 and its Complex with Antiendotoxic Lipid IVa. *Science* **2007**, *316*, 1632−1634.

(16) Takayama, K.; Qureshi, N.; Beutler, B.; Kirkland, T. N. Diphosphoryl Lipid A from Rhodopseudomonas sphaeroides ATCC 17023 Blocks Induction of Cachectin in Macrophages by Lipopolysaccharide. *Infect. Immun.* **1989**, *57*, 1336−1338.

(17) Mullarkey, M.; Rose, J.; Bristol, J.; Kawata, T.; Kimura, A.; Kobayashi, S.; Przetak, M.; Chow, J.; Gusovsky, F.; Christ, W.; Rossignol, D. Inhibition of Endotoxin Response by e5564, a Novel Toll-like Receptor 4-directed Endotoxin Antagonist. *J. Pharmacol. Exp. Ther.* **2003**, *304*, 1093−1102.

(18) Rossignol, D.; Lynn, M. Antagonism of in vivo and ex vivo Response to Endotoxin by E5564, a Synthetic Lipid A Analogue. *J. Endotoxin Res.* **2002**, *8*, 483−488.

(19) Johnson, D.; Sowell, C.; Johnson, C.; Livesay, M.; Keegan, D.; Rhodes, M.; Ulrich, J.; Ward, J.; Cantrell, J.; Brookshire, V. Synthesis and Biological Evaluation of a New Class of Vaccine Adjuvants: Aminoalkyl Glucosaminide 4-phosphates (AGPs). *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2273−2278.

(20) Lewicky, J. D.; Ulanova, M.; Jiang, Z. H. Improving the Immunostimulatory Potency of Diethanolamine-containing Lipid A Mimics. *Bioorg. Med. Chem.* **2013**, *21*, 2199−2209.

(21) Funatogawa, K.; Matsuura, M.; Nakano, M.; Kiso, M.; Hasegawa, A. Relationship of Structure and Biological Activity of Monosaccharide Lipid A Analogues to Induction of Nitric Oxide Production by Murine Macrophage RAW264.7 cells. *Infect. Immun.* **1998**, *66*, 5792−5798.

(22) Kim, H. M.; Park, B. S.; Kim, J. I.; Kim, S. E.; Lee, J.; Oh, S. C.; Enkhbayar, P.; Matsushima, N.; Lee, H.; Yoo, O. J.; Lee, J. O. Crystal Structure of the TLR4-MD-2 Complex with Bound Endotoxin Antagonist Eritoran. *Cell* **2007**, *130*, 906−917.

(23) Shirey, K. A.; Lai, W.; Scott, A. J.; Lipsky, M.; Mistry, P.; Pletneva, L. M.; Karp, C. L.; McAlees, J.; Gioannini, T. L.; Weiss, J.; Chen, W. H.; Ernst, R. K.; Rossignol, D. P.; Gusovsky, F.; Blanco, J. C.; Vogel, S. N. The TLR4 Antagonist Eritoran Protects Mice from Lethal Influenza Infection. *Nature* **2013**, *497*, 498−502.

(24) Piazza, M.; Yu, L.; Teghanemt, A.; Gioannini, T.; Weiss, J.; Peri, F. Evidence of a Specific Interaction between New Synthetic Antisepsis Agents and CD14. *Biochemistry* **2009**, *48*, 12337−12344.

(25) De Paola, M.; Sestito, S. E.; Mariani, A.; Memo, C.; Fanelli, R.; Freschi, M.; Bendotti, C.; Calabrese, V.; Peri, F. Synthetic and Natural Small Molecule TLR4 Antagonists Inhibit Motoneuron Death in Cultures from ALS Mouse Model. *Pharmacol. Res.* **2016**, *103*, 180−187.

(26) Shirey, K. A.; Lai, W.; Patel, M. C.; Pletneva, L. M.; Pang, C.; Kurt-Jones, E.; Lipsky, M.; Roger, T.; Calandra, T.; Tracey, K. J.; Al-Abed, Y.; Bowie, A. G.; Fasano, A.; Dinarello, C. A.; Gusovsky, F.; Blanco, J. C.; Vogel, S. N. Novel Strategies For Targeting Innate Immune Responses to Influenza. *Mucosal Immunol.* **2016**, *9*, 1173−1182.

DOI: 10.1021/acs.jmedchem.7b00095
J. Med. Chem. 2017, 60, 4882−4892

(27) Rodriguez Lavado, J.; Sestito, S. E.; Cighetti, R.; Aguilar Moncayo, E. M.; Oblak, A.; Lainšček, D.; Jiménez Blanco, J. L.; García Fernández, J. M.; Ortiz Mellet, C.; Jerala, R.; Calabrese, V.; Peri, F. Trehalose- and Glucose-derived Glycoamphiphiles: Small-molecule and Nanoparticle Toll-like Receptor 4 (TLR4) Modulators. *J. Med. Chem.* **2014**, *57*, 9105−9123.
(28) Giuliani, M.; Morbioli, I.; Sansone, F.; Casnati, A. Moulding Calixarenes for Biomacromolecule Targeting. *Chem. Commun.* **2015**, *51*, 14140−14159.
(29) Salvio, R.; Volpi, S.; Cacciapaglia, R.; Casnati, A.; Mandolini, L.; Sansone, F. Ribonuclease Activity of an Artificial Catalyst That Combines a Ligated Cu(II) Ion and a Guanidinium Group at the Upper Rim of a cone-Calix[4]arene Platform. *J. Org. Chem.* **2015**, *80*, 5887−5893.
(30) Avvakumova, S.; Fezzardi, P.; Pandolfi, L.; Colombo, M.; Sansone, F.; Casnati, A.; Prosperi, D. Gold Nanoparticles Decorated by Clustered Multivalent Cone-Glycocalixarenes Actively Improve the Targeting Efficiency Toward Cancer Cells. *Chem. Commun.* **2014**, *50*, 11029−11032.
(31) Mochizuki, S.; Nishina, K.; Fujii, S.; Sakurai, K. The Transfection Efficiency of Calix[4]arene-based Lipids: The Role of the Alkyl Chain Length. *Biomater. Sci.* **2015**, *3*, 317−322.
(32) Chen, X.; Dings, R. P.; Nesmelova, I.; Debbert, S.; Haseman, J. R.; Maxwell, J.; Hoye, T. R.; Mayo, K. H. Topomimetics of Amphipathic Beta-sheet and Helix-forming Bactericidal Peptides Neutralize Lipopolysaccharide Endotoxins. *J. Med. Chem.* **2006**, *49*, 7754−7765.
(33) Cighetti, R.; Ciaramelli, C.; Sestito, S. E.; Zanoni, I.; Kubik, L.; Ardá-Freire, A.; Calabrese, V.; Granucci, F.; Jerala, R.; Martín-Santamaría, S.; Jiménez-Barbero, J.; Peri, F. Modulation of CD14 and TLR4-MD-2 Activities by a Synthetic Lipid A Mimetic. *ChemBioChem* **2014**, *15*, 250−258.
(34) Ciaramelli, C.; Calabrese, V.; Sestito, S. E.; Pérez-Regidor, L.; Klett, J.; Oblak, A.; Jerala, R.; Piazza, M.; Martín-Santamaría, S.; Peri, F. Glycolipid-based TLR4Modulators and Fluorescent Probes: Rational Design, Synthesis, and Biological Properties. *Chem. Biol. Drug Des.* **2016**, *88*, 217−229.
(35) Bagnacani, V.; Sansone, F.; Donofrio, G.; Baldini, L.; Casnati, A.; Ungaro, R. Macrocyclic Nonviral Vectors: High Cell Transfection Efficiency and Low Toxicity in a Lower Rim Guanidinium Calix[4]-arene. *Org. Lett.* **2008**, *10*, 3953−3956.
(36) Sansone, F.; Dudic, M.; Donofrio, G.; Rivetti, C.; Baldini, L.; Casnati, A.; Cellai, S.; Ungaro, R. DNA Condensation and Cell Transfection Properties of guanidinocalixarenes: Dependence on Macrocycle Lipophilicity, Size, and Conformation. *J. Am. Chem. Soc.* **2006**, *128*, 14528−14536.
(37) Baldini, L.; Cacciapaglia, R.; Casnati, A.; Mandolini, L.; Salvio, R.; Sansone, F.; Ungaro, R. Upper Rim Guanidinocalix[4]arenes as Artificial Phosphodiesterases. *J. Org. Chem.* **2012**, *77*, 3381−3389.
(38) Barboso, S.; Garcia Carrera, A.; Matthews, S. E.; Arnaud-Neu, F.; Böhmer, V.; Dozol, J.-F.; Rouquette, H.; Schwing-Weill, M.-J. Calix[4]arenes with CMPO Functions at the Narrow Rim. Synthesis and Extraction Properties. *J. Chem. Soc., Perkin Trans. 2* **1999**, 719−724.
(39) Nomura, E.; Hosoda, A.; Takagaki, M.; Mori, H.; Miyake, Y.; Shibakami, M.; Taniguchi, H. Self-organized Honeycomb-patterned Microporous Polystyrene thin Films Fabricated by calix[4]arene Derivatives. *Langmuir* **2010**, *26*, 10266−10270.
(40) Gallego-Yerga, L.; Lomazzi, M.; Franceschi, V.; Sansone, F.; Ortiz Mellet, C.; Donofrio, G.; Casnati, A.; García Fernández, J. M. Cyclodextrin- and Calixarene-based Polycationic Amphiphiles as Gene Delivery Systems: a Structure-activity Relationship Study. *Org. Biomol. Chem.* **2015**, *13*, 1708−1723.
(41) Unitt, J.; Hornigold, D. Plant Lectins are Novel Toll-like Receptor Agonists. *Biochem. Pharmacol.* **2011**, *81*, 1324−1328.
(42) Ohto, U.; Fukase, K.; Miyake, K.; Shimizu, T. Structural Basis of Species-specific Endotoxin Sensing by Innate Immune Receptor TLR4/MD-2. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 7421−7426.
(43) Matsuura, M.; Kiso, M.; Hasegawa, A. Activity of Monosaccharide Lipid A Analogues in Human Monocytic Cells as Agonists or Antagonists of Bacterial Lipopolysaccharide. *Infect. Immun.* **1999**, *67*, 6286−6292.
(44) Ulrich, J.; Myers, K. Monophosphoryl Lipid A as an Adjuvant. Past Experiences and New Directions. *Pharm. Biotechnol.* **1995**, *6*, 495−524.
(45) Resman, N.; Gradisar, H.; Vasl, J.; Keber, M.; Pristovsek, P.; Jerala, R. Taxanes Inhibit Human TLR4 Signaling by Binding to MD-2. *FEBS Lett.* **2008**, *582*, 3929−3934.
(46) Morin, M. D.; Wang, Y.; Jones, B. T.; Su, L.; Surakattula, M. M.; Berger, M.; Huang, H.; Beutler, E. K.; Zhang, H.; Beutler, B.; Boger, D. L. Discovery and Structure-Activity Relationships of the Neoseptins: A New Class of Toll-like Receptor-4 (TLR4) Agonists. *J. Med. Chem.* **2016**, *59*, 4812−4830.
(47) Wang, Y.; Su, L.; Morin, M. D.; Jones, B. T.; Whitby, L. R.; Surakattula, M. M.; Huang, H.; Shi, H.; Choi, J. H.; Wang, K. W.; Moresco, E. M.; Berger, M.; Zhan, X.; Zhang, H.; Boger, D. L.; Beutler, B. TLR4/MD-2 Activation by a Synthetic Agonist with no Similarity to LPS. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113*, E884−E893.
(48) Goligorsky, M. S. TLR4 and HMGB1: Partners in Crime? *Kidney Int.* **2011**, *80*, 450−452.
(49) Park, H. J.; Hong, J. H.; Kwon, H. J.; Kim, Y.; Lee, K. H.; Kim, J. B.; Song, S. K. TLR4-mediated Activation of Mouse Macrophages by Korean Mistletoe Lectin-C (KML-C). *Biochem. Biophys. Res. Commun.* **2010**, *396*, 721−725.
(50) *PyMOL Molecular Graphics System*, version 1.8; Schrodinger, LLC, 2015.
(51) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, J. A., Jr.; Peralta, J. E.; Ogliaro, F.; Bearpark, M. J.; Heyd, J.; Brothers, E. N.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A. P.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, N. J.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, Ö.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J. *Gaussian 09*; Gaussian, Inc.: Wallingford, CT, U.S., 2009.
(52) Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. AutoDock4 and AutoDockTools4: Automated Docking with Selective Receptor Flexibility. *J. Comput. Chem.* **2009**, *30*, 2785−2791.
(53) Trott, O.; Olson, A. J. AutoDock Vina: Improving the Speed and Accuracy of Docking with a New Scoring Function, Efficient Optimization, and Multithreading. *J. Comput. Chem.* **2010**, *31*, 455−461.
(54) Wang, J.; Wang, W.; Kollman, P. A.; Case, D. A. Automatic Atom Type and Bond Type Perception in Molecular Mechanical Calculations. *J. Mol. Graphics Modell.* **2006**, *25*, 247−260.
(55) Case, D.; Babin, V.; Berryman, J.; Betz, R.; Cai, Q.; Cerutti, D.; Cheatham, T., III; Darden, T.; Duke, R.; Gohlke, H.; et al. *AMBER 14*; University of California, San Francisco, 2014.
(56) Wang, J.; Wolf, R. M.; Caldwell, J. W.; Kollman, P. A.; Case, D. A. Development and Testing of a General Amber Force Field. *J. Comput. Chem.* **2004**, *25*, 1157−1174.
(57) Maier, J. A.; Martinez, C.; Kasavajhala, K.; Wickstrom, L.; Hauser, K. E.; Simmerling, C. ff14SB: Improving the Accuracy of Protein Side Chain and Backbone Parameters from ff99SB. *J. Chem. Theory Comput.* **2015**, *11*, 3696−3713.
(58) Baell, J. B.; Holloway, G. A. New Substructure Filters for Removal of Pain Assay Interference Compounds (PAINS) from Screening Libraries and for Their Exclusion in Bioassays. *J. Med. Chem.* **2010**, *53*, 2719−2740.

DOI: 10.1021/acs.jmedchem.7b00095
J. Med. Chem. 2017, 60, 4882−4892

6. BIBLIOGRAPHY

1. Medzhitov, R. Toll-like receptors and innate immunity. *Nature Reviews Immunology* **2001,** 1, 135.
2. Kamada, N.; Seo, S.-U.; Chen, G. Y.; Nunez, G. Role of the gut microbiota in immunity and inflammatory disease. *Nat Rev Immunol* **2013,** 13, 321-335.
3. O'Neill, L. A. J.; Golenbock, D.; Bowie, A. G. The history of Toll-like receptors — redefining innate immunity. **2013,** 13, 453.
4. Li, X.-D.; Chiu, Y.-H.; Ismail, A. S.; Behrendt, C. L.; Wight-Carter, M.; Hooper, L. V.; Chen, Z. J. Mitochondrial antiviral signaling protein (MAVS) monitors commensal bacteria and induces an immune response that prevents experimental colitis. *Proceedings of the National Academy of Sciences of the United States of America* **2011,** 108, 17390-17395.
5. Negishi, H.; Miki, S.; Sarashina, H.; Taguchi-Atarashi, N.; Nakajima, A.; Matsuki, K.; Endo, N.; Yanai, H.; Nishio, J.; Honda, K.; Taniguchi, T. Essential contribution of IRF3 to intestinal homeostasis and microbiota-mediated Tslp gene induction. *Proceedings of the National Academy of Sciences of the United States of America* **2012,** 109, 21016-21021.
6. Hayden, M.; Ghosh, S. *NF-κB in immunobiology*. 2011; Vol. 21, p 223-44.
7. Kumar, H.; Kawai, T.; Akira, S. Pathogen recognition by the innate immune system. *International reviews of immunology* **2011,** 30, 16-34.
8. Kumar, H.; Kawai, T.; Akira, S. Toll-like receptors and innate immunity. *Biochemical and biophysical research communications* **2009,** 388, 621-625.
9. Mogensen, T. H. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical microbiology reviews* **2009,** 22, 240-73, Table of Contents.
10. Oosting, M.; Cheng, S.-C.; Bolscher, J. M.; Vestering-Stenger, R.; Plantinga, T. S.; Verschueren, I. C.; Arts, P.; Garritsen, A.; van Eenennaam, H.; Sturm, P.; Kullberg, B.-J.; Hoischen, A.; Adema, G. J.; van der Meer, J. W. M.; Netea, M. G.; Joosten, L. A. B. Human TLR10 is an anti-inflammatory pattern-recognition receptor. *Proceedings of the National Academy of Sciences of the United States of America* **2014,** 111, E4478-E4484.
11. Morger, J.; Bajnok, J.; Boyce, K.; Craig, P. S.; Rogan, M. T.; Lun, Z.-R.; Hide, G.; Tschirren, B. Naturally occurring Toll-like receptor 11 (TLR11) and Toll-like receptor 12 (TLR12) polymorphisms are not associated with

Toxoplasma gondii infection in wild wood mice. *Infection, Genetics and Evolution* **2014,** 26, 180-184.
12. McCarthy, C. G.; Goulopoulou, S.; Wenceslau, C. F.; Spitler, K.; Matsumoto, T.; Webb, R. C. Toll-like receptors and damage-associated molecular patterns: novel links between inflammation and hypertension. *American Journal of Physiology - Heart and Circulatory Physiology* **2014,** 306, H184-H196.
13. Mann, D. L. THE EMERGING ROLE OF INNATE IMMUNITY IN THE HEART AND VASCULAR SYSTEM: FOR WHOM THE CELL TOLLS. *Circulation research* **2011,** 108, 1133-1145.
14. Akira, S.; Hoshino, K. Myeloid Differentiation Factor 88—Dependent and —Independent Pathways in Toll-Like Receptor Signaling. *The Journal of Infectious Diseases* **2003,** 187, S356-63.
15. Zanoni, I.; Ostuni, R.; Marek, L. R.; Barresi, S.; Barbalat, R.; Barton, G. M.; Granucci, F.; Kagan, J. C. CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* **2011,** 147, 868-80.
16. Karin, M.; Delhase, M. The IκB kinase (IKK) and NF-κB: key elements of proinflammatory signalling. *Seminars in Immunology* **2000,** 12, 85-98.
17. Kawai, T.; Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* **2010,** 11, 373-384.
18. Poltorak, A.; He, X.; Smirnova, I.; Liu, M.-Y.; Huffel, C. V.; Du, X.; Birdwell, D.; Alejos, E.; Silva, M.; Galanos, C.; Freudenberg, M.; Ricciardi-Castagnoli, P.; Layton, B.; Beutler, B. Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in Tlr4 Gene. *Science* **1998,** 282, 2085.
19. Zanin-Zhorov, A.; Tal-Lapidot, G.; Cahalon, L.; Cohen-Sfady, M.; Pevsner-Fischer, M.; Lider, O.; Cohen, I. R. Cutting Edge: T Cells Respond to Lipopolysaccharide Innately via TLR4 Signaling. *The Journal of Immunology* **2007,** 179, 41.
20. Reynolds, J. M.; Martinez, G. J.; Chung, Y.; Dong, C. Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation. *Proceedings of the National Academy of Sciences of the United States of America* **2012,** 109, 13064-13069.
21. Triantafilou, M.; Triantafilou, K. Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. *Trends in Immunology* **2002,** 23, 301-304.
22. Kim, H. M.; Park, B. S.; Kim, J.-I.; Kim, S. E.; Lee, J.; Oh, S. C.; Enkhbayar, P.; Matsushima, N.; Lee, H.; Yoo, O. J.; Lee, J.-O. Crystal Structure of the TLR4-MD-2 Complex with Bound Endotoxin Antagonist Eritoran. *Cell* **2007,** 130, 906-917.

23. Ohto, U.; Yamakawa, N.; Akashi-Takamura, S.; Miyake, K.; Shimizu, T. Structural Analyses of Human Toll-like Receptor 4 Polymorphisms D299G and T399I. *The Journal of biological chemistry* **2012,** 287, 40611-40617.
24. Botos, I.; Segal, D. M.; Davies, D. R. The Structural Biology of Toll-Like Receptors. *Structure (London, England : 1993)* **2011,** 19, 447-459.
25. Bella, J.; Hindle, K. L.; McEwan, P. A.; Lovell, S. C. The leucine-rich repeat structure. *Cellular and Molecular Life Sciences* **2008,** 65, 2307-2333.
26. Jerala, R. Structural biology of the LPS recognition. *International journal of medical microbiology : IJMM* **2007,** 297, 353-63.
27. Gruber, A.; Mancek, M.; Wagner, H.; Kirschning, C. J.; Jerala, R. Structural model of MD-2 and functional role of its basic amino acid clusters involved in cellular lipopolysaccharide recognition. *The Journal of biological chemistry* **2004,** 279, 28475-82.
28. Park, B. S.; Song, D. H.; Kim, H. M.; Choi, B. S.; Lee, H.; Lee, J. O. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* **2009,** 458, 1191-5.
29. Billod, J.-M.; Lacetera, A.; Guzmán-Caldentey, J.; Martín-Santamaría, S. Computational Approaches to Toll-Like Receptor 4 Modulation. *Molecules* **2016,** 21.
30. Holst, O.; Ulmer, A. J.; Brade, H.; Flad, H.-D.; Rietschel, E. T. Biochemistry and cell biology of bacterial endotoxins. *FEMS Immunology & Medical Microbiology* **1996,** 16, 83-104.
31. Erridge, C.; Bennett-Guerrero, E.; Poxton, I. R. Structure and function of lipopolysaccharides. *Microbes and Infection* **2002,** 4, 837-851.
32. Qiao, S.; Luo, Q.; Zhao, Y.; Zhang, X. C.; Huang, Y. Structural basis for lipopolysaccharide insertion in the bacterial outer membrane. *Nature* **2014,** 511, 108-111.
33. Medzhitov, R.; Janeway, C. Innate Immunity. *New England Journal of Medicine* **2000,** 343, 338-344.
34. Hoffmann, J. A.; Kafatos, F. C.; Janeway, C. A.; Ezekowitz, R. A. B. Phylogenetic Perspectives in Innate Immunity. *Science* **1999,** 284, 1313.
35. Rietschel, E. T.; Kirikae, T.; Schade, F. U.; Ulmer, A. J.; Holst, O.; Brade, H.; Schmidt, G.; Mamat, U.; Grimmecke, H.-D.; Kusumoto, S.; Zähringer, U. The chemical structure of bacterial endotoxin in relation to bioactivity. *Immunobiology* **1993,** 187, 169-190.
36. Alexander, C.; Rietschel, E. T. Invited review: Bacterial lipopolysaccharides and innate immunity. *Journal of Endotoxin Research* **2001,** 7, 167-202.

37. Matsuura, M. Structural Modifications of Bacterial Lipopolysaccharide that Facilitate Gram-Negative Bacteria Evasion of Host Innate Immunity. *Frontiers in immunology* **2013,** 4, 109.
38. Gioannini, T. L.; Teghanemt, A.; Zhang, D.; Coussens, N. P.; Dockstader, W.; Ramaswamy, S.; Weiss, J. P. Isolation of an endotoxin–MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. *Proceedings of the National Academy of Sciences* **2004,** 101, 4186-4191.
39. Park, B. S.; Lee, J.-O. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Experimental & Molecular Medicine* **2013,** 45, e66.
40. Miyake, K. Innate recognition of lipopolysaccharide by CD14 and toll-like receptor 4-MD-2: unique roles for MD-2. *International Immunopharmacology* **2003,** 3, 119-128.
41. Heumann, D.; Lauener, R.; Ryffel, B. The dual role of LBP and CD14 in response to Gram-negative bacteria or Gram-negative compounds. *Journal of Endotoxin Research* **2003,** 9, 381-384.
42. Gioannini, T. L.; Weiss, J. P. Regulation of interactions of Gram-negative bacterial endotoxins with mammalian cells. *Immunologic Research* **2007,** 39, 249-260.
43. Lu, Y.-C.; Yeh, W.-C.; Ohashi, P. S. LPS/TLR4 signal transduction pathway. *Cytokine* **2008,** 42, 145-151.
44. Carpenter, S.; Neill, Luke A. J. Recent insights into the structure of Toll-like receptors and post-translational modifications of their associated signalling proteins. *Biochemical Journal* **2009,** 422, 1.
45. Ohto, U.; Fukase, K.; Miyake, K.; Satow, Y. Crystal Structures of Human MD-2 and Its Complex with Antiendotoxic Lipid IVa. *Science* **2007,** 316, 1632.
46. Kagan, J. C.; Medzhitov, R. Phosphoinositide-Mediated Adaptor Recruitment Controls Toll-like Receptor Signaling. *Cell* **2006,** 125, 943-955.
47. Bonham, Kevin S.; Orzalli, Megan H.; Hayashi, K.; Wolf, Amaya I.; Glanemann, C.; Weninger, W.; Iwasaki, A.; Knipe, David M.; Kagan, Jonathan C. A Promiscuous Lipid-Binding Protein Diversifies the Subcellular Sites of Toll-like Receptor Signal Transduction. *Cell* **2014,** 156, 705-716.
48. Lin, S.-C.; Lo, Y.-C.; Wu, H. Helical assembly in the MyD88–IRAK4–IRAK2 complex in TLR/IL-1R signalling. *Nature* **2010,** 465, 885-890.
49. Kagan, J. C.; Magupalli, V. G.; Wu, H. SMOCs: supramolecular organizing centres that control innate immunity. *Nat Rev Immunol* **2014,** 14, 821-826.
50. Medzhitov, R.; Horng, T. Transcriptional control of the inflammatory response. *Nat Rev Immunol* **2009,** 9, 692-703.

51. Tan, Y.; Zanoni, I.; Cullen, T. W.; Goodman, A. L.; Kagan, J. C. Mechanisms of Toll-like Receptor 4 Endocytosis Reveal a Common Immune-Evasion Strategy Used by Pathogenic and Commensal Bacteria. *Immunity* **2015,** 43, 909-22.
52. Kagan, J. C.; Su, T.; Horng, T.; Chow, A.; Akira, S.; Medzhitov, R. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-β. *Nature immunology* **2008,** 9, 361-368.
53. Lien, E.; Means, T. K.; Heine, H.; Yoshimura, A.; Kusumoto, S.; Fukase, K.; Fenton, M. J.; Oikawa, M.; Qureshi, N.; Monks, B.; Finberg, R. W.; Ingalls, R. R.; Golenbock, D. T. Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. *Journal of Clinical Investigation* **2000,** 105, 497-504.
54. Poltorak, A.; Ricciardi-Castagnoli, P.; Citterio, S.; Beutler, B. Physical contact between lipopolysaccharide and Toll-like receptor 4 revealed by genetic complementation. *Proceedings of the National Academy of Sciences of the United States of America* **2000,** 97, 2163-2167.
55. Akashi, S.; Nagai, Y.; Ogata, H.; Oikawa, M.; Fukase, K.; Kusumoto, S.; Kawasaki, K.; Nishijima, M.; Hayashi, S.; Kimoto, M.; Miyake, K. Human MD-2 confers on mouse Toll-like receptor 4 species-specific lipopolysaccharide recognition. *International Immunology* **2001,** 13, 1595-1599.
56. Muroi, M.; Ohnishi, T.; Tanamoto, K.-i. MD-2, a Novel Accessory Molecule, Is Involved in Species-Specific Actions of Salmonella Lipid A. *Infection and immunity* **2002,** 70, 3546-3550.
57. Hajjar, A. M.; Ernst, R. K.; Tsai, J. H.; Wilson, C. B.; Miller, S. I. Human Toll-like receptor 4 recognizes host-specific LPS modifications. *Nat Immunol* **2002,** 3, 354-359.
58. Scior, T.; Lozano-Aponte, J.; Figueroa-Vazquez, V.; Yunes-Rojas, J. A.; Zähringer, U.; Alexander, C. Three-dimensional mapping of differential amino acids of human, murine, canine and equine TLR4/MD-2 receptor complexes conferring endotoxic activation by lipid A, antagonism by Eritoran and species-dependent activities of Lipid IVA in the mammalian LPS sensor system. *Computational and Structural Biotechnology Journal* **2013,** 7, e201305003.
59. Srikrishna, G.; Freeze, H. H. Endogenous Damage-Associated Molecular Pattern Molecules at the Crossroads of Inflammation and Cancer. *Neoplasia (New York, N.Y.)* **2009,** 11, 615-628.
60. Tolle, L. B.; Standiford, T. J. Danger-associated molecular patterns (DAMPs) in acute lung injury. *The Journal of Pathology* **2013,** 229, 145-156.
61. Scaffidi, P.; Misteli, T.; Bianchi, M. E. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* **2002,** 418, 191.

62. van Zoelen, M. A. D.; Yang, H.; Florquin, S.; Meijers, J. C. M.; Akira, S.; Arnold, B.; Nawroth, P. P.; Bierhaus, A.; Tracey, K. J.; van der Poll, T. ROLE OF TOLL-LIKE RECEPTORS 2 AND 4, AND THE RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS IN HIGH-MOBILITY GROUP BOX 1–INDUCED INFLAMMATION IN VIVO. *Shock (Augusta, Ga.)* **2009,** 31, 280-284.
63. Vogl, T.; Tenbrock, K.; Ludwig, S.; Leukert, N.; Ehrhardt, C.; van Zoelen, M. A. D.; Nacken, W.; Foell, D.; van der Poll, T.; Sorg, C.; Roth, J. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nature Medicine* **2007,** 13, 1042.
64. Hiratsuka, S.; Watanabe, A.; Sakurai, Y.; Akashi-Takamura, S.; Ishibashi, S.; Miyake, K.; Shibuya, M.; Akira, S.; Aburatani, H.; Maru, Y. The S100A8–serum amyloid A3–TLR4 paracrine cascade establishes a pre-metastatic phase. *Nature Cell Biology* **2008,** 10, 1349.
65. Okamura, Y.; Watari, M.; Jerud, E. S.; Young, D. W.; Ishizaka, S. T.; Rose, J.; Chow, J. C.; Strauss, J. F. The Extra Domain A of Fibronectin Activates Toll-like Receptor 4. *Journal of Biological Chemistry* **2001,** 276, 10229-10233.
66. Lefebvre, J. S.; Lévesque, T.; Picard, S.; Paré, G.; Gravel, A.; Flamand, L.; Borgeat, P. Extra domain A of fibronectin primes leukotriene biosynthesis and stimulates neutrophil migration through activation of Toll-like receptor 4. *Arthritis & Rheumatism* **2011,** 63, 1527-1533.
67. Jiang, D.; Liang, J.; Fan, J.; Yu, S.; Chen, S.; Luo, Y.; Prestwich, G. D.; Mascarenhas, M. M.; Garg, H. G.; Quinn, D. A.; Homer, R. J.; Goldstein, D. R.; Bucala, R.; Lee, P. J.; Medzhitov, R.; Noble, P. W. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nature Medicine* **2005,** 11, 1173.
68. Lugrin, J.; Rosenblatt-Velin, N.; Parapanov, R.; Liaudet, L. The role of oxidative stress during inflammatory processes. In *Biological chemistry*, 2014; Vol. 395, p 203.
69. Lorne, E.; Dupont, H.; Abraham, E. Toll-like receptors 2 and 4: initiators of non-septic inflammation in critical care medicine? *Intensive care medicine* **2010,** 36, 1826-1835.
70. Stewart, C. R.; Stuart, L. M.; Wilkinson, K.; van Gils, J. M.; Deng, J.; Halle, A.; Rayner, K. J.; Boyer, L.; Zhong, R.; Frazier, W. A.; Lacy-Hulbert, A.; El Khoury, J.; Golenbock, D. T.; Moore, K. J. CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. *Nature immunology* **2010,** 11, 155-161.
71. Yang, K.; Zhang, X. J.; Cao, L. J.; Liu, X. H.; Liu, Z. H.; Wang, X. Q.; Chen, Q. J.; Lu, L.; Shen, W. F.; Liu, Y. Toll-Like Receptor 4 Mediates Inflammatory Cytokine Secretion in Smooth Muscle Cells Induced by Oxidized Low-Density Lipoprotein. *PloS one* **2014,** 9, e95935.

72. Erridge, C.; Kennedy, S.; Spickett, C. M.; Webb, D. J. Oxidized Phospholipid Inhibition of Toll-like Receptor (TLR) Signaling Is Restricted to TLR2 and TLR4: ROLES FOR CD14, LPS-BINDING PROTEIN, AND MD2. *The Journal of biological chemistry* **2008,** 283, 24748-24759.
73. Manček-Keber, M.; Frank-Bertoncelj, M.; Hafner-Bratkovič, I.; Smole, A.; Zorko, M.; Pirher, N.; Hayer, S.; Kralj-Iglič, V.; Rozman, B.; Ilc, N.; Horvat, S.; Jerala, R. Toll-like receptor 4 senses oxidative stress mediated by the oxidation of phospholipids in extracellular vesicles. *Science signaling* **2015,** 8, ra60.
74. Erridge, C. Endogenous ligands of TLR2 and TLR4: agonists or assistants? *Journal of leukocyte biology* **2010,** 87, 989-999.
75. Ketloy, C.; Engering, A.; Srichairatanakul, U.; Limsalakpetch, A.; Yongvanitchit, K.; Pichyangkul, S.; Ruxrungtham, K. Expression and function of Toll-like receptors on dendritic cells and other antigen presenting cells from non-human primates. *Veterinary Immunology and Immunopathology* **2008,** 125, 18-30.
76. Muzio, M.; Bosisio, D.; Polentarutti, N.; D'amico, G.; Stoppacciaro, A.; Mancinelli, R.; van't Veer, C.; Penton-Rol, G.; Ruco, L. P.; Allavena, P.; Mantovani, A. Differential Expression and Regulation of Toll-Like Receptors (TLR) in Human Leukocytes: Selective Expression of TLR3 in Dendritic Cells. *The Journal of Immunology* **2000,** 164, 5998.
77. Mita, Y.; Dobashi, K.; Endou, K.; Kawata, T.; Shimizu, Y.; Nakazawa, T.; Mori, M. Toll-like receptor 4 surface expression on human monocytes and B cells is modulated by IL-2 and IL-4. *Immunology Letters* **2002,** 81, 71-75.
78. Zarember, K. A.; Godowski, P. J. Tissue Expression of Human Toll-Like Receptors and Differential Regulation of Toll-Like Receptor mRNAs in Leukocytes in Response to Microbes, Their Products, and Cytokines. *The Journal of Immunology* **2002,** 168, 554.
79. Bsibsi, M.; Ravid, R.; Gveric, D.; van Noort, J. M. Broad Expression of Toll-Like Receptors in the Human Central Nervous System. *Journal of Neuropathology & Experimental Neurology* **2002,** 61, 1013-1021.
80. Vaure, C.; Liu, Y. A Comparative Review of Toll-Like Receptor 4 Expression and Functionality in Different Animal Species. *Frontiers in immunology* **2014,** 5, 316.
81. Leow-Dyke, S.; Allen, C.; Denes, A.; Nilsson, O.; Maysami, S.; Bowie, A. G.; Rothwell, N. J.; Pinteaux, E. Neuronal toll-like receptor 4 signaling induces brain endothelial activation and neutrophil transmigration in vitro. *Journal of Neuroinflammation* **2012,** 9, 230-230.

82. Wadachi, R.; Hargreaves, K. M. Trigeminal Nociceptors Express TLR-4 and CD14: a Mechanism for Pain due to Infection. *Journal of Dental Research* **2006,** 85, 49-53.
83. Ye, Y.; Xu, H.; Zhang, X.; Li, Z.; Jia, Y.; He, X.; Huang, J. H. Association between Toll-Like Receptor 4 Expression and Neural Stem Cell Proliferation in the Hippocampus Following Traumatic Brain Injury in Mice. *International Journal of Molecular Sciences* **2014,** 15, 12651-12664.
84. Rolls, A.; Shechter, R.; London, A.; Ziv, Y.; Ronen, A.; Levy, R.; Schwartz, M. Toll-like receptors modulate adult hippocampal neurogenesis. *Nature Cell Biology* **2007,** 9, 1081.
85. Zeuner, M.; Bieback, K.; Widera, D. Controversial Role of Toll-like Receptor 4 in Adult Stem Cells. *Stem Cell Reviews and Reports* **2015,** 11, 621-634.
86. Anstadt, E. J.; Fujiwara, M.; Wasko, N.; Nichols, F.; Clark, R. B. TLR Tolerance as a Treatment for Central Nervous System Autoimmunity. *The Journal of Immunology* **2016,** 197, 2110.
87. Garay-Malpartida, H. M.; Mourão, R. F.; Mantovani, M.; Santos, I. A.; Sogayar, M. C.; Goldberg, A. C. Toll-like receptor 4 (TLR4) expression in human and murine pancreatic beta-cells affects cell viability and insulin homeostasis. *BMC Immunology* **2011,** 12, 18.
88. Jia, L.; Vianna, C. R.; Fukuda, M.; Berglund, E. D.; Liu, C.; Tao, C.; Sun, K.; Liu, T.; Harper, M. J.; Lee, C. E.; Lee, S.; Scherer, P. E.; Elmquist, J. K. Hepatocyte Toll-like receptor 4 regulates obesity-induced inflammation and insulin resistance. *Nature Communications* **2014,** 5, 3878.
89. Bès-Houtmann, S.; Roche, R.; Hoareau, L.; Gonthier, M.-P.; Festy, F.; Caillens, H.; Gasque, P.; Lefebvre d'Hellencourt, C.; Cesari, M. Presence of functional TLR2 and TLR4 on human adipocytes. *Histochemistry and Cell Biology* **2007,** 127, 131-137.
90. Eißler, R.; Schmaderer, C.; Rusai, K.; Kühne, L.; Sollinger, D.; Lahmer, T.; Witzke, O.; Lutz, J.; Heemann, U.; Baumann, M. Hypertension augments cardiac Toll-like receptor 4 expression and activity. *Hypertension Research* **2011,** 34, 551.
91. Pryshchep, O.; Ma-Krupa, W.; Younge, B. R.; Goronzy, J. J.; Weyand, C. M. Vessel-specific Toll-like receptor profiles in human medium and large arteries. *Circulation* **2008,** 118, 1276-1284.
92. Cario, E.; Podolsky, D. K. Differential Alteration in Intestinal Epithelial Cell Expression of Toll-Like Receptor 3 (TLR3) and TLR4 in Inflammatory Bowel Disease. *Infection and immunity* **2000,** 68, 7010-7017.

93. Faure, E.; Equils, O.; Sieling, P. A.; Thomas, L.; Zhang, F. X.; Kirschning, C. J.; Polentarutti, N.; Muzio, M.; Arditi, M. Bacterial Lipopolysaccharide Activates NF-κB through Toll-like Receptor 4 (TLR-4) in Cultured Human Dermal Endothelial Cells: DIFFERENTIAL EXPRESSION OF TLR-4 AND TLR-2 IN ENDOTHELIAL CELLS. *Journal of Biological Chemistry* **2000,** 275, 11058-11063.
94. Cario, E. Toll-like receptors in inflammatory bowel diseases: a decade later. *Inflammatory bowel diseases* **2010,** 16, 1583-97.
95. Lynn, L. S.; Gerene, M. D.; Neal, L. W. Endotoxin, TLR4 Signaling and Vascular Inflammation: Potential Therapeutic Targets in Cardiovascular Disease. *Current Pharmaceutical Design* **2006,** 12, 4229-4245.
96. Curtiss, L. K.; Tobias, P. S. Emerging role of Toll-like receptors in atherosclerosis. *Journal of Lipid Research* **2009,** 50, S340-S345.
97. Stifano, G.; Affandi, A. J.; Mathes, A. L.; Rice, L. M.; Nakerakanti, S.; Nazari, B.; Lee, J.; Christmann, R. B.; Lafyatis, R. Chronic Toll-like receptor 4 stimulation in skin induces inflammation, macrophage activation, transforming growth factor beta signature gene expression, and fibrosis. *Arthritis Research & Therapy* **2014,** 16, R136-R136.
98. Panzer, R.; Blobel, C.; Fölster-Holst, R.; Proksch, E. TLR2 and TLR4 expression in atopic dermatitis, contact dermatitis and psoriasis. *Experimental Dermatology* **2014,** 23, 364-366.
99. Abdollahi-Roodsaz, S.; Joosten, L. A. B.; Roelofs, M. F.; Radstake, T. R. D. J.; Matera, G.; Popa, C.; van der Meer, J. W. M.; Netea, M. G.; van den Berg, W. B. Inhibition of toll-like receptor 4 breaks the inflammatory loop in autoimmune destructive arthritis. *Arthritis & Rheumatism* **2007,** 56, 2957-2967.
100. Cao, L.; Tanga, F. Y.; DeLeo, J. A. The Contributing Role of CD14 in Toll-Like Receptor 4 Dependent Neuropathic Pain. *Neuroscience* **2009,** 158, 896-903.
101. Casula, M.; Iyer, A. M.; Spliet, W. G. M.; Anink, J. J.; Steentjes, K.; Sta, M.; Troost, D.; Aronica, E. Toll-like receptor signaling in amyotrophic lateral sclerosis spinal cord tissue. *Neuroscience* **2011,** 179, 233-243.
102. Singer, M.; Deutschman, C. S.; Seymour, C. W.; Shankar-Hari, M.; Annane, D.; Bauer, M.; Bellomo, R.; Bernard, G. R.; Chiche, J.-D.; Coopersmith, C. M.; Hotchkiss, R. S.; Levy, M. M.; Marshall, J. C.; Martin, G. S.; Opal, S. M.; Rubenfeld, G. D.; van der Poll, T.; Vincent, J.-L.; Angus, D. C. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* **2016,** 315, 801-810.
103. Angus, D. C.; Linde-Zwirble, W. T.; Lidicker, J.; Clermont, G.; Carcillo, J.; Pinsky, M. R. Epidemiology of severe sepsis in the United States: Analysis of

incidence, outcome, and associated costs of care. *Critical Care Medicine* **2001,** 29.
104. Ramachandran, G. Gram-positive and gram-negative bacterial toxins in sepsis: A brief review. *Virulence* **2014,** 5, 213-218.
105. Morrison, D. C.; Ryan, J. L. Endotoxins and disease mechanisms. *Annual Review of Medicine* **1987,** 38, 417-432.
106. Delvos, U.; Janssen, B.; Müller-Berghaus, G. Effect of lipopolysaccharides on cultured human endothelial cells. *Blut* **1987,** 55, 101-108.
107. Hoshino, K.; Takeuchi, O.; Kawai, T.; Sanjo, H.; Ogawa, T.; Takeda, Y.; Takeda, K.; Akira, S. Cutting Edge: Toll-Like Receptor 4 (TLR4)-Deficient Mice Are Hyporesponsive to Lipopolysaccharide: Evidence for TLR4 as the Lps Gene Product. *The Journal of Immunology* **1999,** 162, 3749.
108. Sørensen, L. K.; Havemose-Poulsen, A.; Sønder, S. U.; Bendtzen, K.; Holmstrup, P. Blood Cell Gene Expression Profiling in Subjects With Aggressive Periodontitis and Chronic Arthritis. *Journal of Periodontology* **2008,** 79, 477-485.
109. Kim, K.-W.; Cho, M.-L.; Lee, S.-H.; Oh, H.-J.; Kang, C.-M.; Ju, J. H.; Min, S.-Y.; Cho, Y.-G.; Park, S.-H.; Kim, H.-Y. Human rheumatoid synovial fibroblasts promote osteoclastogenic activity by activating RANKL via TLR-2 and TLR-4 activation. *Immunology Letters* **2007,** 110, 54-64.
110. Huang, Q.-Q.; Pope, R. M. ROLE OF TOLL LIKE RECEPTORS IN RHEUMATOID ARTHRITIS. *Current rheumatology reports* **2009,** 11, 357-364.
111. Dasu, M. R.; Devaraj, S.; Park, S.; Jialal, I. Increased Toll-Like Receptor (TLR) Activation and TLR Ligands in Recently Diagnosed Type 2 Diabetic Subjects. *Diabetes Care* **2010,** 33, 861-868.
112. Kim, J. J.; Sears, D. D. *TLR4 and Insulin Resistance*. 2010; Vol. 2010, p 11.
113. Cani, P. D.; Amar, J.; Iglesias, M. A.; Poggi, M.; Knauf, C.; Bastelica, D.; Neyrinck, A. M.; Fava, F.; Tuohy, K. M.; Chabo, C.; Waget, A.; Delmée, E.; Cousin, B.; Sulpice, T.; Chamontin, B.; Ferrières, J.; Tanti, J.-F.; Gibson, G. R.; Casteilla, L.; Delzenne, N. M.; Alessi, M. C.; Burcelin, R. Metabolic Endotoxemia Initiates Obesity and Insulin Resistance. *Diabetes* **2007,** 56, 1761.
114. Patrice, D. C.; Nathalie, M. D. The Role of the Gut Microbiota in Energy Metabolism and Metabolic Disease. *Current Pharmaceutical Design* **2009,** 15, 1546-1558.
115. Brun, P.; Castagliuolo, I.; Leo, V. D.; Buda, A.; Pinzani, M.; Palù, G.; Martines, D. Increased intestinal permeability in obese mice: new evidence in

the pathogenesis of nonalcoholic steatohepatitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **2007,** 292, G518-G525.

116. Erridge, C.; Attina, T.; Spickett, C. M.; Webb, D. J. A high-fat meal induces low-grade endotoxemia: evidence of a novel mechanism of postprandial inflammation. *The American Journal of Clinical Nutrition* **2007,** 86, 1286-1292.

117. Ghoshal, S.; Witta, J.; Zhong, J.; de Villiers, W.; Eckhardt, E. Chylomicrons promote intestinal absorption of lipopolysaccharides. *Journal of Lipid Research* **2009,** 50, 90-97.

118. Walter, S.; Letiembre, M.; Liu, Y.; Heine, H.; Penke, B.; Hao, W.; Bode, B.; Manietta, N.; Walter, J.; Schulz-Schüffer, W.; Fassbender, K. Role of the Toll-Like Receptor 4 in Neuroinflammation in Alzheimer's Disease. *Cellular Physiology and Biochemistry* **2007,** 20, 947-956.

119. Frantz, S.; Ertl, G.; Bauersachs, J. Mechanisms of Disease: Toll-like receptors in cardiovascular disease. *Nature Clinical Practice Cardiovascular Medicine* **2007,** 4, 444.

120. Bomfim, Gisele F.; Santos, Rosangela A. D.; Oliveira, Maria A.; Giachini, Fernanda R.; Akamine, Eliana H.; Tostes, Rita C.; Fortes, Zuleica B.; Webb, R. C.; Carvalho, Maria Helena C. Toll-like receptor 4 contributes to blood pressure regulation and vascular contraction in spontaneously hypertensive rats. *Clinical Science* **2012,** 122, 535.

121. Liang, C.-F.; Liu, J. T. C.; Wang, Y.; Xu, A.; Vanhoutte, P. M. Toll-Like Receptor 4 Mutation Protects Obese Mice Against Endothelial Dysfunction by Decreasing NADPH Oxidase Isoforms 1 and 4. *Arteriosclerosis, Thrombosis, and Vascular Biology* **2013,** 33, 777.

122. Funda, D. P.; Tučková, L.; Farré, M. A.; Iwase, T.; Moro, I.; Tlaskalová-Hogenová, H. CD14 Is Expressed and Released as Soluble CD14 by Human Intestinal Epithelial Cells In Vitro: Lipopolysaccharide Activation of Epithelial Cells Revisited. *Infection and immunity* **2001,** 69, 3772-3781.

123. Abreu, M. T.; Vora, P.; Faure, E.; Thomas, L. S.; Arnold, E. T.; Arditi, M. Decreased Expression of Toll-Like Receptor-4 and MD-2 Correlates with Intestinal Epithelial Cell Protection Against Dysregulated Proinflammatory Gene Expression in Response to Bacterial Lipopolysaccharide. *The Journal of Immunology* **2001,** 167, 1609.

124. Smith, P. D.; Smythies, L. E.; Mosteller-Barnum, M.; Sibley, D. A.; Russell, M. W.; Merger, M.; Sellers, M. T.; Orenstein, J. M.; Shimada, T.; Graham, M. F.; Kubagawa, H. Intestinal Macrophages Lack CD14 and CD89 and Consequently Are Down-Regulated for LPS- and IgA-Mediated Activities. *The Journal of Immunology* **2001,** 167, 2651.

125. Abreu, M. T.; Arnold, E. T.; Thomas, L. S.; Gonsky, R.; Zhou, Y.; Hu, B.; Arditi, M. TLR4 and MD-2 Expression Is Regulated by Immune-mediated Signals in Human Intestinal Epithelial Cells. *Journal of Biological Chemistry* **2002,** 277, 20431-20437.
126. M. C. GRIMM, P. P., E. VAN DE POL & W. F. DOE. Evidence for a CD14+ population of monocytes in inflammatory bowel disease mucosa-implications for pathogenesis. *Clin Exp Immunol* **1995,** 100, 291-297.
127. Cribbs, S. K.; Martin, G. S. Expanding the global epidemiology of sepsis*. *Critical Care Medicine* **2007,** 35.
128. Andreu, D.; Rivas, L. Animal antimicrobial peptides: An overview. *Peptide Science* **1998,** 47, 415-433.
129. David, S. A. Towards a rational development of anti-endotoxin agents: novel approaches to sequestration of bacterial endotoxins with small molecules. **2001**.
130. Burns, M. R.; Jenkins, S. A.; Kimbrell, M. R.; Balakrishna, R.; Nguyen, T. B.; Abbo, B. G.; David, S. A. Polycationic Sulfonamides for the Sequestration of Endotoxin. *Journal of medicinal chemistry* **2007,** 50, 877-888.
131. Mullarkey, M.; Rose, J. R.; Bristol, J.; Kawata, T.; Kimura, A.; Kobayashi, S.; Przetak, M.; Chow, J.; Gusovsky, F.; Christ, W. J.; Rossignol, D. P. Inhibition of Endotoxin Response by E5564, a Novel Toll-Like Receptor 4-Directed Endotoxin Antagonist. *Journal of Pharmacology and Experimental Therapeutics* **2003,** 304, 1093.
132. Takayama, K.; Qureshi, N.; Beutler, B.; Kirkland, T. N. Diphosphoryl lipid A from Rhodopseudomonas sphaeroides ATCC 17023 blocks induction of cachectin in macrophages by lipopolysaccharide. *Infection and immunity* **1989,** 57, 1336-1338.
133. Rossignol, D. P.; Lynn, M. Antagonism of in vivo and ex vivo response to endotoxin by E5564, a synthetic lipid A analogue. *Journal of Endotoxin Research* **2002,** 8, 483-488.
134. Lewicky, J. D.; Ulanova, M.; Jiang, Z.-H. Improving the immunostimulatory potency of diethanolamine-containing lipid A mimics. *Bioorganic & Medicinal Chemistry* **2013,** 21, 2199-2209.
135. De Paola, M.; Sestito, S. E.; Mariani, A.; Memo, C.; Fanelli, R.; Freschi, M.; Bendotti, C.; Calabrese, V.; Peri, F. Synthetic and natural small molecule TLR4 antagonists inhibit motoneuron death in cultures from ALS mouse model. *Pharmacological research* **2016,** 103, 180-7.
136. Shirey, K. A.; Lai, W.; Patel, M. C.; Pletneva, L. M.; Pang, C.; Kurt-Jones, E.; Lipsky, M.; Roger, T.; Calandra, T.; Tracey, K.; Al-Abed, Y.; Bowie, A. G.; Fasano, A.; Dinarello, C.; Gusovsky, F.; Blanco, J. C. G.; Vogel, S. N. Novel

Strategies for Targeting Innate Immune Responses to Influenza. *Mucosal immunology* **2016,** 9, 1173-1182.
137. Johnson, D.; Gregory Sowell, C.; L. Johnson, C.; T. Livesay, M.; S. Keegan, D.; Rhodes, M.; Terry Ulrich, J.; R. Ward, J.; L. Cantrell, J.; G. Brookshire, V. *Synthesis and biological evaluation of a new class of vaccine adjuvants: Aminoalkyl glucosaminide 4-phosphates (AGPs)*. 1999; Vol. 9, p 2273-8.
138. Yeaman, M. R.; Yount, N. Y. Mechanisms of Antimicrobial Peptide Action and Resistance. *Pharmacological Reviews* **2003,** 55, 27.
139. Rifkind, D. Studies on the interaction between endotoxin and polymyxin B. *journal of infectious diseases* **1967,** 117, 433-438.
140. Lee, J. Y.; Boman, A.; Sun, C. X.; Andersson, M.; Jörnvall, H.; Mutt, V.; Boman, H. G. Antibacterial peptides from pig intestine: isolation of a mammalian cecropin. *Proceedings of the National Academy of Sciences of the United States of America* **1989,** 86, 9159-9162.
141. Zasloff, M.; Martin, B.; Chen, H. C. Antimicrobial activity of synthetic magainin peptides and several analogues. *Proceedings of the National Academy of Sciences of the United States of America* **1988,** 85, 910-913.
142. Agerberth, B.; Lee, J.-Y.; Bergman, T.; Carlquist, M.; Boman, H. G.; Mutt, V.; JÖRnvall, H. Amino acid sequence of PR-39. *European Journal of Biochemistry* **1991,** 202, 849-854.
143. Kawano, K.; Yoneya, T.; Miyata, T.; Yoshikawa, K.; Tokunaga, F.; Terada, Y.; Iwanaga, S. Antimicrobial peptide, tachyplesin I, isolated from hemocytes of the horseshoe crab (Tachypleus tridentatus). NMR determination of the beta-sheet structure. *Journal of Biological Chemistry* **1990,** 265, 15365-15367.
144. Lehrer, R. I.; Ganz, T.; Selsted, M. E. Defensins: Endogenous antibiotic peptides of animal cells. *Cell* **1991,** 64, 229-230.
145. Japelj, B.; Pristovšek, P.; Majerle, A.; Jerala, R. Structural Origin of Endotoxin Neutralization and Antimicrobial Activity of a Lactoferrin-based Peptide. *Journal of Biological Chemistry* **2005,** 280, 16955-16961.
146. Mayo, K. H.; Haseman, J.; Young, H. C.; Mayo, J. W. Structure-function relationships in novel peptide dodecamerswith broad-spectrum bactericidal and endotoxin-neutralizing activities. *Biochemical Journal* **2000,** 349, 717-728.
147. Moore, A. J.; Beazley Wd Fau - Bibby, M. C.; Bibby Mc Fau - Devine, D. A.; Devine, D. A. Antimicrobial activity of cecropins. *Journal of Antimicrobial Chemotherapy* **1996,** 37, 1077-89.
148. Steiner, H.; Hultmark, D.; Engström, Å.; Bennich, H.; Boman, H. G. Pillars Article: Sequence and Specificity of Two Antibacterial Proteins Involved

in Insect Immunity. Nature 1981. 292: 246–248. *The Journal of Immunology* **2009,** 182, 6635.
149. De Lucca, A. J.; Jacks, T. J.; Brogden, K. A. Binding between lipopolysaccharide and cecropin A. *Molecular and Cellular Biochemistry* **1995,** 151, 141-148.
150. Zanetti, M.; Gennaro, R.; Romeo, D. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Letters* **1995,** 374, 1-5.
151. Cowland, J. B.; Johnsen, A. H.; Borregaard, N. hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules. *FEBS Letters* **1995,** 368, 173-176.
152. Wan, M.; van der Does, A. M.; Tang, X.; Lindbom, L.; Agerberth, B.; Haeggstrom, J. Z. Antimicrobial peptide LL-37 promotes bacterial phagocytosis by human macrophages. *Journal of leukocyte biology* **2014,** 95, 971-81.
153. De, Y.; Chen, Q.; Schmidt, A. P.; Anderson, G. M.; Wang, J. M.; Wooters, J.; Oppenheim, J. J.; Chertov, O. Ll-37, the Neutrophil Granule–And Epithelial Cell–Derived Cathelicidin, Utilizes Formyl Peptide Receptor–Like 1 (Fprl1) as a Receptor to Chemoattract Human Peripheral Blood Neutrophils, Monocytes, and T Cells. *The Journal of Experimental Medicine* **2000,** 192, 1069-1074.
154. Golec, M. Cathelicidin LL-37: LPS-neutralizing, pleiotropic peptide. **2007**.
155. Scott, A.; Weldon, S.; Buchanan, P. J.; Schock, B.; Ernst, R. K.; McAuley, D. F.; Tunney, M. M.; Irwin, C. R.; Elborn, J. S.; Taggart, C. C. Evaluation of the ability of LL-37 to neutralise LPS in vitro and ex vivo. *PloS one* **2011,** 6, e26525.
156. Raj, P. A.; Dentino, A. R. Current status of defensins and their role in innate and adaptive immunity. *FEMS Microbiology Letters* **2002,** 206, 9-18.
157. Izadpanah, A.; Gallo, R. L. Antimicrobial peptides. *Journal of the American Academy of Dermatology* **2005,** 52, 381-390.
158. Kaconis, Y.; Kowalski, I.; Howe, J.; Brauser, A.; Richter, W.; Razquin-Olazarán, I.; Iñigo-Pestaña, M.; Garidel, P.; Rössle, M.; Martinez de Tejada, G.; Gutsmann, T.; Brandenburg, K. Biophysical Mechanisms of Endotoxin Neutralization by Cationic Amphiphilic Peptides. *Biophysical Journal* **2011,** 100, 2652-2661.
159. Morrison, D. C.; Jacobs, D. M. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. *Immunochemistry* **1976,** 13, 813-818.
160. Moore, R. A.; Bates, N. C.; Hancock, R. E. Interaction of polycationic antibiotics with Pseudomonas aeruginosa lipopolysaccharide and lipid A studied by using dansyl-polymyxin. *Antimicrobial Agents and Chemotherapy* **1986,** 29, 496-500.

161. From, A. H.; Fong, J. S.; Good, R. A. Polymyxin B sulfate modification of bacterial endotoxin: effects on the development of endotoxin shock in dogs. *Infection and immunity* **1979,** 23, 660-664.
162. Corrigan, J. J.; Kiernat, J. F. Effect of Polymyxin B Sulfate on Endotoxin Activity in a Gram-Negative Septicemia Model. *Pediatr Res* **1979,** 13, 48-51.
163. Rifkind, D. Prevention by polymyxin B of endotoxin lethality in mice. *Journal of Bacteriology* **1967,** 93, 1463-1464.
164. Falagas, M. E.; Kasiakou, S. K. Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. *Critical Care* **2006,** 10, R27-R27.
165. Danner, R. L.; Joiner, K. A.; Rubin, M.; Patterson, W. H.; Johnson, N.; Ayers, K. M.; Parrillo, J. E. Purification, toxicity, and antiendotoxin activity of polymyxin B nonapeptide. *Antimicrobial Agents and Chemotherapy* **1989,** 33, 1428-1434.
166. Koch-Weser, J.; Sidel, V. W.; Federman, E. B.; Kanarek, P.; Finer, D. C.; Eaton, A. E. Adverse effects of sodium colistimethate: Manifestations and specific reaction rates during 317 courses of therapy. *Annals of Internal Medicine* **1970,** 72, 857-868.
167. Price, D. J. E.; Graham, D. I. Effects of Large Doses of Colistin Sulphomethate Sodium on Renal Function. *British Medical Journal* **1970,** 4, 525-527.
168. Vinnicombe J Fau - Stamey, T. A.; Stamey, T. A. The relative nephrotoxicities of polymyxin B sulfate, sodium sulfomethyl-polymyxin B, sodium sulfomethyl-colistin (colymycin), and neomycin sulfate. **1969,** 6, 505-519.
169. Miller, K. A.; Kumar, E. V. K. S.; Wood, S. J.; Cromer, J. R.; Datta, A.; David*, S. A. Lipopolysaccharide Sequestrants: Structural Correlates of Activity and Toxicity in Novel Acylhomospermines. *Journal of medicinal chemistry* **2005,** 48, 2589-2599.
170. Scott, M. G.; Davidson, D. J.; Gold, M. R.; Bowdish, D.; Hancock, R. E. W. The Human Antimicrobial Peptide LL-37 Is a Multifunctional Modulator of Innate Immune Responses. *The Journal of Immunology* **2002,** 169, 3883.
171. Travis, S. M.; Anderson, N. N.; Forsyth, W. R.; Espiritu, C.; Conway, B. D.; Greenberg, E. P.; McCray, P. B.; Lehrer, R. I.; Welsh, M. J.; Tack, B. F. Bactericidal Activity of Mammalian Cathelicidin-Derived Peptides. *Infection and immunity* **2000,** 68, 2748-2755.
172. Nell, M. J.; Tjabringa, G. S.; Wafelman, A. R.; Verrijk, R.; Hiemstra, P. S.; Drijfhout, J. W.; Grote, J. J. Development of novel LL-37 derived antimicrobial

peptides with LPS and LTA neutralizing and antimicrobial activities for therapeutic application. *Peptides* **2006,** 27, 649-60.
173. Wang, G. Structures of Human Host Defense Cathelicidin LL-37 and Its Smallest Antimicrobial Peptide KR-12 in Lipid Micelles. *Journal of Biological Chemistry* **2008,** 283, 32637-32643.
174. Oh, D.; Shin Sy Fau - Kang, J. H.; Kang Jh Fau - Hahm, K. S.; Hahm Ks Fau - Kim, K. L.; Kim Kl Fau - Kim, Y.; Kim, Y. NMR structural characterization of cecropin A(1-8) - magainin 2(1-12) and cecropin A (1-8) - melittin (1-12) hybrid peptides. *Journal of Peptide Research* **1999,** 53, 578-589.
175. Andreu, D.; Ubach J Fau - Boman, A.; Boman A Fau - Wahlin, B.; Wahlin B Fau - Wade, D.; Wade D Fau - Merrifield, R. B.; Merrifield Rb Fau - Boman, H. G.; Boman, H. G. Shortened cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity. *FEBS Letters* **1992,** 296, 190-194.
176. Chen, Y.; Mant, C. T.; Farmer, S. W.; Hancock, R. E. W.; Vasil, M. L.; Hodges, R. S. Rational Design of α-Helical Antimicrobial Peptides with Enhanced Activities and Specificity/Therapeutic Index. *The Journal of biological chemistry* **2005,** 280, 12316-12329.
177. Lai, Y.; Gallo, R. L. AMPed Up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends in immunology* **2009,** 30, 131-141.
178. Matsuzaki, K. Why and how are peptide–lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1999,** 1462, 1-10.
179. Shai, Y. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1999,** 1462, 55-70.
180. Yang, L.; Weiss, T. M.; Lehrer, R. I.; Huang, H. W. Crystallization of Antimicrobial Pores in Membranes: Magainin and Protegrin. *Biophysical Journal* **2000,** 79, 2002-2009.
181. Wilkinson, T. S.; Dhaliwal, K.; Hamilton, T. W.; Lipka, A. F.; Farrell, L.; Davidson, D. J.; Duffin, R.; Morris, A. C.; Haslett, C.; Govan, J. R. W.; Gregory, C. D.; Sallenave, J.-M.; Simpson, A. J. Trappin-2 Promotes Early Clearance of Pseudomonas aeruginosa through CD14-Dependent Macrophage Activation and Neutrophil Recruitment. *The American journal of pathology* **2009,** 174, 1338-1346.
182. Oppenheim, J.; Biragyn, A.; Kwak, L.; Yang, D. Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. *Annals of the Rheumatic Diseases* **2003,** 62, ii17-ii21.

183. Di Nardo, A.; Braff, M. H.; Taylor, K. R.; Na, C.; Granstein, R. D.; McInturff, J. E.; Krutzik, S.; Modlin, R. L.; Gallo, R. L. Cathelicidin Antimicrobial Peptides Block Dendritic Cell TLR4 Activation and Allergic Contact Sensitization. *The Journal of Immunology* **2007,** 178, 1829.
184. Dallegri, F.; Ottonello, L. Tissue injury in neutrophilic inflammation. *Inflammation Research* **1997,** 46, 382-391.
185. Williams, Steven E.; Brown, Thomas I.; Roghanian, A.; Sallenave, J.-M. SLPI and elafin: one glove, many fingers. *Clinical Science* **2006,** 110, 21.
186. Phoenix, D. A.; Dennison, S. R.; Harris, F. Antimicrobial Peptides: Their History, Evolution, and Functional Promiscuity. In *Antimicrobial Peptides*, Wiley-VCH Verlag GmbH & Co. KGaA: 2013; pp 1-37.
187. Zaiou, M. Multifunctional antimicrobial peptides: therapeutic targets in several human diseases. *Journal of Molecular Medicine* **2007,** 85, 317-329.
188. Aoki, W.; Ueda, M. Characterization of Antimicrobial Peptides toward the Development of Novel Antibiotics. *Pharmaceuticals* **2013,** 6, 1055-1081.
189. Frew, L.; Stock, S. J. Antimicrobial peptides and pregnancy. *Reproduction* **2011,** 141, 725-735.
190. Bulusu, M. A.; Waldstatten P Fau - Hildebrandt, J.; Hildebrandt J Fau - Schutze, E.; Schutze E Fau - Schulz, G.; Schulz, G. Acyclic analogues of lipid A: synthesis and biological activities. **1996**.
191. Calabrese, V.; Cighetti, R.; Peri, F. Molecular simplification of lipid A structure: TLR4-modulating cationic and anionic amphiphiles. *Molecular immunology* **2015,** 63, 153-61.
192. Kawata, T.; Bristol, J. R.; Rossignol, D. P.; Rose, J. R.; Kobayashi, S.; Yokohama, H.; Ishibashi, A.; Christ, W. J.; Katayama, K.; Yamatsu, I.; Kishi, Y. E5531, a synthetic non-toxic lipid A derivative blocks the immunobiological activities of lipopolysaccharide. *British Journal of Pharmacology* **1999,** 127, 853-862.
193. Rose, J. R.; Mullarkey, M. A.; Christ, W. J.; Hawkins, L. D.; Lynn, M.; Kishi, Y.; Wasan, K. M.; Peteherych, K.; Rossignol, D. P. Consequences of Interaction of a Lipophilic Endotoxin Antagonist with Plasma Lipoproteins. *Antimicrobial Agents and Chemotherapy* **2000,** 44, 504-510.
194. Opal, S. M.; Laterre, P.; Francois, B.; et al. Effect of eritoran, an antagonist of md2-tlr4, on mortality in patients with severe sepsis: The access randomized trial. *JAMA* **2013,** 309, 1154-1162.
195. Imoto, M.; Yoshimura, H.; Shimamoto, T.; Sakaguchi, N.; Kusumoto, S.; Shiba, T. Total Synthesis of Escherichia coli Lipid A, the Endotoxically Active Principle of Cell-Surface Lipopolysaccharide. *Bulletin of the Chemical Society of Japan* **1987,** 60, 2205-2214.

196. David, A. J. Synthetic TLR4-active Glycolipids as Vaccine Adjuvants and Stand-alone Immunotherapeutics. *Current Topics in Medicinal Chemistry* **2008,** 8, 64-79.
197. Danner, R. L.; Joiner, K. A.; Parrillo, J. E. Inhibition of endotoxin-induced priming of human neutrophils by lipid X and 3-Aza-lipid X. *The Journal of Clinical Investigation* **1987,** 80, 605-612.
198. Funatogawa, K.; Matsuura, M.; Nakano, M.; Kiso, M.; Hasegawa, A. Relationship of Structure and Biological Activity of Monosaccharide Lipid A Analogues to Induction of Nitric Oxide Production by Murine Macrophage RAW264.7 Cells. *Infection and immunity* **1998,** 66, 5792-5798.
199. Matsuura, M.; Kiso, M.; Hasegawa, A. Activity of Monosaccharide Lipid A Analogues in Human Monocytic Cells as Agonists or Antagonists of Bacterial Lipopolysaccharide. *Infection and immunity* **1999,** 67, 6286-6292.
200. Lonez, C.; Vandenbranden, M.; Ruysschaert, J.-M. Cationic lipids activate intracellular signaling pathways. *Advanced Drug Delivery Reviews* **2012,** 64, 1749-1758.
201. Tanaka, T.; Legat, A.; Adam, E.; Steuve, J.; Gatot, J.-S.; Vandenbranden, M.; Ulianov, L.; Lonez, C.; Ruysschaert, J.-M.; Muraille, E.; Tuynder, M.; Goldman, M.; Jacquet, A. DiC14-amidine cationic liposomes stimulate myeloid dendritic cells through Toll-like receptor 4. *European Journal of Immunology* **2008,** 38, 1351-1357.
202. Vangasseri, D. P.; Cui, Z.; Chen, W.; Hokey, D. A.; Falo, L. D.; Huang, L. Immunostimulation of dendritic cells by cationic liposomes. *Molecular Membrane Biology* **2006,** 23, 385-395.
203. Leon-Ponte, M.; Kirchhof, M. G.; Sun, T.; Stephens, T.; Singh, B.; Sandhu, S.; Madrenas, J. Polycationic lipids inhibit the pro-inflammatory response to LPS. *Immunology Letters* **2005,** 96, 73-83.
204. Piazza, M.; Yu, L.; Teghanemt, A.; Gioannini, T.; Weiss, J.; Peri, F. Evidence of a specific interaction between new synthetic antisepsis agents and CD14. *Biochemistry* **2009,** 48, 12337-44.
205. Piazza, M.; Calabrese, V.; Baruffa, C.; Gioannini, T.; Weiss, J.; Peri, F. The cationic amphiphile 3,4-bis(tetradecyloxy)benzylamine inhibits LPS signaling by competing with endotoxin for CD14 binding. *Biochemical pharmacology* **2010,** 80, 2050-6.
206. Rodriguez Lavado, J.; Sestito, S. E.; Cighetti, R.; Aguilar Moncayo, E. M.; Oblak, A.; Lainscek, D.; Jimenez Blanco, J. L.; Garcia Fernandez, J. M.; Ortiz Mellet, C.; Jerala, R.; Calabrese, V.; Peri, F. Trehalose- and glucose-derived glycoamphiphiles: small-molecule and nanoparticle Toll-like receptor 4 (TLR4) modulators. *Journal of medicinal chemistry* **2014,** 57, 9105-23.

207. Cighetti, R.; Ciaramelli, C.; Sestito, S. E.; Zanoni, I.; Kubik, L.; Arda-Freire, A.; Calabrese, V.; Granucci, F.; Jerala, R.; Martin-Santamaria, S.; Jimenez-Barbero, J.; Peri, F. Modulation of CD14 and TLR4.MD-2 activities by a synthetic lipid A mimetic. *Chembiochem : a European journal of chemical biology* **2014,** 15, 250-8.
208. Artner, D.; Oblak, A.; Ittig, S.; Garate, J. A.; Horvat, S.; Arrieumerlou, C.; Hofinger, A.; Oostenbrink, C.; Jerala, R.; Kosma, P.; Zamyatina, A. Conformationally Constrained Lipid A Mimetics for Exploration of Structural Basis of TLR4/MD-2 Activation by Lipopolysaccharide. *ACS Chemical Biology* **2013,** 8, 2423-2432.
209. Imoto, M.; Yoshimura, H.; Sakaguchi, N.; Kusumoto, S.; Shiba, T. Total synthesis of escherichia coli lipid A. *Tetrahedron Letters* **1985,** 26, 1545-1548.
210. Perrin-Cocon, L.; Aublin-Gex, A.; Sestito, S. E.; Shirey, K. A.; Patel, M. C.; Andre, P.; Blanco, J. C.; Vogel, S. N.; Peri, F.; Lotteau, V. TLR4 antagonist FP7 inhibits LPS-induced cytokine production and glycolytic reprogramming in dendritic cells, and protects mice from lethal influenza infection. *Scientific reports* **2017,** 7, 40791.
211. Xavier, R. J.; Podolsky, D. K. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* **2007,** 448, 427-434.
212. Abraham, C.; Cho, J. H. Inflammatory Bowel Disease. *The New England journal of medicine* **2009,** 361, 2066-2078.
213. de Souza, H. S. P.; Fiocchi, C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol* **2016,** 13, 13-27.
214. Rutgeerts, P. Crohn's disease recurrence can be prevented after ileal resection. *Gut* **2002,** 51, 152-153.
215. Cho, J. H. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol* **2008,** 8, 458-466.
216. Podolsky, D. K. Inflammatory Bowel Disease. *New England Journal of Medicine* **2002,** 347, 417-429.
217. Bouma, G.; Strober, W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* **2003,** 3, 521-533.
218. Strober, W.; Fuss, I.; Mannon, P. The fundamental basis of inflammatory bowel disease. *Journal of Clinical Investigation* **2007,** 117, 514-521.
219. Cosnes, J.; Gower–Rousseau, C.; Seksik, P.; Cortot, A. Epidemiology and Natural History of Inflammatory Bowel Diseases. *Gastroenterology* **2011,** 140, 1785-1794.e4.
220. Molodecky, N. A.; Soon, I. S.; Rabi, D. M.; Ghali, W. A.; Ferris, M.; Chernoff, G.; Benchimol, E. I.; Panaccione, R.; Ghosh, S.; Barkema, H. W.;

Kaplan, G. G. Increasing Incidence and Prevalence of the Inflammatory Bowel Diseases With Time, Based on Systematic Review. *Gastroenterology* **2012,** 142, 46-54.e42.
221. Ponder, A.; Long, M. D. A clinical review of recent findings in the epidemiology of inflammatory bowel disease. *Clinical Epidemiology* **2013,** 5, 237-247.
222. Parente, J. M. L.; Coy, C. S. R.; Campelo, V.; Parente, M. P. P. D.; Costa, L. A.; da Silva, R. M.; Stephan, C.; Zeitune, J. M. R. Inflammatory bowel disease in an underdeveloped region of Northeastern Brazil. *World Journal of Gastroenterology : WJG* **2015,** 21, 1197-1206.
223. Fofanova, T. Y.; Petrosino, J. F.; Kellermayer, R. Microbiome-Epigenome Interactions and the Environmental Origins of Inflammatory Bowel Diseases. *Journal of pediatric gastroenterology and nutrition* **2016,** 62, 208-219.
224. Benchimol, E. I.; Bernstein, C. N.; Bitton, A.; Carroll, M. W.; Singh, H.; Otley, A. R.; Vutcovici, M.; El-Matary, W.; Nguyen, G. C.; Griffiths, A. M.; Mack, D. R.; Jacobson, K.; Mojaverian, N.; Tanyingoh, D.; Cui, Y.; Nugent, Z. J.; Coulombe, J.; Targownik, L. E.; Jones, J. L.; Leddin, D.; Murthy, S. K.; Kaplan, G. G. Trends in Epidemiology of Pediatric Inflammatory Bowel Disease in Canada: Distributed Network Analysis of Multiple Population-Based Provincial Health Administrative Databases. *Am J Gastroenterol* **2017,** 112, 1120-1134.
225. Burisch, J.; Munkholm, P. The epidemiology of inflammatory bowel disease. *Scandinavian Journal of Gastroenterology* **2015,** 50, 942-951.
226. Kaplan, G. G. The global burden of IBD: from 2015 to 2025. *Nat Rev Gastroenterol Hepatol* **2015,** 12, 720-727.
227. Halme, L.; Paavola-Sakki, P.; Turunen, U.; Lappalainen, M.; Färkkilä, M.; Kontula, K. Family and twin studies in inflammatory bowel disease. *World Journal of Gastroenterology : WJG* **2006,** 12, 3668-3672.
228. Gregersen, P. K.; Olsson, L. M. Recent Advances in the Genetics of Autoimmune Disease. *Annual review of immunology* **2009,** 27, 363-391.
229. Jostins, L.; Ripke, S.; Weersma, R. K.; Duerr, R. H.; McGovern, D. P.; Hui, K. Y.; Lee, J. C.; Schumm, L. P.; Sharma, Y.; Anderson, C. A.; Essers, J.; Mitrovic, M.; Ning, K.; Cleynen, I.; Theatre, E.; Spain, S. L.; Raychaudhuri, S.; Goyette, P.; Wei, Z.; Abraham, C.; Achkar, J.-P.; Ahmad, T.; Amininejad, L.; Ananthakrishnan, A. N.; Andersen, V.; Andrews, J. M.; Baidoo, L.; Balschun, T.; Bampton, P. A.; Bitton, A.; Boucher, G.; Brand, S.; Büning, C.; Cohain, A.; Cichon, S.; D'Amato, M.; De Jong, D.; Devaney, K. L.; Dubinsky, M.; Edwards, C.; Ellinghaus, D.; Ferguson, L. R.; Franchimont, D.; Fransen, K.; Gearry, R.; Georges, M.; Gieger, C.; Glas, J.; Haritunians, T.; Hart, A.; Hawkey, C.; Hedl, M.; Hu, X.; Karlsen, T. H.; Kupcinskas, L.; Kugathasan, S.; Latiano, A.; Laukens, D.;

Lawrance, I. C.; Lees, C. W.; Louis, E.; Mahy, G.; Mansfield, J.; Morgan, A. R.; Mowat, C.; Newman, W.; Palmieri, O.; Ponsioen, C. Y.; Potocnik, U.; Prescott, N. J.; Regueiro, M.; Rotter, J. I.; Russell, R. K.; Sanderson, J. D.; Sans, M.; Satsangi, J.; Schreiber, S.; Simms, L. A.; Sventoraityte, J.; Targan, S. R.; Taylor, K. D.; Tremelling, M.; Verspaget, H. W.; De Vos, M.; Wijmenga, C.; Wilson, D. C.; Winkelmann, J.; Xavier, R. J.; Zeissig, S.; Zhang, B.; Zhang, C. K.; Zhao, H.; The International, I. B. D. G. C.; Silverberg, M. S.; Annese, V.; Hakonarson, H.; Brant, S. R.; Radford-Smith, G.; Mathew, C. G.; Rioux, J. D.; Schadt, E. E.; Daly, M. J.; Franke, A.; Parkes, M.; Vermeire, S.; Barrett, J. C.; Cho, J. H. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* **2012,** 491, 119-124.

230. Liu, T.-C.; Stappenbeck, T. S. GENETICS AND PATHOGENESIS OF INFLAMMATORY BOWEL DISEASE. *Annual review of pathology* **2016,** 11, 127-148.

231. McCarroll, S. A.; Huett, A.; Kuballa, P.; Chilewski, S. D.; Landry, A.; Goyette, P.; Zody, M. C.; Hall, J. L.; Brant, S. R.; Cho, J. H.; Duerr, R. H.; Silverberg, M. S.; Taylor, K. D.; Rioux, J. D.; Altshuler, D.; Daly, M. J.; Xavier, R. J. Deletion polymorphism upstream of IRGM associated with altered IRGM expression and Crohn's disease. *Nature genetics* **2008,** 40, 1107-1112.

232. Van Limbergen, J.; Wilson, D. C.; Satsangi, J. The Genetics of Crohn's Disease. *Annual Review of Genomics and Human Genetics* **2009,** 10, 89-116.

233. Parkes, M.; Barrett, J. C.; Prescott, N.; Tremelling, M.; Anderson, C. A.; Fisher, S. A.; Roberts, R. G.; Nimmo, E. R.; Cummings, F. R.; Soars, D.; Drummond, H.; Lees, C. W.; Khawaja, S. A.; Bagnall, R.; Burke, D. A.; Todhunter, C. E.; Ahmad, T.; Onnie, C. M.; McArdle, W.; Strachan, D.; Bethel, G.; Bryan, C.; Deloukas, P.; Forbes, A.; Sanderson, J.; Jewell, D. P.; Satsangi, J.; Mansfield, J. C.; the Wellcome Trust Case Control, C.; Cardon, L.; Mathew, C. G. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn disease susceptibility. *Nature genetics* **2007,** 39, 830-832.

234. Hampe, J.; Franke, A.; Rosenstiel, P.; Till, A.; Teuber, M.; Huse, K.; Albrecht, M.; Mayr, G.; De La Vega, F. M.; Briggs, J.; Gunther, S.; Prescott, N. J.; Onnie, C. M.; Hasler, R.; Sipos, B.; Folsch, U. R.; Lengauer, T.; Platzer, M.; Mathew, C. G.; Krawczak, M.; Schreiber, S. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet* **2007,** 39, 207-211.

235. Abraham, C.; Cho, J. H. Functional consequences ofNOD2(CARD15) mutations. *Inflammatory bowel diseases* **2006,** 12.

236. Brant, S. R. Update on the heritability of inflammatory bowel disease: The importance of twin studies. *Inflammatory bowel diseases* **2011,** 17.
237. Strober, W.; Fuss, I. J.; Blumberg, R. S. The Immunology of Mucosal Models of Inflammation. *Annual Review of Immunology* **2002,** 20, 495-549.
238. Zeng, M. Y.; Inohara, N.; Nunez, G. Mechanisms of inflammation-driven bacterial dysbiosis in the gut. *Mucosal immunology* **2017,** 10, 18-26.
239. Salzman, N. H.; Hung, K.; Haribhai, D.; Chu, H.; Karlsson-Sjöberg, J.; Amir, E.; Teggatz, P.; Barman, M.; Hayward, M.; Eastwood, D.; Stoel, M.; Zhou, Y.; Sodergren, E.; Weinstock, G. M.; Bevins, C. L.; Williams, C. B.; Bos, N. A. Enteric defensins are essential regulators of intestinal microbial ecology. *Nature immunology* **2010,** 11, 76-83.
240. Macpherson, A. J.; Uhr, T. Induction of Protective IgA by Intestinal Dendritic Cells Carrying Commensal Bacteria. *Science* **2004,** 303, 1662.
241. Hooper, L. V.; Macpherson, A. J. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol* **2010,** 10, 159-169.
242. Stecher, B.; Hardt, W.-D. Mechanisms controlling pathogen colonization of the gut. *Current Opinion in Microbiology* **2011,** 14, 82-91.
243. Renz, H.; Brandtzaeg, P.; Hornef, M. The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. *Nat Rev Immunol* **2012,** 12, 9-23.
244. Cario, E.; Podolsky, D. K. Intestinal epithelial Tollerance versus inTollerance of commensals. *Molecular immunology* **2005,** 42, 887-893.
245. Smith, P. D.; Smythies, L. E.; Shen, R.; Greenwell-Wild, T.; Gliozzi, M.; Wahl, S. M. Intestinal macrophages and response to microbial encroachment. *Mucosal immunology* **2011,** 4, 31-42.
246. Littman, D. R.; Pamer, E. G. Role of the commensal microbiota in normal and pathogenic host immune responses. *Cell host & microbe* **2011,** 10, 311-323.
247. Honda, K.; Littman, D. R. The Microbiome in Infectious Disease and Inflammation. *Annual review of immunology* **2012,** 30, 759-795.
248. Suzuki, K.; Meek, B.; Doi, Y.; Muramatsu, M.; Chiba, T.; Honjo, T.; Fagarasan, S. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proceedings of the National Academy of Sciences* **2004,** 101, 1981-1986.
249. Sartor, R. B. Microbial Influences in Inflammatory Bowel Diseases. *Gastroenterology* **2008,** 134, 577-594.
250. Consortium, H. M. P. Structure, function and diversity of the healthy human microbiome. *Nature* **2012,** 486, 207-214.

251. Faust, K.; Sathirapongsasuti, J. F.; Izard, J.; Segata, N.; Gevers, D.; Raes, J.; Huttenhower, C. Microbial Co-occurrence Relationships in the Human Microbiome. *PLOS Computational Biology* **2012,** 8, e1002606.
252. Arthur, J. C.; Perez-Chanona, E.; Mühlbauer, M.; Tomkovich, S.; Uronis, J. M.; Fan, T.-J.; Campbell, B. J.; Abujamel, T.; Dogan, B.; Rogers, A. B.; Rhodes, J. M.; Stintzi, A.; Simpson, K. W.; Hansen, J. J.; Keku, T. O.; Fodor, A. A.; Jobin, C. Intestinal inflammation targets cancer-inducing activity of the microbiota. *Science (New York, N.Y.)* **2012,** 338, 120-123.
253. Barnich, N.; Darfeuille-Michaud, A. Adherent-invasive Escherichia coli and Crohn's disease. *Current Opinion in Gastroenterology* **2007,** 23.
254. Lupp, C.; Robertson, M. L.; Wickham, M. E.; Sekirov, I.; Champion, O. L.; Gaynor, E. C.; Finlay, B. B. Host-Mediated Inflammation Disrupts the Intestinal Microbiota and Promotes the Overgrowth of Enterobacteriaceae. *Cell Host & Microbe* **2007,** 2, 119-129.
255. Haag, L.-M.; Fischer, A.; Otto, B.; Plickert, R.; Kühl, A. A.; Göbel, U. B.; Bereswill, S.; Heimesaat, M. M. Intestinal Microbiota Shifts towards Elevated Commensal Escherichia coli Loads Abrogate Colonization Resistance against Campylobacter jejuni in Mice. *PloS one* **2012,** 7, e35988.
256. Ayres, J. S.; Trinidad, N. J.; Vance, R. E. Lethal inflammasome activation by a multi-drug resistant pathobiont upon antibiotic disruption of the microbiota. *Nature medicine* **2012,** 18, 799-806.
257. Darfeuille-Michaud, A.; Boudeau, J.; Bulois, P.; Neut, C.; Glasser, A.-L.; Barnich, N.; Bringer, M.-A.; Swidsinski, A.; Beaugerie, L.; Colombel, J.-F. High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. *Gastroenterology* **2004,** 127, 412-421.
258. De la Fuente, M.; Franchi, L.; Araya, D.; Díaz-Jiménez, D.; Olivares, M.; Álvarez-Lobos, M.; Golenbock, D.; González, M.-J.; López-Kostner, F.; Quera, R.; Núñez, G.; Vidal, R.; Hermoso, M. A. Escherichia coli Isolates from Inflammatory Bowel Diseases Patients Survive in Macrophages and Activate NLRP3 Inflammasome. *International journal of medical microbiology : IJMM* **2014,** 304, 384-392.
259. Gophna, U.; Sommerfeld, K.; Gophna, S.; Doolittle, W. F.; Veldhuyzen van Zanten, S. J. O. Differences between Tissue-Associated Intestinal Microfloras of Patients with Crohn's Disease and Ulcerative Colitis. *Journal of Clinical Microbiology* **2006,** 44, 4136-4141.
260. Frank, D. N.; St. Amand, A. L.; Feldman, R. A.; Boedeker, E. C.; Harpaz, N.; Pace, N. R. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proceedings of*

the National Academy of Sciences of the United States of America **2007,** 104, 13780-13785.
261. Baumgart, M.; Dogan, B.; Rishniw, M.; Weitzman, G.; Bosworth, B.; Yantiss, R.; Orsi, R. H.; Wiedmann, M.; McDonough, P.; Kim, S. G.; Berg, D.; Schukken, Y.; Scherl, E.; Simpson, K. W. Culture independent analysis of ileal mucosa reveals a selective increase in invasive Escherichia coli of novel phylogeny relative to depletion of Clostridiales in Crohn's disease involving the ileum. *ISME J* **2007,** 1, 403-418.
262. Buffie, C. G.; Jarchum, I.; Equinda, M.; Lipuma, L.; Gobourne, A.; Viale, A.; Ubeda, C.; Xavier, J.; Pamer, E. G. Profound Alterations of Intestinal Microbiota following a Single Dose of Clindamycin Results in Sustained Susceptibility to Clostridium difficile-Induced Colitis. *Infection and immunity* **2012,** 80, 62-73.
263. Zeng, M. Y.; Cisalpino, D.; Varadarajan, S.; Hellman, J.; Warren, H. S.; Cascalho, M.; Inohara, N.; Núñez, G. Gut Microbiota-Induced Immunoglobulin G Controls Systemic Infection by Symbiotic Bacteria and Pathogens. *Immunity* **2016,** 44, 647-658.
264. Wallace, J. L.; Syer, S.; Denou, E.; de Palma, G.; Vong, L.; McKnight, W.; Jury, J.; Bolla, M.; Bercik, P.; Collins, S. M.; Verdu, E.; Ongini, E. Proton Pump Inhibitors Exacerbate NSAID-Induced Small Intestinal Injury by Inducing Dysbiosis. *Gastroenterology* **2011,** 141, 1314-1322.e5.
265. Akira, S.; Takeda, K. Toll-like receptor signalling. *Nat Rev Immunol* **2004,** 4, 499-511.
266. Salim, S. a. Y.; Söderholm, J. D. Importance of disrupted intestinal barrier in inflammatory bowel diseases. *Inflammatory bowel diseases* **2011,** 17.
267. Malamud, D.; Wahl, S. M. THE MOUTH: A GATEWAY OR A TRAP FOR HIV? *AIDS (London, England)* **2010,** 24, 5-16.
268. Fukata, M.; Vamadevan, A. S.; Abreu, M. T. Toll-like receptors (TLRs) and Nod-like receptors (NLRs) in inflammatory disorders. *Seminars in Immunology* **2009,** 21, 242-253.
269. Bortoluci, K. R.; Medzhitov, R. Control of infection by pyroptosis and autophagy: role of TLR and NLR. *Cellular and Molecular Life Sciences* **2010,** 67, 1643-1651.
270. Smythies, L. E.; Sellers, M.; Clements, R. H.; Mosteller-Barnum, M.; Meng, G.; Benjamin, W. H.; Orenstein, J. M.; Smith, P. D. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *Journal of Clinical Investigation* **2005,** 115, 66-75.

271. Cerovic, V.; Jenkins, C. D.; Barnes, A. G. C.; Milling, S. W. F.; MacPherson, G. G.; Klavinskis, L. S. Hyporesponsiveness of Intestinal Dendritic Cells to TLR Stimulation Is Limited to TLR4. *The Journal of Immunology* **2009,** 182, 2405.
272. Smith, P. D.; Janoff, E. N.; Mosteller-Barnum, M.; Merger, M.; Orenstein, J. M.; Kearney, J. F.; Graham, M. F. Isolation and purification of CD14-negative mucosal macrophages from normal human small intestine. *Journal of Immunological Methods* **1997,** 202, 1-11.
273. Smythies, L. E.; Shen, R.; Bimczok, D.; Novak, L.; Clements, R. H.; Eckhoff, D. E.; Bouchard, P.; George, M. D.; Hu, W. K.; Dandekar, S.; Smith, P. D. Inflammation anergy in human intestinal macrophages is due to Smad-induced IkappaBalpha expression and NF-kappaB inactivation. *The Journal of biological chemistry* **2010,** 285, 19593-604.
274. Zigmond, E.; Varol, C.; Farache, J.; Elmaliah, E.; Satpathy, Ansuman T.; Friedlander, G.; Mack, M.; Shpigel, N.; Boneca, Ivo G.; Murphy, Kenneth M.; Shakhar, G.; Halpern, Z.; Jung, S. Ly6Chi Monocytes in the Inflamed Colon Give Rise to Proinflammatory Effector Cells and Migratory Antigen-Presenting Cells. *Immunity* **2012,** 37, 1076-1090.
275. Bain, C. C.; Mowat, A. M. Macrophages in intestinal homeostasis and inflammation. *Immunological Reviews* **2014,** 260, 102-117.
276. Naiki, Y.; Michelsen, K. S.; Zhang, W.; Chen, S.; Doherty, T. M.; Arditi, M. Transforming Growth Factor-β Differentially Inhibits MyD88-dependent, but Not TRAM- and TRIF-dependent, Lipopolysaccharide-induced TLR4 Signaling. *Journal of Biological Chemistry* **2005,** 280, 5491-5495.
277. Hirotani, T.; Lee, P. Y.; Kuwata, H.; Yamamoto, M.; Matsumoto, M.; Kawase, I.; Akira, S.; Takeda, K. The Nuclear IκB Protein IκBNS Selectively Inhibits Lipopolysaccharide-Induced IL-6 Production in Macrophages of the Colonic Lamina Propria. *The Journal of Immunology* **2005,** 174, 3650.
278. Friedman, A. D. Transcriptional control of granulocyte and monocyte development. *Oncogene* **2007,** 26, 6816-6828.
279. Grimm, M. C.; Pullman, W. E.; Bennett, G. M.; Sullivan, P. J.; Pavli, P.; Doe, W. F. Direct evidence of monocyte recruitment to inflammatory bowel disease mucosa. *Journal of gastroenterology and hepatology* **1995,** 10, 387-395.
280. Kamada, N.; Hisamatsu, T.; Okamoto, S.; Chinen, H.; Kobayashi, T.; Sato, T.; Sakuraba, A.; Kitazume, M. T.; Sugita, A.; Koganei, K.; Akagawa, K. S.; Hibi, T. Unique CD14(+) intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-γ axis. *The Journal of Clinical Investigation* **2008,** 118, 2269-2280.

281. Thiesen, S.; Janciauskiene, S.; Uronen-Hansson, H.; Agace, W.; Högerkorp, C.-M.; Spee, P.; Håkansson, K.; Grip, O. CD14hiHLA-DRdim macrophages, with a resemblance to classical blood monocytes, dominate inflamed mucosa in Crohn's disease. *Journal of leukocyte biology* **2014,** 95, 531-541.
282. Cario, E.; Rosenberg, I. M.; Brandwein, S. L.; Beck, P. L.; Reinecker, H.-C.; Podolsky, D. K. Lipopolysaccharide Activates Distinct Signaling Pathways in Intestinal Epithelial Cell Lines Expressing Toll-Like Receptors. *The Journal of Immunology* **2000,** 164, 966.
283. Walton, K. L. W.; Holt, L.; Sartor, R. B. Lipopolysaccharide activates innate immune responses in murine intestinal myofibroblasts through multiple signaling pathways. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **2009,** 296, G601-G611.
284. Ortega-Cava, C. F.; Ishihara, S.; Rumi, M. A. K.; Kawashima, K.; Ishimura, N.; Kazumori, H.; Udagawa, J.; Kadowaki, Y.; Kinoshita, Y. Strategic Compartmentalization of Toll-Like Receptor 4 in the Mouse Gut. *The Journal of Immunology* **2003,** 170, 3977.
285. Otte, J.-M.; Cario, E.; Podolsky, D. K. Mechanisms of cross hyporesponsiveness to toll-like receptor bacterial ligands in intestinal epithelial cells. *Gastroenterology* **2004,** 126, 1054-1070.
286. Rumio, C.; Besusso, D.; Palazzo, M.; Selleri, S.; Sfondrini, L.; Dubini, F.; Ménard, S.; Balsari, A. Degranulation of Paneth Cells via Toll-Like Receptor 9. *The American journal of pathology* **2004,** 165, 373-381.
287. Bogunovic, M.; Davé, S. H.; Tilstra, J. S.; Chang, D. T. W.; Harpaz, N.; Xiong, H.; Mayer, L. F.; Plevy, S. E. Enteroendocrine cells express functional Toll-like receptors. *American journal of physiology. Gastrointestinal and liver physiology* **2007,** 292, G1770-G1783.
288. Otte, J.-M.; Rosenberg, I. M.; Podolsky, D. K. Intestinal myofibroblasts in innate immune responses of the intestine. *Gastroenterology* **2003,** 124, 1866-1878.
289. Hausmann, M.; Kiessling, S.; Mestermann, S.; Webb, G.; Spöttl, T.; Andus, T.; Schölmerich, J.; Herfarth, H.; Ray, K.; Falk, W.; Rogler, G. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. *Gastroenterology* **2002,** 122, 1987-2000.
290. Hart, A. L.; Al-Hassi, H. O.; Rigby, R. J.; Bell, S. J.; Emmanuel, A. V.; Knight, S. C.; Kamm, M. A.; Stagg, A. J. Characteristics of Intestinal Dendritic Cells in Inflammatory Bowel Diseases. *Gastroenterology* **2005,** 129, 50-65.

291. Cerovic, V.; Jenkins, C. D.; Barnes, A. G.; Milling, S. W.; MacPherson, G. G.; Klavinskis, L. S. Hyporesponsiveness of intestinal dendritic cells to TLR stimulation is limited to TLR4. *Journal of immunology* **2009,** 182, 2405-15.
292. Tomita, T.; Kanai, T.; Fujii, T.; Nemoto, Y.; Okamoto, R.; Tsuchiya, K.; Totsuka, T.; Sakamoto, N.; Akira, S.; Watanabe, M. MyD88-Dependent Pathway in T Cells Directly Modulates the Expansion of Colitogenic CD4⁺ T Cells in Chronic Colitis. *The Journal of Immunology* **2008,** 180, 5291.
293. Fukata, M.; Breglio, K.; Chen, A.; Vamadevan, A. S.; Goo, T.; Hsu, D.; Conduah, D.; Xu, R.; Abreu, M. T. The myeloid differentiation factor 88 (MyD88) is required for CD4+ T cell effector function in a murine model of inflammatory bowel disease. *Journal of immunology (Baltimore, Md. : 1950)* **2008,** 180, 1886-1894.
294. Rodríguez, L. A. G.; Ruigómez, A.; Panés, J. Acute Gastroenteritis Is Followed by an Increased Risk of Inflammatory Bowel Disease. *Gastroenterology* **2006,** 130, 1588-1594.
295. Meijssen, M. A. C.; Brandwein, S. L.; Reinecker, H.-C.; Bhan, A. K.; Podolsky, D. K. Alteration of gene expression by intestinal epithelial cells precedes colitis in interleukin-2-deficient mice. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **1998,** 274, G472.
296. Suzuki, M.; Hisamatsu, T.; Podolsky, D. K. Gamma Interferon Augments the Intracellular Pathway for Lipopolysaccharide (LPS) Recognition in Human Intestinal Epithelial Cells through Coordinated Up-Regulation of LPS Uptake and Expression of the Intracellular Toll-Like Receptor 4-MD-2 Complex. *Infection and immunity* **2003,** 71, 3503-3511.
297. de Mattos, B. R.; Garcia, M. P.; Nogueira, J. B.; Paiatto, L. N.; Albuquerque, C. G.; Souza, C. L.; Fernandes, L. G.; Tamashiro, W. M.; Simioni, P. U. Inflammatory Bowel Disease: An Overview of Immune Mechanisms and Biological Treatments. *Mediators of inflammation* **2015,** 2015, 493012.
298. Carter, M.; Lobo, A.; Travis, S. Guidelines for the management of inflammatory bowel disease in adults. *Gut* **2004,** 53, v1-v16.
299. Rufo, P. A.; Bousvaros, A. Current Therapy of Inflammatory Bowel Disease in Children. *Pediatric Drugs* **2006,** 8, 279-302.
300. Reichel, C.; Streit, J.; Ott, K.; Wunsch, S. Appropriateness of Crohn's Disease Therapy in Gastroenterological Rehabilitation. *Digestion* **2010,** 82, 239-245.
301. Targan, S. R.; Hanauer, S. B.; van Deventer, S. J. H.; Mayer, L.; Present, D. H.; Braakman, T.; DeWoody, K. L.; Schaible, T. F.; Rutgeerts, P. J. A Short-

Term Study of Chimeric Monoclonal Antibody cA2 to Tumor Necrosis Factor α for Crohn's Disease. *New England Journal of Medicine* **1997,** 337, 1029-1036.
302. Thomas, S.; Baumgart, D. C. Targeting leukocyte migration and adhesion in Crohn's disease and ulcerative colitis. *Inflammopharmacology* **2012,** 20, 1-18.
303. Hanauer, S. B. Risks and benefits of combining immunosuppressives and biological agents in inflammatory bowel disease: is the synergy worth the risk? *Gut* **2007,** 56, 1181-1183.
304. van Schouwenburg, P. A.; Rispens, T.; Wolbink, G. J. Immunogenicity of anti-TNF biologic therapies for rheumatoid arthritis. *Nat Rev Rheumatol* **2013,** 9, 164-172.
305. Sestito, S. E.; Facchini, F. A.; Morbioli, I.; Billod, J.-M.; Martin-Santamaria, S.; Casnati, A.; Sansone, F.; Peri, F. Amphiphilic Guanidinocalixarenes Inhibit Lipopolysaccharide (LPS)- and Lectin-Stimulated Toll-like Receptor 4 (TLR4) Signaling. *Journal of medicinal chemistry* **2017,** 60, 4882-4892.
306. Giuliani, M.; Morbioli, I.; Sansone, F.; Casnati, A. Moulding calixarenes for biomacromolecule targeting. *Chemical Communications* **2015,** 51, 14140-14159.
307. Salvio, R.; Volpi, S.; Cacciapaglia, R.; Casnati, A.; Mandolini, L.; Sansone, F. Ribonuclease Activity of an Artificial Catalyst That Combines a Ligated CuII Ion and a Guanidinium Group at the Upper Rim of a cone-Calix[4]arene Platform. *The Journal of Organic Chemistry* **2015,** 80, 5887-5893.
308. Avvakumova, S.; Fezzardi, P.; Pandolfi, L.; Colombo, M.; Sansone, F.; Casnati, A.; Prosperi, D. Gold nanoparticles decorated by clustered multivalent cone-glycocalixarenes actively improve the targeting efficiency toward cancer cells. *Chemical Communications* **2014,** 50, 11029-11032.
309. Mochizuki, S.; Nishina, K.; Fujii, S.; Sakurai, K. The transfection efficiency of calix[4]arene-based lipids: the role of the alkyl chain length. *Biomaterials Science* **2015,** 3, 317-322.
310. Chen, X.; Dings, R. P. M.; Nesmelova, I.; Debbert, S.; Haseman, J. R.; Maxwell, J.; Hoye, T. R.; Mayo, K. H. TOPOMIMETICS OF AMPHIPATHIC β-SHEET AND HELIX-FORMING BACTERICIDAL PEPTIDES NEUTRALIZE LIPOPOLYSACCHARIDE ENDOTOXINS. *Journal of medicinal chemistry* **2006,** 49, 7754-7765.
311. Unitt, J.; Hornigold, D. Plant lectins are novel Toll-like receptor agonists. *Biochemical pharmacology* **2011,** 81, 1324-8.
312. Schrodinger, L. T. P. M. G. S., Version 1.8. In 2015.

313. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Petersson, G. A.; Nakatsuji, H.; Li, X.; Caricato, M.; Marenich, A. V.; Bloino, J.; Janesko, B. G.; Gomperts, R.; Mennucci, B.; Hratchian, H. P.; Ortiz, J. V.; Izmaylov, A. F.; Sonnenberg, J. L.; Williams; Ding, F.; Lipparini, F.; Egidi, F.; Goings, J.; Peng, B.; Petrone, A.; Henderson, T.; Ranasinghe, D.; Zakrzewski, V. G.; Gao, J.; Rega, N.; Zheng, G.; Liang, W.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Throssell, K.; Montgomery Jr, J. A.; Peralta, J. E.; Ogliaro, F.; Bearpark, M. J.; Heyd, J. J.; Brothers, E. N.; Kudin, K. N.; Staroverov, V. N.; Keith, T. A.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A. P.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Millam, J. M.; Klene, M.; Adamo, C.; Cammi, R.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Farkas, O.; Foresman, J. B.; Fox, D. J. *Gaussian 16*, Wallingford, CT, 2016.

314. Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. AutoDock4 and AutoDockTools4: Automated Docking with Selective Receptor Flexibility. *Journal of computational chemistry* **2009,** 30, 2785-2791.

315. Trott, O.; Olson, A. J. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. *Journal of computational chemistry* **2010,** 31, 455-461.

316. Wang, J.; Wang, W.; Kollman, P. A.; Case, D. A. Automatic atom type and bond type perception in molecular mechanical calculations. *Journal of Molecular Graphics and Modelling* **2006,** 25, 247-260.

317. Wang, J.; Wolf, R. M.; Caldwell, J. W.; Kollman, P. A.; Case, D. A. Development and testing of a general amber force field. *Journal of Computational Chemistry* **2004,** 25, 1157-1174.

318. Maier, J. A.; Martinez, C.; Kasavajhala, K.; Wickstrom, L.; Hauser, K. E.; Simmerling, C. ff14SB: Improving the Accuracy of Protein Side Chain and Backbone Parameters from ff99SB. *Journal of Chemical Theory and Computation* **2015,** 11, 3696-3713.

319. Baell, J. B.; Holloway, G. A. New Substructure Filters for Removal of Pan Assay Interference Compounds (PAINS) from Screening Libraries and for Their Exclusion in Bioassays. *Journal of medicinal chemistry* **2010,** 53, 2719-2740.

320. Peri, F.; Calabrese, V. Toll-like receptor 4 (TLR4) modulation by synthetic and natural compounds: an update. *Journal of medicinal chemistry* **2014,** 57, 3612-22.

321. Rosenfeld, Y.; Papo, N.; Shai, Y. Endotoxin (lipopolysaccharide) neutralization by innate immunity host-defense peptides. Peptide properties

and plausible modes of action. *The Journal of biological chemistry* **2006,** 281, 1636-43.
322. Wade, D.; Boman A Fau - Wahlin, B.; Wahlin B Fau - Drain, C. M.; Drain Cm Fau - Andreu, D.; Andreu D Fau - Boman, H. G.; Boman Hg Fau - Merrifield, R. B.; Merrifield, R. B. All-D amino acid-containing channel-forming antibiotic peptides. *Proceedings of the National Academy of Sciences of the United States of America* **1990,** 87, 4761-4765.
323. Fernández-Reyes, M.; Díaz, D.; de la Torre, B. G.; Cabrales-Rico, A.; Vallès-Miret, M.; Jiménez-Barbero, J.; Andreu, D.; Rivas, L. Lysine Nε-Trimethylation, a Tool for Improving the Selectivity of Antimicrobial Peptides. *Journal of medicinal chemistry* **2010,** 53, 5587-5596.
324. Chicharro, C.; Granata, C.; Lozano, R.; Andreu, D.; Rivas, L. N-Terminal Fatty Acid Substitution Increases the Leishmanicidal Activity of CA(1-7)M(2-9), a Cecropin-Melittin Hybrid Peptide. *Antimicrobial Agents and Chemotherapy* **2001,** 45, 2441-2449.
325. Gudmundsson, G. H.; Magnusson, K. P.; Chowdhary, B. P.; Johansson, M.; Andersson, L.; Boman, H. G. Structure of the gene for porcine peptide antibiotic PR-39, a cathelin gene family member: comparative mapping of the locus for the human peptide antibiotic FALL-39. *Proceedings of the National Academy of Sciences of the United States of America* **1995,** 92, 7085-7089.
326. Wu, J.; Mu, L.; Zhuang, L.; Han, Y.; Liu, T.; Li, J.; Yang, Y.; Yang, H.; Wei, L. A cecropin-like antimicrobial peptide with anti-inflammatory activity from the black fly salivary glands. *Parasites & vectors* **2015,** 8, 561.
327. Resman, N.; Gradisar, H.; Vasl, J.; Keber, M. M.; Pristovsek, P.; Jerala, R. Taxanes inhibit human TLR4 signaling by binding to MD-2. *FEBS Lett* **2008,** 582, 3929-34.
328. Monteleone, G.; Monteleone, I.; Fina, D.; Vavassori, P.; Del Vecchio Blanco, G.; Caruso, R.; Tersigni, R.; Alessandroni, L.; Biancone, L.; Naccari, G. C.; MacDonald, T. T.; Pallone, F. Interleukin-21 enhances T-helper cell type I signaling and interferon-γ production in Crohn's disease. *Gastroenterology* **2005,** 128, 687-694.
329. Franchi, L.; Monteleone, I.; Hao, L. Y.; Spahr, M. A.; Zhao, W.; Liu, X.; Demock, K.; Kulkarni, A.; Lesch, C. A.; Sanchez, B.; Carter, L.; Marafini, I.; Hu, X.; Mashadova, O.; Yuan, M.; Asara, J. M.; Singh, H.; Lyssiotis, C. A.; Monteleone, G.; Opipari, A. W.; Glick, G. D. Inhibiting Oxidative Phosphorylation In Vivo Restrains Th17 Effector Responses and Ameliorates Murine Colitis. *Journal of immunology* **2017,** 198, 2735-2746.
330. Ohto, U.; Fukase, K.; Miyake, K.; Shimizu, T. Structural basis of species-specific endotoxin sensing by innate immune receptor TLR4/MD-2.

Proceedings of the National Academy of Sciences of the United States of America **2012,** 109, 7421-7426.

331. Ulrich, J. T.; Myers, K. R. Monophosphoryl lipid A as an adjuvant. Past experiences and new directions. *Pharmaceutical Biotechnology* **1999,** 6, 495-524.

332. Goligorsky, M. S. TLR4 and HMGB1: partners in crime? *Kidney International* 80, 450-452.

333. Morin, M. D.; Wang, Y.; Jones, B. T.; Su, L.; Surakattula, M. M. R. P.; Berger, M.; Huang, H.; Beutler, E. K.; Zhang, H.; Beutler, B.; Boger, D. L. Discovery and Structure–Activity Relationships of the Neoseptins: A New Class of Toll-like Receptor-4 (TLR4) Agonists. *Journal of medicinal chemistry* **2016,** 59, 4812-4830.

334. Park, H.-J.; Hong, J.-h.; Kwon, H.-J.; Kim, Y.; Lee, K.-H.; Kim, J.-B.; Song, S. K. TLR4-mediated activation of mouse macrophages by Korean mistletoe lectin-C (KML-C). *Biochemical and biophysical research communications* **2010,** 396, 721-725.

335. Yu, L.; Tan, M.; Ho, B.; Ding, J. L.; Wohland, T. Determination of critical micelle concentrations and aggregation numbers by fluorescence correlation spectroscopy: Aggregation of a lipopolysaccharide. *Analytica Chimica Acta* **2006,** 556, 216-225.

336. Levin, A.; Shibolet, O. Toll-like receptors in inflammatory bowel disease-stepping into uncharted territory. *World Journal of Gastroenterology : WJG* **2008,** 14, 5149-5153.

7. UNKNOWLEDGMENTS

Anche questo percorso si conclude e sono tante le persone che devo ringraziare:

Grazie Prof., o spero da qui in poi Francesco. Il mio percorso di dottorato nel suo gruppo è forse nato un po' per caso, un po' in modo imprevisto. Le sono piombato in studio chidendole disperatamente una possibilità e lei me la ha semplicemente data (anche se con non pochi sacrifici). Mi ricordo ancora le sue parole al mio inizio: " Fabio dovrai avere molta intraprendenza e saperti adattare, insomma io ti butterò nell'oceano….starà a te imparare a nuotare o annegare…". Se all'inizio mi fecero paura, mi diederò subito dopo quella grinta ed energia necessaria per compiere questo percorso tortuoso! Grazie di cuore.

Grazie a tutte le persone con cui ho condiviso parte o l'intero percorso di questi lunghi 3 anni. Andando in ordine:

Grazie Stefania per avermi accolto, aiutato, insegnato e per essere sempre disponibile in qualsiasi momento.

Grazie Vale, il mio punto di riferimento in labortorio. Non sai quanto ancora sorrido a ripensare a tutti i bei momenti passati insieme, alle risate fino a piangere e ai confronti che abbiamo avuto!

Grazie Laura, la mia salvezza! Non oso immaginare il numero delle ore che abbiamo passato al telefono o davanti al pc insieme! Se non fosse stato per te, probabilmente molte cose oggi sarebbero andate diversamente…mi dispiace solo poterti vedere così raramente.

Grazie Florent (o più semplicemnte FLO). La nostra amicizia tra colleghi è forse iniziata al primo congresso insieme, e se ripenso a Marbella Toll2015 ancora oggi non riesco a smettere di ridere! Grazie davvero per tutti i bei momenti passati insieme: sia per i caffè, i pranzi (all-you-can-eat e non) e le birre sia per tutto l'aiuto che forse non sai di darmi in laboratorio ogni volta che parliamo e ci confrontiamo. Merci beaucoup!

Grazie Andrea, arrivato anche tu forse un po' per caso sei diventato subito un perno insostituibile del nostro laboratorio! Grazie per avermi ascoltato sempre con reale attenzione ogni volta che abbiamo parlato, anche quando le cose per te non erano facili (non sono molte le persone così); grazie per tutti i confronti utili che spesso abbiamo, ma soprattutto per le birre e le sane risate che ci facciamo anche al di fuori del lavoro. Scusa se ogni tanto ti sembro un po' "assente", ma purtroppo fa un po' parte del mio carattere. Spero prima o poi di dimostrarti tutta la mia gratitudine….ma direi che da qui in avanti di tempo ne abbiamo! XD Grazie davvero!

Grazie Silvia (e qua dovrò usare le parole con molta cautela XD). Scherzi a parte…so di non essere un granché ad esprimere affetto e gratituidine, ma ci proverò in poche righe: grazie davvero del percorso di crescita che TU mi hai fatto fare, e di quello che abbiamo fatto insieme in quel fantastico anno ormai passato. Ci sono stati alti e bassi per entrambi (forse un po' più bassi per me XD), ma siamo riusciti ad affrontare tutte le difficoltà insieme una dopo l'altra! Grazie perché sei riuscita ad insegnarmi davvero molto, come ad esempio che ahimè non ho sempre ragione, anche se ovviamente ho le idee migliori XD….
Grazie davvero tanto Gina, tutto sommato ti voglio bene!!!

Grazie Luca, anche con te un anno davvero intenso passato insieme! Grazie per aver avuto la pazienza di ascoltare le miei lunghe filippiche senza aver mai mai dato cenno di pazzia. Grazie per avermi fatto capire che la musica è soprattutto fatta da ciò che non ascolto, ma soprattuto per il percorso di crescita che, insieme a Silvia, mi hai fatto fare! Grazie davvero e in bocca al lupo con la musica!

Grazie Lenny, grazie al mio zione preferito! Grazie per aver passato con me tutti i giorni (o quasi) di questi lunghi 3 anni, sorbendoti i miei deliri e le mie pazzie! Tu, forse meglio di chiunque altro, sai cosa mi succede quando passo troppe ore in laboratorio trasformandomi da Dr. Jekyll a Mr. Hide! Grazie per tutte le cagate (si può dire?! si cazzo diciamolo) che siamo riusciti a concepire insieme e che ci hanno fatto ridere fino a piangere!

Grazie Fede, grazie per tutto il tempo che abbiamo e stiamo passando insieme! Sei una persona davvero speciale! Hai saputo aiutarmi quando vedevi che ero davvero in difficoltà, mi hai ascoltato quando capivi che ne avevo bisogno e mi hai consigliato quando cercavo un confronto. Grazie davvero!

Grazie Mati, grazie per avermi sempre ascoltato e supportato, per aver avuto la pazienza di insegnarmi, guidarmi e consigliarmi per tutta la durata del mio viaggio! Grazie per avermi fatto sempre sentire una persona di valore e per avermi aiutato a credere sempre nelle mie capacità! Grazie di cuore

Grazie Prof. Fusi, prima o poi riuscirò a dire grazie Paola! Grazie semplicemente per esserci sempre stata per me. Un punto di riferimento importante, una guida, una persona che ha sempre creduto in me! Grazie

Grazie Monica, la mia babbazza permalosa preferita! Scherzi a parte…ne abbiamo passato di tempo insieme io e te! Abbiamo parlato e discusso, ci siamo confidati e scornati (XD)….ma soprattuto abbiamo imparato a conoscerci dando vita ad una bella amicizia! Grazie davvero per tutto!

Grazie Maya, beh insomma è inutile scriverlo…credo tu sappia già quanti grazie ti devo. Se non fosse stato per te io di certo non avrei mai iniziato il dottorato e non sarei mai giunto a questo importante traguardo. Grazie per avermi sempre spronato, grazie per aver convertito le mie insicurezze in sicurezze ed essere il mio più importante punto di riferimento...Grazie…

Grazie a tutte le persone che hanno condiviso (chi più e chi meno) questi lunghi 3 anni con me, anche se non vi ho citato esplicitamente sappiate che ho bene in mente ognuno di voi!
Grazie a tutti!