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Receptors involved in cell activation by defined uronic acid polymers and bacterial components

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PREFACE

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CONTENTS

1 INNATE IMMUNITY AND THE INFLAMMATORY RESPONSE 6

1.1 SEPSIS 7

2 BACTERIAL TRIGGERS OF THE INFLAMMATORY RESPONSE 8

2.1 CELL WALL COMPONENTS 8
2.1.1 Gram-positive bacteria 8
2.1.2 Gram-negative bacteria 9
2.1.3 Other bacteria 11

3 ALGINATE 12

3.1 STRUCTURE AND FUNCTIONAL PROPERTIES 13
3.2 IMMUNOMODULATING PROPERTIES 13
3.3 BIOMEDICAL APPLICATIONS 14

4 RECEPTORS INVOLVED IN ACTIVATION OF IMMUNE CELLS BY BACTERIAL CELL WALL COMPONENTS 15

4.1 CD14 17
4.1.1 Specificity of CD14 17
4.2 β2-INTEGRINS 18
4.2.1 Specificity of β2-integrins 18
4.3 TOLL-LIKE RECEPTORS (TLRs) 19
4.3.1 Specificity of TLRs 20
4.3.2 TLR signaling 21

5 AIMS OF THE STUDY 23

6 SUMMARY OF WORK 24

7 GENERAL DISCUSSION 27

7.1 ADVANTAGES AND LIMITATIONS WITH *IN VITRO* STRATEGIES AND THE USE OF CELL LINES 27
7.2 HOW ARE BACTERIAL CELL WALL COMPONENTS RECOGNIZED? 28
7.2.1 Differential activity due to physicochemical differences of ligands 29
7.2.2 Differential receptor usage due to physicochemical differences of ligands 30
7.2.3 Composition of signaling receptor complexes 31
7.2.4 Expression and regulation of TLRs 33
7.3 RECEPTOR-DIRECTED STRATEGIES AND IMMUNOMODULATORY POLYSACCHARIDES TO INHIBIT SYSTEMIC INFLAMMATION 35
7.3.1 Receptor-directed strategies 35
7.3.2 Immunomodulatory polysaccharides 36

8 REFERENCES 37

URN:NBN:no-6359

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[1]The contribution of the first two authors should be considered as equal.

URN:NBN:no-6359

ABBREVIATIONS

-/-	knockout
Ab	antibody
β2-integrins	CD11a-c/CD18
C6OXY	cellulose oxidized in C6-position
CHO	Chinese hamster ovary
CR3	complement receptor 3 (CD11b/CD18)
CR4	complement receptor 4 (CD11c/CD18)
DC	dendritic cell
DLPS	detoxified/deacylated LPS
G	guluronic acid
G-	gram-negative
G+	gram-positive
GBS	group B streptococci
GPI	glycosylphosphatidyl-inositol
Hsp	heat-shock protein
IL	interleukin
IL-1Ra	IL-1 receptor antagonist
kDa	kiloDalton
LAM	lipoarabinomannan
LBP	LPS binding protein
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LTA	lipoteichoic acid
M	mannuronic acid
mAb	monoclonal antibody
MODS	multi organ dysfunction syndrome
Mw	molecular weight
NF-κB	nuclear factor-κB
NK	natural killer
PAMP	pathogen-associated molecular pattern
PGN	peptidoglycan
PRR	pattern recognition receptor
R	receptor
sCD14	soluble CD14
SIRS	systemic inflammatory response syndrome
TGF	transforming growth factor
TIR	toll/IL-1R
TLR	toll-like receptor
TNF	tumor necrosis factor

URN:NBN:no-6359

1 INNATE IMMUNITY AND THE INFLAMMATORY RESPONSE

Innate immunity refers to the first-line host defense against invading pathogens and is evolutionary conserved between vertebrate and invertebrate species[1]. The term "pattern recognition receptors" (PRRs) was suggested by Janeway to describe the non-clonal, germ-line encoded molecules recognizing structural features (pathogen-associated molecular patterns, PAMPs) common to a variety of pathogens, but different from host components[2-4]. PRRs can be secreted, endocytic, or signaling. Among the secreted PRRs used by the host to limit infection are the complement system[5], collectins[6] and lipopolysaccharide (LPS)-binding proteins[7]. The endocytic and activating PRRs are expressed on innate immune cells like monocytes/macrophages, neutrophils, B-cells and dendritic cells (DCs). Endocytosis through PRRs enables phagocytes to process and present antigens to T-cells, whereas activation through PRRs can induce expression of costimulatory surface molecules needed for efficient activation of the T-cells (reviewed in refs.[1,4]). Thus, the innate immune cells provide a link to the subsequent response of acquired immunity, which is adaptive, restricted to vertebrates, and rely on clonal expansion of T- and B-lymphocytes with induction of immunological memory.

In response to an infection, trauma or tissue injury, the immune system mounts an inflammatory response in order to combat, limit and repair the damage. Proinflammatory mediators like tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-8, IL-12, interferon-γ, platelet-activating factor, various chemokines and subsequently arachidonic acid metabolites (leukotrienes, prostaglandins, thromboxanes) are released into the microenvironment by mononuclear phagocytes and endothelial cells[8-12]. The complement and coagulation cascades become activated. The combined effect of all these agents increases capillary permeability and expression of adhesion molecules on vascular endothelium, with concomitant influx of leukocytes to the infected tissue. Antimicrobial mediators are released from recruited neutrophils, phagocytic activity and further cytokine production is activated in surrounding cells, and tissue is regenerated. The intensity and duration of the inflammatory response is closely regulated through production of compensatory anti-inflammatory cytokines (IL-4, IL-10, transforming growth factor (TGF)-β) and mediators like soluble TNF receptors (TNFR) and IL-1 receptor antagonist (IL-1Ra)[8-12].

URN:NBN:no-6359

1.1 SEPSIS

In cases of severe infection, microbes, microbial products or other inflammatory mediators may enter the circulation and the patient exhibits a systemic inflammatory response syndrome (SIRS)[10,13]. SIRS is eventually counteracted by a compensatory anti-inflammatory response syndrome, but if the balance is out of control, the patient may die from immediate shock or subsequent multi organ dysfunction syndrome (MODS)[10,13]. Sepsis is defined as SIRS caused by infection, and is further subdivided into severe sepsis or septic shock according to the severity of clinical manifestations like hypoperfusion, hypotension and organ dysfunction[13]. Advances in medical practice and technology, together with a growing number of resistant microorganisms due to unrestricted use of antibiotics, have increased the risk of sepsis[14]. In USA, 400-500 000 septic episodes is estimated to occur annually, and the numbers are rising[15]. Much of this change is caused by hospital-acquired infections[14,16]. A current estimate is that about 25% of intensive care unit (ICU)-patients have sepsis, whereas severe sepsis occurs in 2-3% of ward patients and 10-15% of ICU-patients[16]. The 28-day mortality is approximately 10% (SIRS), 20% (sepsis), 20-40% (severe sepsis) and 40-60% (septic shock). The outcome of sepsis is determined by the severity of underlying diseases and the presence of shock and MODS[16].

Most of the approaches used in developing sepsis therapies have been towards the pro-inflammatory response[17]. TNF was among the first cytokines implicated in the pathogenesis of sepsis, and is rapidly induced together with IL-1 in response to infection or injury[18-21]. Still, clinical trials with antibodies to TNF, soluble TNFR chimeras or IL-1Ra have failed to show significant improvement[17]. Lipopolysaccharide (LPS) is a main mediator of gram-negative (G-) sepsis, but clinical studies with antibodies to LPS have yielded inconsistent results[22]. Other anti-LPS strategies with LPS-mimetic antagonists, LPS inhibitors like bactericidal/permeability protein (BPI), and hemoperfusion with polymyxin B, are currently in trials[17,23,24]. Among several ongoing studies[17], coagulation inhibitors like activated protein C (APC), tissue-factor pathway inhibitor and antithrombin III show promising results[25,26]. Recently, phase III clinical trials with recombinant human APC were stopped as significant improvement was achieved[27]. Despite the failure of most clinical trials on sepsis, retrospective studies have shown significant benefit in better-characterized subgroups of the patients included. A better understanding of the mechanisms underlying the systemic inflammatory response leading to sepsis will help in development of new therapies, as well as in early identification of patients who are more likely to respond to new modalities (better diagnostic tools).

URN:NBN:no-6359

2 BACTERIAL TRIGGERS OF THE INFLAMMATORY RESPONSE

In the pre-antibiotic era, gram-positive (G+) organisms were the most common agents of infections, whereas in the period 1950-1980, sepsis caused by gram-negative (G-) bacteria dominated[15,16]. Lately there has been a growing incidence of G+ microbes, especially in hospital-acquired infections, and at the moment about equal proportions of G+ and G- bacteria are causing sepsis[16].

During infection, bacterial components are spontaneously released into the microenvironment and may reach the circulation. This also occurs as a result of antibiotic treatment and/or lysis of the bacteria. Several of the bacterial mediators are known to be pro-inflammatory, and thus could be involved in triggering the sepsis cascade (Table 1 and reviewed in refs.[9,28,29]). Enterotoxins and exotoxins are proteins released by various bacterial species that in addition to their native functions (e.g. enzymes, superantigens, porins), can be highly potent cytokine inducers[9,30]. Also, bacterial DNA has immunostimulatory properties and is recognized by host cells through unmethylated CpG dinucleotide motifs[31,32]. Still, the major bacterial cytokine inducers are the diverse cell wall components composed of sugars, lipids and proteins.

TABLE 1 BACTERIAL MEDIATORS

Microorganism	Inflammatory component
General	DNA (CpG) lipoproteins, lipopeptides peptidoglycan (PGN) cell wall associated proteins heat shock proteins (Hsp)
G+ and G- bacteria	exotoxins capsular polysaccharides
G+ bacteria	teichoic acids lipoteichoic acid (LTA) superantigens*
G- bacteria	lipopolysaccharide (LPS) outer membrane proteins fimbriae/pili*
Mycobacteria	lipoarabinomannan (LAM) lipomannans

* Not restricted to, but mainly expressed by either G+ or G- bacteria

2.1 CELL WALL COMPONENTS

2.1.1 Gram-positive bacteria

G+ bacteria most often implicated in sepsis are *Staphylococcus aureus, Streptococcus pneumoniae,* coagulase-negative staphylococci, β-hemolytic streptococci and enterococci[9,16]. However, Group B Streptococci (GBS) are the most common cause of life-threatening neonatal infections, causing sepsis, meningitis and pneumonia with an incidence of 0.5-3 cases per 1000 live births[33-35]. The case-fatality rate is 5-15%, and although the incidence is low among adults, the case-fatality rates are higher than in the newborn[34,35]. *Listeria monocytogenes* is a food-borne G+ bacteria causing listeriosis with clinical manifestations including sepsis and

URN:NBN:no-6359

meningitis[36,37]. As with GBS, persons at greatest risk are pregnants, neonates, immunocompromised people and the elderly, and even if the incidence is low (ca. 0.5 per 100 000), the mortality is high (15-20%)[36,37].

The mechanisms underlying G+ sepsis are not clear, but probably involve released toxins and cell wall components with pro-inflammatory activity[9]. The G+ cell wall is composed of several layers of peptidoglycan (PGN, N-acetyl glucosamine and N-acetyl muramic acid crosslinked by peptide bridges) where proteins and carbohydrates like teichoic acids (poly glycero- or ribitol-phosphate substituted by sugars and D-Ala), teichuronic acids (polymers of alternating uronic acid and a hexose/hexosamine) and capsular polysaccharides are covalently attached[38] (Figure 1). Lipoteichoic acids (LTA) are teichoic acids with a glycolipid anchor, and are found inserted into the bacterial cell membrane together with other glycolipids and lipoproteins. Several G+ cell wall structures are pro-inflammatory[9,28,29]. PGNs and cell wall fragments are released from dying bacteria or bacteria treated with antibiotics, and these components were early known to be immunostimulatory[39-42]. LTA from different strains has varying capacity to stimulate monocyte cytokine release, and deacylation abolishes the activity, pointing to the importance of the lipid part[41,43-45]. The amount of capsular and other cell wall associated polysaccharides differs between G+ strains, and in addition to their role as virulence factors, they often induce cytokine synthesis[45-47]. Substantial amounts of lipoproteins are present in G+ bacteria[48], and although no reports exist on their cytokine-inducing properties, some of the immunostimulating membrane proteins reported could be lipoproteins[28,49,50].

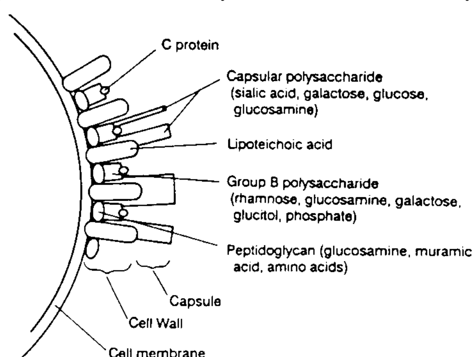

FIGURE 1 Schematic diagram of a gram-positive bacterial cell wall (Group B streptococci), from ref. 9.

2.1.2 Gram-negative bacteria

The most prevalent bacteria involved in G- sepsis are *Escherichia coli, Pseudomonas aeruginosa,* Klebsiella species and enterobacteria[16]. The membrane glycolipid lipopolysaccharide (LPS) was early considered the main agent responsible for G- sepsis, although exotoxins and other cell wall components also contribute in triggering the septic cascade. Different from G+ bacteria, only a thin (mono) layer of PGN is surrounding the plasma membrane of G- bacteria, and a second, outer membrane is covering the PGN layer[38]. Like G+

URN:NBN:no-6359

bacteria, several G- bacteria are encapsulated, and various proteins, lipoproteins and carbohydrates are attached to the plasma membrane or to PGN (Figure 2A). Fimbriae, or pili, are originating in the cytoplasmic membrane and mediate bacterial adherence. LPS is anchored in the outer membrane together with various proteins, some of them lipoproteins linking the outer membrane to the PGN layer. Fimbriae/pili[51], membrane proteins[52], lipoproteins[53,54] and capsular polysaccharides[55,56] all stimulate cytokine synthesis in mononuclear phagocytes. However, due to the potency in stimulating release of proinflammatory mediators and the ability to reconstruct the clinical events of sepsis, most of the research done on G- bacterial components has been on LPS[18,19,57].

LPS is an amphiphilic molecule composed of a lipid A part, a core oligosaccharide and an O-antigenic polysaccharide part[58-61] (Figure 2), and exists as aggregates in aqueous solutions above the critical micelle concentration[62]. The O-specific chain is a highly variable heteropolymer made up from 0-50 repeating oligosaccharide units. The core region is divided into an outer and an inner core, where 3-deoxy-D-*manno*-octulosonic acid (KDO) links the sugar part of LPS to lipid A in an acid labile linkage. Lipid A is a phosphorylated β-(1-6) di-glucosamine, N- and O-substituted with acyl chains of different nature, numbers and length, and is the most conserved part of LPS. Although some studies suggest the importance also for the core- and O-antigenic chains[63-66], the endotoxic activity of LPS is considered to be in the lipid A part (Figure 2B), and is dependent on the structure and conformation of lipid A[67-69]. Full endotoxic activity requires a bisphosphorylated di-glucosamine with six saturated fatty acids in a defined location, as in *E. coli* lipid A. Partial structures have reduced or no stimulatory activity and can act as LPS antagonists[70-73], as does lipid A from the non-pathogenic bacteria *Rhodobacter sphaeroides*[74] and *R. capsulatus*[75].

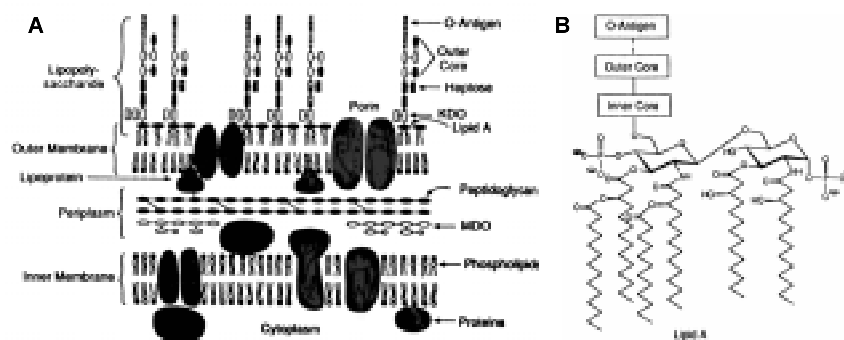

FIGURE 2 Schematic representation of the *E. coli* envelope (A) and lipid A (B), from ref. 58.

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2.1.3 Other bacteria

Mycobacteria are the causative agents of diseases like tuberculosis and leprae. Several lipids, glycolipids and lipoproteins covering the mycobacterial cell wall can stimulate immune cells, but the main focus of research has been on lipoarabinomannan (LAM)[76,77]. The spirochetes *Borrelia burgdorferi* and *Treponema pallidum* are implicated in Lyme disease and syphilis, respectively, and lipoproteins are considered the principle spirochete components activating an immune response[78-81].

URN:NBN:no-6359

3 ALGINATE

P. aeruginosa is an opportunistic pathogen to patients with cystic fibrosis, where the bacteria undergo mucoid conversion and colonize the patient's lungs[82]. The capsule is made of a family of polysaccharides called alginate, and promotes adhesion of the bacteria to the tracheal epithelium. The alginate capsule forms a protective barrier against opsonization and phagocytosis, and it is also closely associated with virulence[83]. Thus, the inflammatory properties of alginate are of importance, also because of an increasing interest in the use of alginate in biomedical and pharmaceutical applications[84-86].

Alginates are linear co-polymers of 1-4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) (Figure 3) with highly variable composition and sequential structure depending on the source from where they are isolated[87,88]. In nature, alginate is found mainly as the structure substance of marine brown seaweed, but also as an exopolysaccharide produced by soil bacteria of the *Azotobacter* spp.[89], and several *Pseudomonas* spp.[90]. Because of its viscosity, its ability to retain water, and its gelling and stabilizing properties, alginate has found a variety of industrial applications, with an annual production of about 35 000 metric tons. The main applications are in textile print pastes, in the pharmaceutical and food industry, and for immobilization of living cells[84-86,88,91]. Different designations are used to discriminate between alginates isolated from various sources and with different composition, and the names employed are listed in Table 2 together with some average characteristics.

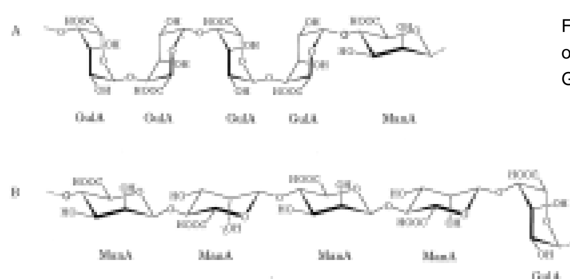

FIGURE 3 Schematic representation of the alginate structures G-blocks (A) and M-blocks (B).

TABLE 2 DESIGNATION AND TYPICAL CHARACTERISTICS OF SELECTED ALGINATES

Polymer	Source (example)	Mw (kDa)	Composition
High-M alginate	Algae *(A. nodosum)*	6-500	80-90% D-ManA
Mannuronan	Bacteria *(P. aeruginosa)*	50-500	100% D-ManA
Poly-M	Bacteria *(P. aeruginosa)*	50-500	90-99% D-ManA, 1-9% L-GulA
M-blocks	Algae, bacteria *(P. aeruginosa)*	< 6	94-99% D-ManA, 6-9% L-GulA
G-blocks	Algae, bacteria *(A. vinelandii)*	< 6	90-99% L-GulA, 1-9% D-ManA

URN:NBN:no-6359

3.1 STRUCTURE AND FUNCTIONAL PROPERTIES

Alginates do not have any regular repeating unit; thus the knowledge of the monomeric M/G composition is not sufficient to determine the sequential structure of the polymers (reviewed in ref.[88]). Some sequential parameters (monad, diad and triad frequencies) can be obtained from ^{1}H-NMR spectroscopy, making it possible to estimate average block lengths of homopolymeric regions[92,93]. This is important, as the functional properties of alginates are strongly correlated with the structure. Di-axially linked G-blocks form cavities favoring binding of multivalent cations like calcium, strontium and barium, accounting for the capacity of oligomeric G-regions to form ionotropic gels[88,94]. The alginate composition varies between algal species and between various tissues in the same plant[87], with high-M alginate found in *Ascophyllum nodosum* fruiting bodies[88]. Bacterial alginate differ from the algal polymers in that it is O-acetylated[95], and *Pseudomonas* spp. make alginate with more than 90% M and lacking G-block sequences (poly-M). As first demonstrated by Larsen and Haug for *A. vinelandii*, alginate is synthesized in both algae and bacteria as homopolymeric mannuronan[96,97]. Varying amounts of the M-residues in the polymer are then epimerized to G-residues by mannuronan C5-epimerases, and several C5-epimerases yielding different epimerization patterns have been isolated from bacteria[98-100]. The epimerases can use both algal and bacterial alginate substrates, and recombinant production of these enzymes may be used to design alginate polymers with desired composition and structure best fit for various applications[100,101].

3.2 IMMUNOMODULATING PROPERTIES

Several years ago, a Japanese group fractionated polysaccharides with anti-tumor activity against murine sarcomas and carcinomas from the seaweed species *Laminaria* and *Sargassum*, and found the active extract to be alginate[102-104]. Best anti-tumor activity was obtained with high-M alginate[103], and the researchers suggested that structural differences of the alginates resulting in different conformations could be a possible explanation[104]. Alginate from *P. aeruginosa* (poly-M) is shown to stimulate murine macrophages to IL-1 production, and to be a potent polyclonal B-cell mitogen[56]. Several reports from Otterlei *et al*. demonstrate that alginates isolated from algae share the immunostimulating properties of bacterial alginates[55,105,106]. The cytokine-inducing effect increases with molecular size and the content of mannuronic acid in the polymers, with poly-M being the most active[55]. Moreover, oxidation of cellulose in C6-position (C6OXY) introduces D-glucuronic acid in the β-(1-4) linked D-glucose-polymer, resulting in a 3D-structure resembling that of poly-M. And like poly-M, C6OXY-cellulose induces monocyte TNF-production, although with lower efficiency[55]. Homooligomeric G-blocks do not induce cell activation, but rather inhibit the cytokine production induced by poly-M and LPS, as well as binding of poly-M and LPS to monocytes[55].

URN:NBN:no-6359

Poly-M is the first non-LPS molecule shown to bind to the myeloid surface molecule CD14 and to activate cells in a CD14-dependent manner[106]. Moreover, poly-M given prophylactically protects mice from lethal bacterial infections (T. Espevik and G. Skjåk-Bræk, unpublished results) and radiation[107], and stimulates murine haematopoiesis[107]. These results support the potential use of poly-M as a general immunostimulator for therapeutic purposes.

3.3 BIOMEDICAL APPLICATIONS

The immunomodulating properties of alginates are particularly important as these polymers are used in several biomedical applications (reviewed in refs.[84-86]). Dressings of calcium alginate are used in wound healing and as surgical hemostats[86], and alginate-based raft-formulations are used for symptomatic treatment of heartburn and oesophagitis[108]. Another application of alginate is in sustained drug-release[86]. Living cells immobilized in calcium alginate beads are used as biocatalysts in several industrial processes, ranging from ethanol production by yeast cells, production of monoclonal antibodies from hybridoma cells, and production of recombinant cytokines, factor IX or human growth hormone from transfected fibroblasts[84,91]. Alginate has also potential as implantation material for hormone producing cells, and encapsulated Langerhans islets are currently being evaluated as a bio-artificial endocrine pancreas[85,109,110]. In all the abovementioned applications, the biological activities of different alginates are of outmost importance. When alginate is used for encapsulation of living cells, the immunostimulating property is unwanted. However, immunostimulatory poly-M is of relevance for treatment of patients at high risk of infections.

URN:NBN:no-6359

4 RECEPTORS INVOLVED IN ACTIVATION OF IMMUNE CELLS BY BACTERIAL CELL WALL COMPONENTS

LPS has become a reference bacterial component, and most of what is known about the mechanisms involved in cellular activation by other bacterial components, come from comparison with LPS. A selection of cellular receptors and some of their bacterial ligands is given in Table 3. Whereas some receptors are proposed to be involved in phagocytosis and/or as binding molecules in signaling complexes (mannose receptor, scavenger receptors, β2-integrins, CD14), others probably function as signal transducers (TLRs) that not necessarily bind to the ligand. Some of the receptors involved in cell activation are presented in the following sections.

TABLE 3 RECEPTORS INVOLVED IN RECOGNITION OF BACTERIAL COMPONENTS

Bacterial component	Pathogen	Receptor	A, B*	Selected refs.
LPS, lipid A and most intact bacteria	G- bacteria	CD14	A, B	111-114
		TLR2	A, B?	115-118
		TLR4	A, B?	118-123
		β2-integrins	A, B	124-128
		Scavenger R	B	129-131
		Mannose R	B	132,133
		Moesin	A	134,135
		DAF (CD55)	A, B	136,137
		K+ channel	A	138
		Nod1, Nod2	A, B	139
		216 kD protein	A?, B	140
		P2X7	A	141
Intact bacteria	G+ bacteria	CD14	A	142
		TLR2	A	143-148
		TLR6	A	148
		β2-integrins	A, B	149
		Scavenger R	B	131,150-152
		Mannose R	B	133
	mycobacteria	CD14	A, B	153,154
		TLR2	A	145,155,156
		TLR4	A	155
		β2-integrins	B	154,157-161
		Scavenger R	B	154,162
		Mannose R	B	154,157,163
	spirochetes	CD14	A, B	78
		TLR2	A	145
		β2-integrins	B	164
		proteoglycans	B	165

URN:NBN:no-6359

Bacterial component	Pathogen	Receptor	A, B*	Selected refs.
Cell walls	G+ bacteria	CD14	A, B	[166-169]
		TLR2	A	[170,171]
	mycobacteria	TLR2, TLR4	A	[172]
Peptidoglycan (PGN)	most bacteria	CD14	A, B	[42,173-175]
		TLR2	A	[143,144,148,170,171]
		TLR6	A	[148]
		PGRP	B	[176]
		Nod2	A	[139]
Lipoproteins, lipopeptides	most bacteria	CD14	A, B	[80,81,177]
		TLR2	A	[117,145,178-180]
DNA (CpG)	most bacteria	TLR9	A	[181]
Heat shock proteins (Hsp)	most bacteria	CD14	A, B	[182]
Lipoteichoic acid (LTA)	G+ bacteria	CD14	A, B	[168,183]
		TLR2	A	[143,144]
		TLR4	A	[170]
		Scavenger R	B	[150,151,184]
Rhamnose-glucose polymers	G+ bacteria	CD14	A, B	[46]
		β2-integrins	B	[46]
Glycolipids	G+, spirochete	CD14	A	[185,186]
		TLR2, TLR4	A	[185,186]
Alginate/uronic acid polymers	G- bacteria	CD14	A, B	[106,187]
Lipoarabinomannan (LAM)	mycobacteria	CD14	A, B	[77,167,188]
		TLR2	A	[156,189]
		Mannose R	B	[190-192]

* A = activation, B = binding.

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4.1 CD14

CD14 is a 53-57 kDa glycoprotein highly expressed on monocytes/macrophages[193], with lower amounts present on the surface of neutrophils[193-195], B-cells[196,197] and some epithelial cells[198,199]. It contains extracellular leucine-rich repeats (LRRs) and is linked to the outer cell membrane via a glycosylphosphatidyl-inositol (GPI) anchor[200,201]. CD14 was the first receptor shown to mediate LPS-activation of myeloid cells, facilitated by the 58 kDa plasma acute phase protein, LPS binding protein (LBP)[7,111,202]. LBP shuttles LPS from the bacterial outer membrane to serum high- or low-density lipoproteins (neutralization)[203,204], to CD14 (uptake, activation)[205], and possibly into cell membranes (uptake, activation?)[206,207]. This process is facilitated by soluble CD14 (sCD14) which exists in µg/ml quantities in normal serum[208,209], generated by cleavage of the surface receptor by endogenous or exogenous enzymes, and by secretion from intracellular pools[210-216]. sCD14 mediates LPS-activation of cells lacking membrane CD14[214,217-219], and low concentrations of sCD14 enhance responses even in CD14-expressing cells[63,205,220,221] whereas high concentrations can be inhibitory[222]. Elevated levels of sCD14 are found in serum from patients with various diseases, and in some cases the level of sCD14 correlates with the severity of the disease[223-225].

4.1.1 Specificity of CD14

CD14 mediates binding and cell activation by structurally diverse compounds (bacterial structures listed in Table 3, reviewed in refs.[214,215,226]), and the importance of CD14 in cellular activation by LPS or G- bacteria is obvious. Cells from patients with paroxysmal nocturnal haemoglobinuria lack GPI-anchored proteins and are hyporesponsive to LPS[227], and the same is observed in CD14 knockout (CD14-/-) mice[228]. Moreover, CD14-negative cells gain LPS responsiveness when they are transfected with CD14[229,230]. Anti-CD14 mAbs block cellular cytokine-production by LPS *in vitro*[111] and *in vivo*[231], but high concentrations of LPS induce cellular activation by CD14-independent pathways[228,232]. CD14 is also involved in clearance of LPS and bacteria by internalization/phagocytosis[70,112,233], a process that may also be involved in LPS signaling[234-236].

In addition to LPS, CD14 is involved in responses to G- bacterial heat-shock proteins[182], lipoproteins[177] and capsular polysaccharides, like poly-M[106]. CD14 mediates binding and cellular activation by several G+ bacterial structures (reviewed in ref.[226]): Some intact bacteria[142], cell walls[167-169], PGN[42,173-175], LTA[183], and rhamnose-glucose polymers[46]. However, in contrast to G- bacteria, no difference in survival is observed among CD14+/+ and CD14-/- mice in response to G+ *S. aureus*[237]. Mycobacteria[153,154] and the mycobacterial cell wall structure LAM[77,167,188], spirochetes[78], spirochetal lipoproteins and lipopeptides[80,81], and the yeast WI-1 antigen[238], all signal cell activation through CD14, although CD14-independent

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mechanisms are also employed. Both the binding and cell-activation domains for LPS, PGN and LAM are located within the N-terminal 152 amino acids of CD14, with amino acids 51-64 as a shared region for binding[168,174,239-242]. However, some of the sequences involved in binding and activation of CD14 are distinct, as antibodies or LPS/lipid A partial structures that inhibit activation do not necessarily block binding[70,233,243].

Non-microbial CD14 ligands are also found, and β-(1-4)-linked glucuronic acids and seaweed high-M alginates[106], chitosans[244], respiratory syncytial virus protein F[245], and the plant derived diterpene Taxol[232] all use CD14 in cell activation. The finding that endogenous phospholipids bind to mCD14, and that transfer is facilitated by LBP, suggest a role for LBP/CD14 also in regulation of plasma membrane phospholipid turnover[206,246,247]. Moreover, CD14 mediates clearance of apoptotic cells[248,249], and is engaged by the endogenous 'danger signals' heat-shock proteins 60 (Hsp60) and Hsp70[182,250], and by IL-2[251].

4.2 β2-INTEGRINS

Integrins belong to a widely expressed family of transmembrane adhesion-molecules, mediating cell-cell and cell-matrix interactions[252]. The β2-integrins are expressed exclusively on the surface of leukocytes as heterodimers of an α subunit, CD11a-d, non-covalently associated with a common β subunit, CD18[253,254]. CD11a/CD18 ($\alpha_L\beta 2$, LFA-1) is expressed by virtually all leukocytes, CD11b/CD18 ($\alpha_M\beta 2$, complement receptor 3 (CR3)) and CD11c/CD18 ($\alpha_X\beta 2$, CR4, gp150/95) are normally present on monocytes/macrophages, neutrophils, DCs, and natural killer (NK)-cells, whereas CD11d/CD18 ($\alpha_d\beta 2$) is found expressed on CD8+ lymphocytes and strongly on specialized tissue macrophages[253,255,256]. Expression varies dependent on the state of cell activation and differentiation. In addition to mediate "outside-in" signaling following binding of extracellular ligands, β2-integrins are subject to "inside-out" signaling that changes the avidity and conformation of the receptors (reviewed in refs.[254,257]).

4.2.1 Specificity of β2-integrins

CD11a-c/CD18 are involved in transmigration of leukocytes into inflamed tissues through firm adhesion to endothelial ligands like ICAMs[252,254]. ICAM-1, fibrinogen, factor X, iC3b (generated by cleavage of complement C3) and heparin binds to the I-domain of CD11b[258], whereas β-glucans and various polysaccharides containing mannose, glucose or N-acetyl-D-glucosamine bind to a lectin domain C-terminal to the CD11b I-domain[259]. CR4 appears to share some of the CR3-ligands[254]. CR3 and CR4 also mediate phagocytosis of iC3b-opsonized and non-opsonized particles/pathogens, and CR3 is involved in apoptosis of neutrophils[260].

Leukocyte adhesion deficiency (LAD) I is characterized by no expression or expression of dysfunctional β2-integrins, and patients are predisposed to recurrent infections due to impaired

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transendothelial emigration, phagocytosis and antibody-dependent cytotoxicity[253,261], demonstrating the importance of β2-integrins in host defense. In addition to signal phagocytosis, a series of studies by Wright and colleagues suggested that β2-integrins could also function as signal transducing receptors triggering activation of phagocytes after non-opsonic binding to LPS and G- bacteria[124,125]. But despite demonstrable binding, subsequent experiments showed that phagocytes from LAD patients responded normally to LPS, implicating that the β2-integrins were not involved in signaling LPS-activation[262]. In retrospect, the results can be explained from the normal expression of CD14 and Toll-like receptors (TLRs) on these cells, and later studies by Ingalls *et al*. have showed that LPS can induce nuclear factor (NF)-κB translocation in Chinese hamster ovary (CHO) cells transfected with CR3 or CR4 in the absence of CD14[127,128].

β2-integrins are also differently involved in non-opsonic binding to and/or activation by G+ bacterial components. CR3 contributes to NO-production induced by *S. epidermidis* and heat killed Group B streptococci (GBS)[149]. Binding to CR3/β2-integrins has also been demonstrated for mycobacteria[158-161] and mycobacterial capsular polysaccharides[160], yeast[263,264], the yeast WI-1 antigen[238] and polysaccharides[265], spirochetes[164], *Bordetella pertussis* hemagglutinin[266], Leishmania lipophosphoglycans[267], and oligodeoxynucleotides[268], although the involvement of β2-integrins in cell activation by these ligands has not been reported.

4.3 TOLL-LIKE RECEPTORS (TLRs)

The Toll/IL-1R (TIR) superfamily comprises mammalian, insect, plant and viral proteins which are all engaged in host defense and express a conserved cytosolic TIR-domain (Figure 4, reviewed in refs.[269-272]). The *Drosophila* Toll protein (dToll) mediates dorsoventral polarity of fly embryos[273] and the expression of anti-fungal responses in larvae and adult flies[274] by activating the Rel proteins Dif, Dorsal, and probably Relish[275-279]. Impaired expression of anti-bacterial peptides is observed in larvae mutant in 18-Wheeler[280], another member of the dToll family, but recent reports suggest that none of the 9 Toll-related receptors currently cloned are involved in *Drosophila* anti-bacterial responses[281].

Medzhitov and colleagues discovered the first human homologue of dToll, and demonstrated that constitutive expression of hToll (now TLR4) induced activation of the Rel-protein NF-κB-controlled immune response genes and expression of co-stimulatory molecules (B7.1), suggesting a role for hToll in innate immunity[282]. By now, 9 human Toll-like receptors (TLRs) are cloned[283-287]. Like dToll, they are type I transmembrane proteins with extracellular LRRs and TIR signaling domains (Figure 4). TLRs are differentially expressed in most lymphoid tissues, including peripheral blood leukocytes, but expression is also observed in non-lymphoid tissues like lung, brain, heart, muscle and reproductive organs[115,282-287]. Based on

URN:NBN:no-6359

mRNA expression patterns in human blood leukocytes, TLR1 is proposed to be ubiquitous, TLR2, TLR4 and TLR5 restricted to monocytes, neutrophils and DCs, and TLR3 as more specific to DCs[288]. However, characterization of the expression patterns of the different TLRs is still in its infancy, and also the roles they play in microbe-induced activation of different cell types.

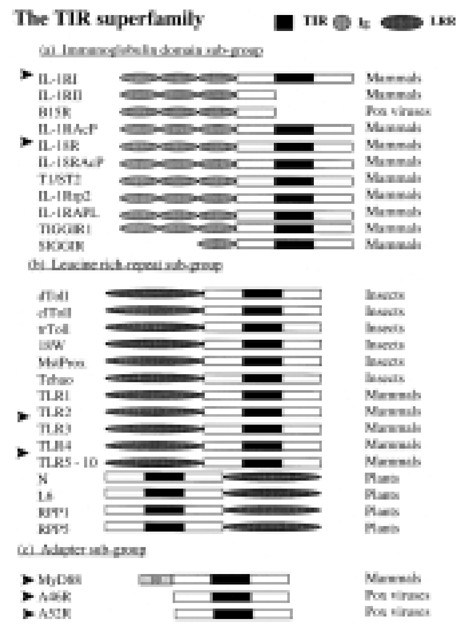

FIGURE 4 The TIR-domain superfamily, ref.269.

4.3.1 Specificity of TLRs

Mammalian TLRs respond to a variety of pathogens with different specificity (bacterial ligands are listed in Table 3), and polymorphism has been demonstrated in human *Tlr2* and *Tlr4* that may influence the susceptibility to infectious diseases[289-292]. Both TLR2 and TLR4 are proposed LPS receptors. Genetic evidences are in support of TLR4, as the defect in LPS hyporesponsive C3H/HeJ and C57BL/10ScCr mice was found to be a dominant negative point mutation and recessive null mutation, respectively, in *Tlr4*[119-121]. The *Tlr4* missense mutation in C3H/HeJ-mice results in failure of TLR4 to associate with an adapter protein, MyD88, necessary for signaling (see below)[293,294]. TLR4 knockout (TLR4-/-) mice are also found to be non-responsive to LPS, whereas TLR2-/- mice respond normally[170]. Moreover, LPS-responses can be blocked by antagonistic TLR4 mAbs[295] or mimicked by agonistic TLR4 polyclonal Abs[296]. Efficient LPS signaling requires the association of TLR4 with a secreted protein, MD-2, but the precise function of MD-2 is unknown at present[295,297,298]. Still, several findings support a role for TLR2 in responses to LPS. Overexpression of TLR2 in cell lines yields LPS-responsiveness[115,116], and a TLR2 mAb has been shown to block LPS-induced activation of monocytes[117]. Furthermore, LPS/lipid A from *Porphyromonas gingivalis* activates the TLR4-deficient C3H/HeJ mice[299,300]. Some of these observations, but not all, can be explained from LPS being contaminated with TLR2 protein/lipoprotein ligands[301,302].

TLR2 seems to have a broader specificity than TLR4, and recognizes a variety of G+ bacteria[143-147], bacterial PGN[143,144,170,171], lipoproteins and lipopeptides[117,145,178-180], as well as spirochetes[145] and yeast zymosan[146]. Similar to LPS, LTA from G+ bacteria is recognized by

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TLR2-transfected cell lines[144], whereas TLR2-/- mice respond normally to LTA and TLR4-/- mice are LTA non-responsive[170]. Moreover, glycolipids isolated from *Enterococcus hirae* LTA-fractions signal cell activation through TLR2 and TLR4[185], whereas Treponeme glycolipids engage TLR2 or TLR4 depending on the structure[186]. Both TLR2 and TLR4 mediate activation by live *Mycobacterium tuberculosis,* while live *M. avium* activates cells via TLR2[145,155,156]. When individual mycobacterial components was examined, it was found that lipoarabinomannan (AraLAM), PGN-complexes and lipoproteins induce TLR2-dependent activation[117,156,189], and *M. tuberculosis* heat-labile cell-associated factors induce activation through TLR4[155]. Recently, bacterial DNA containing unmethylated CpG motifs was found to use TLR9 in mediating cell activation[181].

Non-bacterial components have been found that induce TLR4-dependent signaling, like Taxol[303], respiratory syncytial virus (RSV) protein F[245], and the recombinant human heat-shock proteins Hsp60 and Hsp70[304,305].

4.3.2 TLR signaling

The intracellular signaling pathways from mammalian TLRs, IL-1R and dToll are mediated by the TIR-domain, and share similar or homologue components (Figure 5, reviewed in refs.[269-272,278,279,306]). The adapter myeloid differentiation protein (MyD88) associates with TLRs through TIR-domain interactions, and is required to mount an inflammatory response by most TLR ligands[156,171,179,181,307,308]. However, LPS-induced mitogen activated protein kinase (MAPK) and NF-κB activation is delayed, but not abolished in MyD88-/- mice[309], and MyD88 independent signaling may have a functional role in maturation of DCs[310]. MyD88 also contains a death domain that undergoes homophilic interactions with IL-1R associated kinase (IRAK), which forms a complex with TNFR associated factor 6 (TRAF6)[307,308,311-316]. The subsequent sequence of events is not totally delineated, but the adapter TAB2 bridges TRAF6 to a MAP 3-kinase, TAK-1 (TGFβ activated kinase 1), which then activates inhibitory-κB (I-κB) kinases (IKK)[317,318]. These, in turn, phosphorylate I-κB, leading to degradation of I-κB and translocation of NF-κB to the nucleus[115,116,307,308,311]. A second route that leads to NF-κB activation is from TRAF6 activating another MAP 3-kinase, MEKK-1, through the adapter ECSIT (evolutionary conserved signaling intermediate in Toll pathways). Concomitantly, activator protein (AP)-1 transcription factors like Jun/Fos are also activated[306,307,311,317], but details of the upstream regulation of the MAPKs p38, p42/p44 and JNK by TLRs or IL-1R are not completely worked out[269,298,311,319]. Not only cell activation, but also apoptosis is induced by lipoproteins signaling through TLR2 and MyD88[178,320]. In *Drosophila*, Tube, Pelle, dTRAF, dECSIT, cactus and Dorsal/Dif/Relish are the functional homologues of mammalian MyD88, IRAK, TRAF, ECSIT, I-κB and NF-κB, respectively[269].

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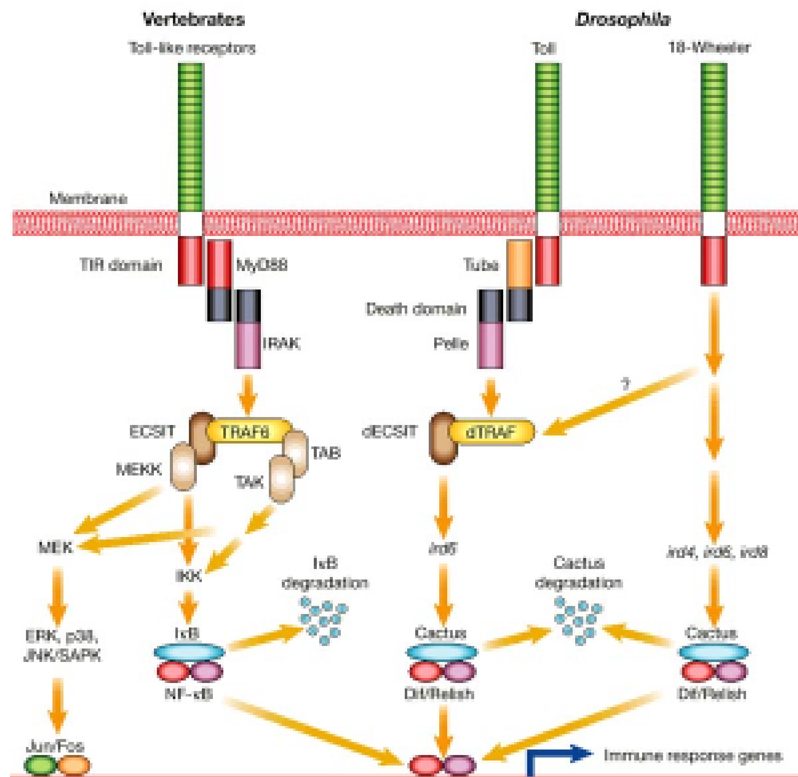

FIGURE 5 Signaling pathways activated by TLRs in vertebrates and in *Drosophila*, from ref. 270.

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5 AIMS OF THE STUDY

The overall aim of the present study was to obtain a better understanding of the receptors involved in cellular activation by bacterial components and defined uronic acid polymers, in soluble forms or covalently attached to particles. Specifically, we intended to:

- study how the presentation form (soluble or particulate) of poly-M and deacylated/detoxified LPS (DLPS) influences the potency and receptor involvement in cell activation. The results from these examinations are presented in papers 1-2.

- examine if TLR2 or TLR4, in addition to CD14, are involved in signaling cell activation by poly-M. The results are found in paper 3.

- compare the receptor usage in activation of cells by different gram-positive bacteria, like group B streptococci type III and *Listeria monocytogenes*. The results are given in papers 4 and 5.

- generate monoclonal Abs to human TLR2 for examination of protein expression and function in response to bacterial stimuli. The results are found in papers 3, 5 and 6.

URN:NBN:no-6359

6 SUMMARY OF WORK

PAPER 1

In the first paper we show that reducing the average molecular weight from ~350 kDa to <6 kDa by acid hydrolysis diminished the cell-stimulating activity of poly-M, measured as TNF-production from human monocytes. However, the activity of the resulting oligomers (M-blocks) was greatly enhanced when covalently attached to particles (plastic beads or biodegradable albumin particles). Similar results were obtained with detoxified/deacylated LPS (DLPS) and glucuronic acid polymers (C6OXY), but not with G-blocks that by themselves are not active. These results suggest that the supramolecular structure affects the potency of polysaccharide stimuli, and that M-blocks attached to biodegradable albumin particles could possibly be exploited as an immunostimulant for protection against various diseases.

PAPER 2

In paper 2, according to the reviewers suggestion, the designation M-polymers of different molecular size was used in place of poly-M (~350 kDa) and M-blocks (~3 kDa).

In this study we demonstrated that M-blocks and DLPS attached to particles engaged different receptors than soluble poly-M and DLPS in activation of monocytes. By using blocking mAbs to CD14, CD11b and CD18, we found that particulate stimuli employed the β2-integrin CD11b/CD18 in addition to the shared CD14 for signaling TNF-production. Moreover, whereas poly-M only bound to CD14-expressing CHO-cells, M-particles preferentially bound to CHO-cells expressing β2-integrins. However, the DLPS- and M-particles failed to activate NF-κB-translocation in CHO-cells co-transfected with CD14 and β2-integrins, suggesting that additional molecules are required for activation of CHO-cells. The major conclusion drawn from this work is that the supramolecular structure, in addition to influence the potency, affects the cellular receptor engagement by carbohydrates like poly-M and DLPS. This points to the importance of comparing the mechanisms involved in activation of immune cells by soluble bacterial components and whole bacteria to achieve a better understanding of inflammatory diseases like sepsis.

URN:NBN:no-6359

PAPER 3

Poly-M activates cells in a CD14-dependent manner, but CD14 is linked to the membrane with a GPI-anchor and mediates activation by interaction with other, signal-transducing molecules, like the TLRs. By using blocking mAbs to TLR2 (generated in our lab, paper 5) and TLR4, we found that both receptors were involved in mediating TNF-production from human monocytes in response to poly-M. Furthermore, TLR4 mutant (C3H/HeJ) and knockout (TLR4-/-) murine macrophages were completely non-responsive to poly-M, whereas TLR2-deficient macrophages showed reduced TNF-responses. These findings indicate that CD14, TLR2 and TLR4 on primary cells all participate in cytokine-induction by poly-M, and that TLR4 may be necessary for activation.

PAPER 4

In addition to CD14, β2-integrins have been implicated in LPS-induced cellular activation, and in this study we compared the involvement of CD14 and β2-integrins in TNF-production and NF-κB-activation induced by LPS and GBS cell wall fragments. With blocking mAbs to CD14 and CD18 we found that LPS and GBS cell walls shared CD14, but in addition the cell walls employed CD11/CD18 in mediating TNF-production from human monocytes. Both stimuli specifically induced NF-κB-translocation in CD14-transfected CHO-cells, but only LPS could activate cells transfected with CD11/CD18. The lack of response to GBS cell walls in CD11/CD18-transfected CHO-cells indicated that the cell walls need CD14 for cell activation. Further in paper 4 we demonstrate the ability of GBS cell walls to activate LPS-hyporesponsive C3H/HeJ mouse macrophages, suggesting that LPS and GBS cell walls employ different receptors/signaling mechanisms in murine macrophages.

PAPER 5

When it was discovered that human TLR2 and TLR4 are involved in microbial recognition, we started to generate a mouse mAb to human TLR2, and in paper 5 we report the production and characterization of the mAb TL2.1. We subsequently used this mAb to evaluate the role of TLR2 in mediating activation by heat-killed GBS and *L. monocytogenes*. *L. monocytogenes*, but not GBS, activated TLR2-transfected CHO-cells to IL-6-production, and the response was inhibited by TL2.1. A CD14 mAb and TL2.1 both inhibited TNF-production from monocytes induced by *L. monocytogenes*, but neither mAb affected the TNF-response triggered by GBS. Our results suggest that CD14 and TLR2 are engaged in cell activation by *L. monocytogenes*, but that neither receptor seem to be involved in activation by GBS. This study was the first to show that human TLR2 can discriminate between two G+ bacteria.

URN:NBN:no-6359

PAPER 6

In paper 6 we report the generation of a new TLR2 mAb, TL2.3, that stained with the same specificity as TL2.1 (anti-TLR2, paper 5). We used these mAbs to investigate the expression of TLR2 protein in human cells. We found that TLR2 was highly expressed in blood monocytes, less in granulocytes, and not present in lymphocytes. The protein level was measured on quiescent and activated cells by extra- and intracellular flow cytometry, and by immunoprecipitation of TLR2 from metabolic S^{35}-labeled cells. Surprisingly, TLR2 protein was detected in activated B-cells located in lymphoid germinal centers, indicating that subsets of lymphocytes may express TLR2. We further show that TLR2 protein was differentially regulated on monocytes and granulocytes after exposure to LPS, pro- or anti-inflammatory cytokines. However, we could not correlate the regulation of TLR2 to cellular responses, as for instance the three anti-inflammatory cytokines TGFβ, IL-4 and IL-10 all inhibited lipopeptide-induced TNF-production, but either did not affect, reduced, or increased the level of surface TLR2, respectively. Thus, the biological significance of TLR2-regulation remains to be found.

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7 GENERAL DISCUSSION

7.1 ADVANTAGES AND LIMITATIONS WITH *IN VITRO* STRATEGIES AND THE USE OF CELL LINES

In vitro studies on isolated cells are often necessary to dissect the individual roles of different receptors or signaling mechanisms in humans. It is difficult to extrapolate results from isolated cells to *in vivo* situations as 1) the complex interplay with neighboring cells and the local environment (paracrine mediators) will influence the ultimate response, and 2) the procedure of isolation and cultivation of so-called primary cells often leave them in a pre-activated state, as reported for adherent monocytes[321,322]. Primary cells have a limited life-span in culture, making them less convenient for genetic manipulations. Specific blocking inhibitors and mAbs are thus invaluable tools for use on primary cells, and in all **papers 2-6** we have used mAbs to study receptor expression or engagement in activation. Still, depending on the degree of overlapping epitopes recognized by the mAb and the ligand, together with mAb affinity, it may be difficult to tell if the particular receptor is crucial for activation unless complete inhibition is obtained. This is clearly seen for poly-M in **paper 3**: The use of blocking mAbs to TLR2 and TLR4 on monocytes demonstrated the participation of both receptors in activation by poly-M, but neither mAb could completely block the response. Experiments with macrophages from TLR2-/- and TLR4-/- mice confirmed these results, but also suggested that TLR4, and not TLR2, was absolutely necessary for activation.

Cell lines are also used to delineate essential components of cell activation and signaling. The obvious advantages with cell lines are reproducibility and ease of cultivation, transfection and genetic manipulation, providing data that would otherwise be difficult to obtain. Most of what is known about the intracellular signaling pathways from TLRs was found by generating deletion-mutants of various signaling components in cell lines[282,307,308,311,320], and the initial finding that human TLR2 could signal LPS-activation was from 293-cells transfected with TLR2[115,116]. However, overexpression studies may overestimate the significance of the transfected component, as later findings in human primary cells (**paper 5**) and mice[122,170] have questioned the biological relevance of TLR2 in LPS activation. An other advantage with cell lines is that some are “natural” mutant in the gene of interest, making it easy to evaluate the importance of one single molecule. But the cell line may lack additional known or unknown molecules that are important to the response measured, leading to wrong conclusions or unexplainable results that doesn’t support results from primary cells (actually putting the other findings into doubt). Poly-M, PGN and LAM do not activate the CD14-negative U373 astrocytoma cell-line, even in the presence of sCD14[106,188,323]. The later discovery that U373-cells in addition lack TLR2, together with the findings that LAM, poly-M and PGN are all TLR2-ligands[143,144,146,155,156,189](**paper 3**), provided a possible explanation. But, as TLR2-

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deficient macrophages still responded to poly-M, albeit with reduced potency (**paper 3**), we do not know if additional molecules are absent in U373-cells that are needed for activation by poly-M, or if U373-cells signal poly-M activation by different mechanisms than macrophages. Thus the major conclusion drawn from the experiments with U373-cells in this paper, was that LPS and poly-M are different with respect to cell activation. Further examples can be found from studies with CHO-cells, which are naturally mutant in TLR2[324] and not responsive to LPS, GBS, poly-M or M-particles. In **paper 2** we found that although mAbs to CD14, CD11b or CD18 inhibited cytokine-production from monocytes in response to M-particles, CHO-cells co-transfected with CD14 and β2-integrins were unresponsive. Although similar results were obtained for GBS cell wall fragments in **paper 4**, activation was observed in CHO/CD14 cells, and not in CHO/CR3 or CHO/CR4 cells, indicating that membrane CD14 could be necessary for activation by GBS cell walls. Common to **papers 2, 4 and 5**, and also shown by others[126-128], is the LPS-activation of CHO-cells transfected with β2-integrins (**paper 2 and 4**) or TLR2 (**paper 5**), despite the fact that these receptors are believed to be of minor importance for cell activation by low concentrations of LPS in primary cells (**paper 2, 4 and 5**)[170,262]. Thus, cell lines overexpressing receptors may yield results where the biological significance needs to be interpreted with caution, but are useful when they support findings in primary cells. This was the case for heat-killed *L. monocytogenes* and GBS: CHO-cells transfected with TLR2 became responsive to *L. monocytogenes*, but not to GBS, supporting the results from monocytes that our TLR2 mAb blocked activation by *L. monocytogenes*, but not by GBS (**paper 5**).

7.2 HOW ARE BACTERIAL CELL WALL COMPONENTS RECOGNIZED?

During inflammation, innate immune cells meet several active components (PAMPs) from/on the invading microbe, with chemical and physical differences that will influence which receptors (PRRs) become engaged in cell activation. Properties like hydrophobicity, polarity, charge and steric hindrance determine the intrinsic conformation of a ligand, and also if it is recognized by a putative receptor. A positive interaction may promote conformational changes in one or both of the molecules that 1) block the receptor, 2) result in activation of the receptor, or 3) induce interactions between the receptor or receptor-ligand complex with another molecule. Obviously, either case 2) or 3) can yield signaling of inflammatory responses. We found that the presentation form of some carbohydrate stimuli (poly-M, C6OXY, DLPS) affected both the cell activating potency (**paper 1**), and the receptors engaged by these ligands when inducing cytokine production (**paper 2**). Both aspects are important with regard to vaccine design and development of therapeutic strategies against inflammation/sepsis.

URN:NBN:no-6359

7.2.1 Differential activity due to physicochemical differences of ligands

Several studies have pointed to the importance of PAMP conformation in cellular recognition and activation. The group of Seydel *et al.* has found that lipid A structures with less than six acyl chains adopt inactive cylindrical structures, whereas fully acylated lipid A has a conical shape and is stimulatory[67,68,325,326]. Moreover, the immunogenic epitope of GBS Type III polysaccharide is conformational, generated by sporadic formation of extended helices in the polymer[327]. High-M alginate and poly-M represent PAMPs that readily stimulate monocytes to TNF-production, whereas G-blocks are without activity[55]. Guluronic acid is the C5-epimer of mannuronic acid, and this has a structural impact on the polymer where M adapts the $^4C_1$ conformation, and G is found in the $^1C_4$ conformation. This results in four possible glycosidic linkages in alginate, with decreasing stiffness in the order α-(1-4) diaxial (GG) > β-(1-4) diequatorial (MM) > β-(1-4) equatorial-axial (MG), α-(1-4) axial-equatorial (GM)[88,94,328] (Figure 2). Our previous[55] and present (**paper 1**) results suggest that cells discriminate between these structures, and that uronic acid polymers with mainly β-(1-4) linkages are the most active. However, when a group at UNIGEN (Trondheim, Norway) managed to knock out the C5-epimerase-gene (*algG*) in *P. aeruginosa* (S. Valla, unpublished data), we found that the resulting mannuronan (100% M) was completely inactive in stimulation of monocytes (L. Ryan, unpublished data). In subsequent experiments, G-residues were reintroduced in mannuronan by use of recombinant C5-epimerases, and the epimerized polymer became immunostimulating (B. L. Strand and T. H. Flo, unpublished observations). Probably, the local sequence is more important than the overall ratio of M to G, and current data suggest that introduction of G-residues in alternating positions, but not G-blocks, increases the activity of mannuronan or poly-M.

We do not know yet what is the optimal immunostimulating conformation of poly-M, but reducing the molecular size of the polymer to <6 kDa yields rather inactive M-blocks[55](**paper 1 and 2**). Similarly, digestion of PGN with lytic enzymes reduces PGN-induced cell activation[174]. When we attached M-blocks to particles, their TNF-inducing potency increased (**paper 1 and 2**), suggesting that optimal activity resides in a certain supramolecular structure. However, as G-blocks attached to particles remained inactive, this further supported the idea that the active epitope is determined by a sequence present in M-blocks, but not in G-blocks. Thus, as discussed in **paper 1**, the stimulating sequence is intrinsic to the molecule, but optimal activity is obtained when it is presented and stabilized in a certain supramolecular structure, as in long chain polymers or attached to particles. Seljelid *et al.* have also demonstrated increased effect of particulate β-glucans in protection against lethal bacterial infections and stimulation of proinflammatory mediators[329,330]. Especially for G+ bacteria, the polysaccharides covalently attached to the cell wall are central in stimulating immune responses, and our results suggest

URN:NBN:no-6359

that this particulate presentation form would increase their immunostimulating potency. In contrast, it is generally believed that particulate LPS has decreased activity as the lipid A part that remains inserted in the bacterial membrane is less available to the cells, although the contrary is also reported[331-335]. A possible explanation of the fact that the potency varies between different supramolecular structures of carbohydrate components, is that the presentation form affects which receptors and signaling mechanisms are triggered in the cells, and the ability to cross-link these receptors. In addition, phagocytosis of particulate stimuli may influence the activity.

7.2.2 Differential receptor usage due to physicochemical differences of ligands

Binding to CD14 has been demonstrated for LPS, lipid A and various lipid A partial structures/mimetics[70,71,113,114], poly-M and G-blocks[55,106,187]. But whereas interactions with LPS and poly-M lead to signaling, inactive lipid A mimetics and G-blocks actually inhibit signaling induced by LPS and poly-M[55,71-73,336](**paper 3**). Thus, ligand conformation is apparently important for activity, but CD14 does not seem to be the discriminating receptor in this regard. CD14 does not have an intracellular part and probably mediates signaling through other membrane proteins[70,126,336-338]. For G-blocks and high concentrations of lipid A analogues, inhibition can be explained by competitive binding to CD14[55,70,71], but low concentrations of LPS antagonists are thought to block interactions with a signal transducer[70,71,336]. Still, some conformational dependency is observed for different ligands interacting with CD14. Although CD14 mAbs inhibited the activity of soluble and particulate poly-M, LPS and DLPS (**paper 1 and 2**)[106,111], we observed binding to CD14 by soluble poly-M whereas M-particles preferentially bound to β2-integrins (**paper 2**). In contrast, enzymatic digestion of PGN reduces both binding to CD14 and cell activation, and soluble muramyl dipeptide (MDP) and GlcNAc-MDP do not bind to CD14 unless immobilized on agarose beads[174,226].

CR3 (CD11b/CD18) has been identified as a receptor for β-glucans, where binding of soluble, low-Mw compounds to the lectin site of CR3 primes NK-cells, neutrophils and macrophages for respiratory burst, phagocytosis and cytokine responses that are triggered by subsequent interaction with iC3b-opsonized target cells and microbes[259,339-342]. However, particulate or high-Mw glucans directly trigger responses after engagement of CR3, and this has been explained by these ligands' ability to cross link receptors[341]. We found that particulate, but not soluble poly-M, bound to CR3 and CR4, and mAbs to CD11b and CD18 inhibited monocyte activation only by particulate poly-M and DLPS (**paper 2**). Moreover, whereas soluble GBS components seem to prefer CD14[343], the more particulate GBS cell wall fragments used both CD14 and CR3 (**paper 4**), and whole GBS use CR3 in activation of monocytes[344]. And while binding to CR3 is demonstrated for GBS Type III polysaccharide[345] and

URN:NBN:no-6359

streptococcal rhamnose-glucose polymers[46], blocking mAbs to CR3 and/or CR4 are without inhibiting effect on monocyte TNF-production[46,343]. β2-integrins also bind to LPS[124-128,262], but even better to whole G- bacteria or LPS particles[346-348]. A recent study demonstrated that heat-killed *E. coli*, but not LPS, induced cytokine production in macrophages from CD14-/- mice through CR3, although higher concentrations of bacteria were needed compared to CD14-mediated signaling[348]. Thus, CD14 and β2-integrins share many PAMPs, but activation of β2-integrins seem to preferentially occur by their particulate presentation forms. Although β2-integrins are involved in cell activation induced by both G+ and G- bacterial components, proinflammatory signaling is probably not mediated by these receptors as deletion of the cytoplasmic domain of CR3 renders it incapable of bacterial internalization without affecting the LPS-induced translocation of NF-κB[127]. Accordingly, β2-integrins may function in the same way as CD14 to concentrate microbial components on the cellular surface to interact with other, signal transducing mechanisms.

The first study to indicate conformational discrimination by TLRs was by Takeuchi *et al*. who showed that the R-isomer of a mycoplasmal lipopeptide, MALP-2, was > 100 times more activate than the S-isomer in activation of macrophages through TLR2[179]. Moreover, TLR4 seems to be the long sought molecule that discriminates between the subtle structural differences of lipid A analogues[122,123,349]. Whether microbes are true TLR ligands is currently not known with certainty[272]. The ligand of dToll is thought to be an endogenous protein, Spätzle[350], generated both during embryogenesis and in response to fungi, although through different proteolytic cascades[279,351,352]. However, as human TLR4 is responsible for species-specific discrimination of lipid A structures, this would suggest that lipid A interacts directly with TLR4[122,123], and recently LPS has been shown to bind TLR4 and MD-2[118]. Low-affinity binding of LPS to TLR2 is also demonstrated[115,118], but several groups have failed to demonstrate binding of LPS to TLRs. Still, each microbial PAMP seems to engage several PRRs, and each PRR recognizes several structurally different PAMPs. This is true for CD14 (section 4.1.1), β2-integrins (section 4.2.1, **paper 2 and 4**), TLR2 and TLR4 (section 4.3.1, **paper 3 and 5**), the receptors of focus in the present work (Table 3). This indicates that specific signaling is probably not confined to a single receptor, but rather to molecular complexes.

7.2.3 Composition of signaling receptor complexes

The compositional requirements of putative receptor complexes signaling through TLRs are not known. CD14 and CR3 are both involved in internalization of bacteria (sections 4.1.1 and 4.2.1). In a recent review, Ross *et al*. suggest that CD14-mediated internalization of *E. coli* occurs by CD14 interacting with CR3[341], supported by the findings that LPS transiently induces CD14 association with CR3[353] and that E-LPS binds to CR3 subsequent to interaction with

URN:NBN:no-6359

CD14[346]. Following this idea that CD14 engages CR3 to signal phagocytosis, CD14 could similarly engage TLRs to signal cytokine production. CD14 is needed for efficient activation by LPS and other microbial components, and is shown to enhance signaling by several PAMPs through TLRs[117,143,144,180,297](**paper 5**). Besides, LPS has been shown to bind TLR4 and MD-2 in the presence of CD14[118], and to promote physical proximity between CD14 and TLR4[354]. A direct correlation between engagement of CD11/CD18 and signaling through TLRs has not been studied. The anti-tumor agent Taxol is an LPS-mimetic in mice that doesn't interact with CD14, but CR3 was recently shown to be involved in Taxol-induced IL-12 from murine macrophages[355]. Moreover, Taxol signals activation through TLR4-MD-2, indicating that β2-integrins may serve a function similar to CD14 as TLR co-receptors[126,127,303].

A range of studies implicate a link between phagocytosis of particles and cytokine production, and recent reports from Aderems group suggest that the presence of TLRs in phagosomes may mediate pro-inflammatory signals during phagocytosis[146]. TLR1, 2, 6 and probably additional TLRs are recruited to phagosomes where they can sample the content and cooperatively induce cytokine responses to specific PAMPs[146,148]. We have found that CD14, TLR2 and TLR4 colocalized in the membrane and intracellular vesicles of monocytes and macrophages ((**paper 6**) and T. Espevik, unpublished results). TLR signaling is believed to occur through receptor dimerization induced by ligand engagement, and several reports support the existence of TLR2 and TLR4 homodimers[146,148,189,308]. However, Aderem *et al*. further suggest that whereas TLR4 dimers are functional signal transducers, TLR2 signals in complex with other TLRs, like TLR1 or TLR6[148]. Yeast zymosan, PGN and several G+ bacteria were shown to employ TLR2-TLR6 heterodimers for signaling[148]. Interestingly, *L. monocytogenes* is one of the G+ bacteria recognized by TLR2-TLR6[148], extending our finding that TLR2 mediated signaling by *L. monocytogenes* (**paper 5**). A functional interaction between TLR1 and TLR2 is supported by recent findings that coexpression of TLR1 and TLR2 enhances responses to soluble *Neisseria menigitidis* LPS complexes[356]. Moreover, our results that poly-M engaged both TLR2 and TLR4 (**paper 3**) suggest the existence of TLR2-TLR4 heterodimers. Apparently, TLR2 homodimers are not involved in poly-M-induced TNF-production as TLR4-deficient macrophages were completely non-responsive to poly-M. However, as TLR2-deficient macrophages were partly responsive to poly-M, it is possible that some signaling is mediated by TLR4 homodimers. Other bacterial components are also shown to interact with both TLR2 and TLR4[155,172,185]. But while live *M. tuberculosis*[155] and *M. bovis* cell wall skeletons[172] expose several PAMPs that may interact with either TLR2 or TLR4, the glycolipids isolated from *E. hirae* are reported to be homogeneous, and like poly-M, still engage both CD14, TLR2 and TLR4[185]. We also found that GBS did not employ TLR2 for cell activation (**paper 5**), and as GBS cell wall fragments readily activated TLR4-deficient C3H/HeJ macrophages (**paper 4**), neither of these TLRs seem to be involved in activation by GBS bacteria. However, the ability

of TLRs to form functional heterodimers leaves the cell with a combinatorial repertoire for specific recognition of different PAMPs. Recently, Goetz *et al*. used fluorescence resonance energy transfer (FRET) technology to show that CD14, CR3, Fcγ-RIII, CD36, TAPA, CD55 and TLR4 are all recruited into a putative LPS receptor complex localized in lipid "raft" microdomains of the cell membrane[357]. Ceramide induced a receptor cluster with different composition, but it remains to be proved that such multimeric complexes represent true cell signaling units specific for different stimuli.

Other molecules, like secreted PRRs, are involved in regulating the specificity and responsiveness of signaling receptors. As discussed in section 4.1, the responses to LPS and several other bacterial components are increased in the presence LBP and/or sCD14, probably by enhanced transfer of the ligands to membrane receptors[111,126,205]. We found that LBP and sCD14 enhanced activation by soluble poly-M and DLPS[187](**paper 2**), GBS polysaccharides[343] and cell walls (**paper 4**), but not by particulate poly-M and DLPS (**paper 2**) or whole GBS bacteria (unpublished observations), indicating that such transport is not needed for particulate stimuli. Possibly, the increased membrane contact area when particulate ligands are phagocytosed yields more efficient receptor engagement and/or aggregation. A soluble form of TLR4 was recently found to be expressed by mouse macrophages, and the secreted protein inhibited LPS-induced cell activation[358]. As mentioned in **paper 6**, we have detected soluble TLR2 in human serum by a TLR2-specific ELISA that are under development, but we cannot yet say what is the function of sTLR2. MD-2 is a secreted protein that localizes to the extracellular domain of TLR4 and increases LPS-mediated signaling[295], presumably by stabilization of TLR4 dimers. Recently, Kawasaki *et al*. showed that although Taxol and LPS both activate cells through murine TLR4-MD-2, the response is species-specific as only LPS, and not Taxol, can activate murine cells transfected with human TLR4-MD-2[303]. In B-cells, an additional TLR protein, RP105, associates with secreted MD-1, and RP105-MD-1 replaces MD-2 in regulating TLR4-mediated LPS signaling[359,360]. It is not known if similar MD-like molecules exist for the other TLRs, although the possibility that MD-2 can function together with TLR2 was suggested at a recent meeting[361]. Moreover, if MD-like molecules show some kind of ligand- and/or cell specificity, this could possibly explain the lack of poly-M-responses in U373/CD14-cells (**paper 3**).

7.2.4 Expression and regulation of TLRs

Differential receptor distribution and regulation may further influence the composition of putative receptor complexes in different cell types and tissues. The expression of CD14 and β2-integrins is well characterized, and both receptors are regulated in response to microbial stimuli or other inflammatory mediators. Similar information about the expression and regulation of

URN:NBN:no-6359

TLRs in various cells and tissues is emerging[283-288,362]. TLR2 mRNA[288] and protein (**paper 6**) is expressed in human blood monocytes and neutrophils, but not in blood lymphocytes, whereas activated germinal center B-cells showed cytoplasmic TLR2 staining (**paper 6**). Detection of TLR2 and TLR4 is also reported in certain murine tissue B-cells[121,297] and T-cells[363,364], suggesting that expression varies in lymphocyte subpopulations. Mokuno *et al.* showed that after i.p. injection of lipid A, peritoneal αβ T-cells expressing marginal levels of TLR2 and TLR4, and liver γδ T-cells only expressing low levels of TLR2, were non-responsive to LPS, whereas γδ-T-cells recruited to the peritoneum expressed high levels of TLR2 and signaled TLR2-mediated lipid A activation[364]. In contrast, TLR2 presumably cannot replace TLR4 in LPS-activation of murine macrophages or B-cells, as TLR2 expression is higher in macrophages made tolerant to LPS than in responsive cells[365], and both macrophages and B-cells from TLR4-deficient mice are hyporesponsive to LPS[121,297]. Thus, these results indicate that PAMPs do not necessarily engage the same receptors in all cells, but that alternative pathways may exist for signaling activation in specific cell types or subpopulations.

Little is known about TLR-expression during disease. TLR4 is reported to be increased in injured human and murine myocardium, possibly linking innate immunity to the pathophysiology of heart failure[366]. Moreover, TLR3 was downregulated and TLR4 upregulated in intestinal epithelial cells isolated from patients with inflammatory bowels disease, whereas the levels of TLR2 and TLR5 remained unchanged[367]. However, the physiological consequences of TLR regulation are not clear. LPS[364,365,368,370-373](**paper 6**), mycobacteria[368], *L. monocytogenes*[369] and zymosan[370] generally upregulate the expression of TLR2, whereas TLR4 is reported to be downregulated[365,368] or not regulated[374] in murine macrophages, and upregulated in human monocytes[288,354] in response to LPS. Some studies suggest that LPS-induced downregulation of TLR4-MD-2 underlies the induction of LPS tolerance, whereas others argue that tolerance is mainly a result of impaired expression or function of downstream signaling intermediates[365,375,376]. The PAMP-mediated upregulation of TLR2 would presumably regulate the cells sensitivity to TLR2 ligands, and the outcome could be inflammatory or anti-inflammatory as apoptosis is also triggered through TLR2 by lipoproteins[178,320]. One would also have to consider that several PRRs are regulated concomitantly, and not only by PAMPs, but also by inflammatory cytokines[288,368,369,374](**paper 6**). We tried to interpret the biological consequences of changes in TLR2-expression by stimulating monocytes with a TLR2-ligand, lipohexapeptide 47L from *T. pallidum*, subsequent to incubation with cytokines. No apparent correlation was found between the expression of TLR2 and the TNF-response from monocytes (**paper 6**). More studies on the regulation and function of the TLRs during disease are needed to obtain a better understanding

URN:NBN:no-6359

7.3 RECEPTOR-DIRECTED STRATEGIES AND IMMUNOMODULATORY POLYSACCHARIDES TO INHIBIT SYSTEMIC INFLAMMATION

7.3.1 Receptor-directed strategies

In intact pathogens, several PAMPs are present that use different PRRs for cell activation, and most of these PAMPs may come into contact with immune cells during infection as the microbe releases endotoxins and exotoxins, or lyses in the presence of antibiotics. In the case of G- bacteria, LPS would engage CD14 and TLR4, lipoproteins TLR2, peptidoglycan CD14, TLR2 and TLR6, DNA TLR9 and so on. The PRRs could thus provide useful targets for anti-inflammatory therapies, or vaccine strategies. However, before interfering with their function, one has to fully understand the biological role of PRRs, which in most cases are to resolve inflammation. CD14-/- mice are resistant to lethal injections of LPS or live *E. coli*[228], whereas CD14 does not seem to affect shock induced by *S. aureus*[237]. In two rabbit animal models, blockade of CD14 with specific mAbs resulted in improved systemic responses, but increased bacterial load of *E. coli* at the site of infection (the lungs[377]), or increased invasion of the intestinal mucosa by *Shigella*[378]. Thus, although CD14 mAbs are shown to protect primates against LPS-mediated shock[231], anti-CD14 treatment may interfere with host defense mechanisms involved in eradication of live bacteria. This would also be expected from anti-β2-integrin strategies, as these receptors play an important role both in clearance of pathogens and transendothelial migration of phagocytes into the infected tissue (see section 4.2.1). TLRs are involved in signaling the presence of PAMPs, and possibly also in recognition of endogenous heat-shock proteins released from damaged or stressed cells[304,305]. The ongoing trials with LPS antagonists for treatment of sepsis (reviewed in ref.[24]) would interfere with TLR4 signaling, as lipid A mimetics are shown to signal through TLR4[122,123]. However, even if TLR4-/- mice are resistant to LPS-induced shock[170], TLR4-deficient mice are hypersensitive to G- bacterial infections[379], and TLR2-/- mice are highly susceptible to infections with G+ *S. aureus*[147], indicating that signaling through TLRs is necessary for triggering an efficient host response. Anti-receptor therapies could therefore be detrimental if not used together with antibiotics. Still, as antibiotics promote the release of endotoxin and other PAMPs from live bacteria, concomitant administration of anti-receptor agents (inactive PAMPs, PRR mAbs and antagonists) could inhibit a subsequent overwhelming immune response. Timing could turn out to be a problem, as for instance LPS has short half-life with serum levels peaking 2-4 h after initiation, often long before intervention is started[21,380]. Moreover, all patients need not be in a hyper pro-inflammatory state, some may be in an immunosuppressed state where anti-inflammatory agents could be detrimental[10,17]. It should be possible, however, to develop strategies to stimulate TLRs in order to enhance host responses to a wide variety of antigens in vaccines.

URN:NBN:no-6359

7.3.2 Immunomodulatory polysaccharides

The terms immunomodulators, or biological response modifiers (BRMs), are used to describe compounds that are capable of interacting with the immune system to upregulate or downregulate specific aspects of the host response[381]. BRMs would thereby allow the host to better defend itself against invading microbes, and can replace or complement specific treatment like antibiotics. Microbial products, natural and synthetic carbohydrates and endogenous cytokines, represent immunomodulators currently in use[342,381-383].

Polysaccharide immunomodulators are isolated from bacteria, yeast, fungi, mushrooms, lichens and algae, and their mode of action is believed to be priming of immune cells for cytotoxicity and/or cytokine production, and stimulation of bone-marrow haematopoiesis[107,342,382,383]. Most of these polysaccharides are heteroglycans with complex secondary and tertiary structures, but relatively few have been examined in detail with respect to structure-function and mechanism of action. Zwitterionic polysaccharides, like the capsular heteropolysaccharide A of *Bacteroides fragilis*, modulate abscess formation associated with experimental intra-abdominal sepsis[342]. β-(1-3)-glucans, often with (1-6)-β-glycopyranoside branches, can be isolated from yeast, fungi and oriental herbs, and are among the best studied polysaccharide immunomodulators[342,382-384]. Clinical trials with the yeast soluble β-(1,3) PGG-glucan (Betafectin) have proved reduced incidence of infections or death after high-risk gastrointestinal surgery[385-387].

Alginates are negatively charged immunomodulators, and whereas alginates with a high content of M-residues should be avoided for use in encapsulation and transplantation, these polymers could possibly be used in a manner similar to β-glucans for general immunostimulating purposes and protection against disease (**papers 1-3**). Poly-M given prophylactically (-48 and/or –24 h) protects mice from LPS-induced septicemia (T. Espevik and G. Skjåk-Bræk, unpublished results), and no apparent toxicity is observed with injection of clinically relevant doses of poly-M (2-20 mg/kg)[107]. The absorption of poly-M reaches a maximum after 5-6 h with a half-life of about 12.5 h following intraperitoneal injections in mice, whereas no uptake is seen after peroral administration[84]. Glomerular filtration is dependent on both molecular size and charge, with lowest excretion seen for large anionic polymers. As we have shown that covalently linking low-molecular M-blocks to biodegradable albumin particles enhances the cytokine inducing potency compared to soluble, high-molecular poly-M (**paper 1**), lower concentrations of the more easily excreted M-blocks could be exploited. As already discussed in section 7.2.1, the MG-sequence is crucial for the activity of poly-M, and as alginates are naturally heterogeneous, recombinant mannuronan C5-epimerases could possibly be used to design alginates with optimal and reproducible immunostimulatory potency.

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