PERIODICUM BIOLOGORUM
VOL. 114, No 2, 211–220, 2012

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Review

Targeting Toll-Like Receptors: a step closer to effective recombinant subunit vaccines

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Key words: pathogen-associated molecular motif, Toll-like receptors, innate immunity, adaptive immunity, vaccine

Abbreviations:

PAMP	pathogen-associated molecular motif
PRR	pathogen recognition receptor
TLR	Toll-like receptor
TIR	Toll/IL-1 receptor
MYD88	Myeloid differentiation primary response gene 88
TRIF	TIR-domain-containing adapter-inducing interferon-â
mDC	myeloid dendritic cells
pDC	plasmacytoid dendritic cells

Received July 17, 2012.

Abstract

The control and prevention of infectious diseases through immunization is one of the greatest achievements of modern medicine since Edward Jenner pioneered smallpox vaccination.

However, future challenges in improving the efficacy of existing vaccines, development of new prophylactic vaccines for infectious diseases and therapeutic immunization for noninfectious diseases require extensive work to reveal key components of the molecular immune mechanism involved. Successful activation of innate immune response is a prerequisite for successful immunization and activation of adaptive immunity. Innate immune system comprises numerous evolutionary conserved pattern-recognition receptors (PRRs) that bind structural components shared by many pathogens. Upon binding the ligand, cascade reaction results in de novo gene expression required for immediate immune response by innate immunity and the activation of specific immune response mediated by the humoral and cellular mediators. Appropriate selection of specific pattern-recognition receptor ligands (adjuvants) enables formulation of the next generation vaccines, with controlled minimal adverse symptoms and efficient adaptive immunity development.

DISCOVERY OF PATHOGEN RECOGNITION RECEPTORS

In 1980's, obstacles to experimentally induce a development of adaptive immune response inspired Charles A. Janaway Jr. to propose the hypothesis that innate immune cells (dendritic cells and macrophages) recognize *pathogen structural molecules* (adjuvants) and provide necessary help to T and B cells mounting cellular and humoral immunity *(1)*. Recognition of these molecules depends on the presence of constitutively expressed receptors, later called pattern-recognition receptors (PRRs).

First PRR gene, called Toll, was discovered in 1985 by German a biologist Christiane Nüsslein-Volhard who revealed its prominent role in the embryogenesis of the fruit fly *(2)*. Ten years later, similarity in intracellular interleukin (IL)-1 cytokine and Drosophila Toll receptor signaling coupled with NF-kB-dependent gene transcription led Jules A. Hoffmann to directly relate Toll with immune responses to fungal infection in Drosophilla *(3)*

Only a year later Ruslan Medzhitov and Charles A. Janeway, Jr. at Yale University cloned the first human Toll gene homologue, Toll-like

receptor (now called TLR4). TLR4 induced NF-kB activation in a similar way as ligation of the IL-1 receptor and Drosophila Toll receptor *(4)*.

At about the same time, Bruce A. Beutler discovered that mouse TLR4 is the long-sought receptor for lipopolysaccharide (LPS), the active component from endotoxin in Gram-negative bacteria *(5)*. It became obvious that TLRs constitute a family of pattern recognition receptors which bind pathogen-associated molecular patterns and activate immune system as predicted by Charles A. Janeway, Jr. several years earlier.

Discovery of Toll-like receptors and their role concerning activation of innate immunity by Jules A. Hoffman and Bruce A. Beutler was honored by the Nobel Prize in Physiology or Medicine in 2011. Many controversial discussions have been raised since a letter signed by 23 prominent immunologists was published in the Nature, implying that »the seminal contribution of immunologists Charles A. Janeway Jr. and Ruslan Medzhitov« should be acknowledged *(6)*.

DIVERSITY OF PATHOGEN RECOGNITION RECEPTORS

Innate immune mechanisms, due to this widespread distribution and no additional requirements to effectively recognize microbial structural moieties *(7)*, represent a first line of immune defense against microorganisms. The basic mechanisms of pathogen recognition are evolutionarily conserved and include sensors present in the plasma, on cellular membranes, and cytosol (Figure 1), reassembling diversity of different pathogen biology and life cycle.

The array of PRRs contains several families of host germline-encoded receptors with the common denominator of broad specificity to different molecules produced by invading microbes. These structural molecules, referred to as pathogen-associated molecular motifs (PAMPs), have an essential function in the life of a microbe and include LPS as the major component of the outer layer of Gram-negative bacteria, peptidoglycan (PGN) as the major component of the cell wall of Gram-positive bacteria, flagellin and bacterial or viral nucleic acids.

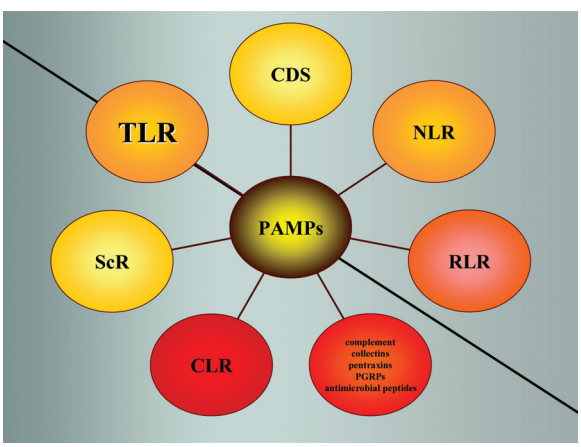

Figure 1. *Pathogen recognition sensors.*
Different host molecules recognize and bind pathogen-derived molecules, neutralize and generate signals that would activate potent innate immune mechanism. TLR, Toll-like recptors; CDS, cytosolic DNA sensors; NLR, Nod-like receptors; RLR, RIG-I-like receptors; PGRP, peptidoglycan recognition proteins; ScR, scavenger receptors; CLR, C-type lectin receptors

PRRs that are able to recognize different PAMPs are classified in five major families: scavenger receptors (ScR), C-type lectin receptors (CLR), Toll-like receptors (TLR), NOD-like receptors (NLR), RIG-I-like receptors (RLR) and cytosolic DNA sensors (CDS) (Figure 2).

The first discovered PRR in 1979 was the scavenger receptor (ScR), identified as a transmembrane receptor capable of binding LPS, acetylated LDL, and certain polynucleotides contributing to endocytosis-mediated clearance *(8)*. Besides this group, CLR family members also recognize a number of pathogen-associated glycan molecular motifs *(9)* and, like ScR, they are primarily involved in endocytosis.

In contrast to ScR and CLR, the families of TLR, NLR, RLR and CDS receptors, upon ligation of structural molecular motifs of potentially harmful pathogenic microorganisms, initiate activation of innate immune mechanisms that would combat the invader and provide a platform for adaptive immune response development. These receptors possess structural domains that bind a

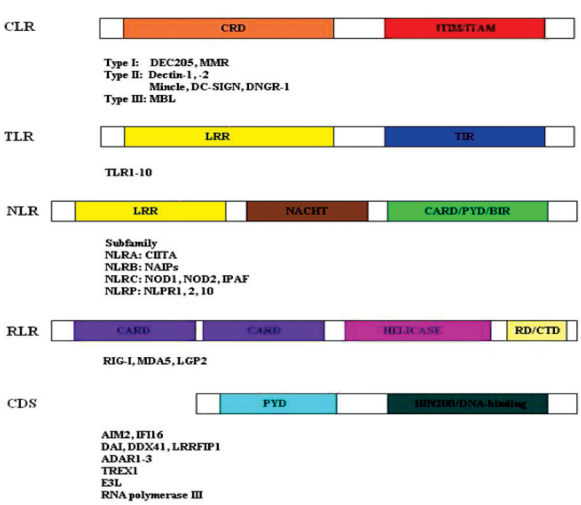

Figure 2. *Pathogen recognition receptors.*
PRRs are membrane or soluble molecules that have ability to recognize different pathogem molecules. They have different domains relevant to recognize distinct pathogen-derived molecular motifs and signaling domains necessary for activation of NF-kB, MAPK and IRF pathways. CRD, carbohydrate recognition domain; ITIM/ITAM, immunoreceptor tyrosine-based inhibitory/activation motif domain; LRR, leucine rich repeats domain; TIR, Toll/Interleukin-1 receptor domain; NACHT (NOD), nucleotide-binding oligomerization domain; PYD, pyrin domain; BIR, Baculovirus inhibitor of apoptosis protein repeat domain; CARD, Caspase activation and recruitment domain; Helicase, helicase domain; RD/CTD, N-terminal receiver /C-terminal effector domain; HIN200, DNA binding domain.

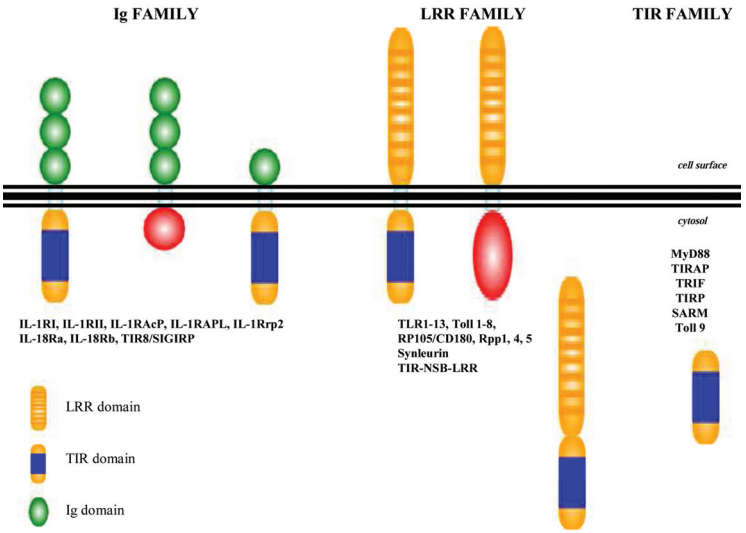

Figure 3. *TIR superfamily.*
The TIR superfamily is defined by a common intracellular TIR domain, involved in the initiation of signaling. Toll/IL-1R (TIR) superfamily members encompasses the Ig domain family (IL-1 receptors, IL-18 receptors, and IL-1R-like receptors), the leucine-rich domain family (the Toll-like receptors and similar receptors), and TIR domain family as a series of TIR domain-containing intracellular adapter molecules, differentially recruited to the Toll/IL-1 receptors and contribute to the specificity of signaling.

pathogenic molecule and the signaling domain required to initiate intracellular cascade in the induction of antimicrobial genes and inflammatory cytokines *(10)*.

NLRs, RLRs and CDSs are multidomain molecules localized in the cytosol and therefore sense intracellular pathogens. NLRs are a family of heterogenic receptors that are involved in the NF-kB and MAPK activation or assembly of inflammasome, a multiprotein oligomer required for the caspase-1-mediated processing of pro-IL-1β and pro-IL-18 *(11, 12)*.

Since 'whole' pathogens are composed of different structural molecules, it is not surprising that multiple PRRs could be triggered simultaneously by a single microorganism. The activation of multiple PRRs results in a combinatorial code that specifically shapes the host response to a particular class of microbes *(13)*.

Besides foreign pathogen molecular motifs, there is mounting evidence that certain PRRs are involved in sensing endogenous non-microbial endogenous 'danger' signals *(14)*. These endogenous adjuvants could amplify the innate immune response or possibly contribute to the development of overwhelmed inflammation due to uncontrolled innate immune response leading to life threatening conditions. In addition, deregulated activation of intracellular TLRs has been associated with the pathogenesis of some autoimmune diseases like systemic lupus erythematosus (SLE), confirming a role of PRRs in adaptive immunity *(15)*.

TOLL-LIKE RECEPTORS

Innate immune defense mechanisms represent a border line where the host senses microbial presence and starts the appropriate response. Sharing the same space, the host has been evolutionally educated to recognize a symbiotic from a virulent pathogen via conserved pathogen recognition receptors shared by different cells and tissues. In a case of infection with a virulent pathogen and the presence of tissue destruction, immune defense mechanisms become activated, beyond activation threshold, by a variety of exogenous pathogen-derived, as well endogenous, molecules. As important role in this process is played by TLRs, the best-characterized PRRs which solely or in combination with other PRRs are capable to recognize common pathogen/host molecular patterns. TLRs are members of the interleukin-1-receptor superfamily (Figure 3) and comprise a leucine-rich repeat (LLR) domain group of transmembrane PRRs that recognize highly conserved microbial molecules *(4, 16, 10)*. Since the discovery of the first Toll-like receptor 4, TLRs have expanded to a family of structurally cohesive receptors found to be widespread in vertebrates, arthropods, and nematodes *(17, 18, 19)*. Overall, 13 members of the TLR family have been described with 10 being functional in humans and 12 in mice *(20)*. TLRs are type 1 transmembrane proteins characterized with amino-terminal extracellular LRR-domain necessary for the recognition of PAMPs. LRR-domains contain a variable number of hydrophobic leucine rich modules 20-30 amino acids in length. The extracellular portion of TLRs, due to LRRs, has a horseshoe shape as a common structural finding of all family members *(21)*. Recognition of different ligands is primarily determined by the amino acid sequence, but post-translation modification, micro-environmental factors like proteolysis-mediated activation and variable adaptor molecule availability contribute to the diversity in ligand recognition.

The controlled microbial recognition requires interplay of TLRs with different accessory molecules *(22)*. A number of different accessory molecules cooperate in the full activation of TLRs. The binding of LPS by TLR4 additionally requires lymphocyte antigen 96 (MD-2), serum LPS-binding protein (LBP) and CD14 that accelerate intracellular signalosome formation and downstream signaling *(23)*.

The members of the TLR family have acquired the ability to recognize different PAMPs that was evolutionary driven from ancestral TLR gene via a molecular

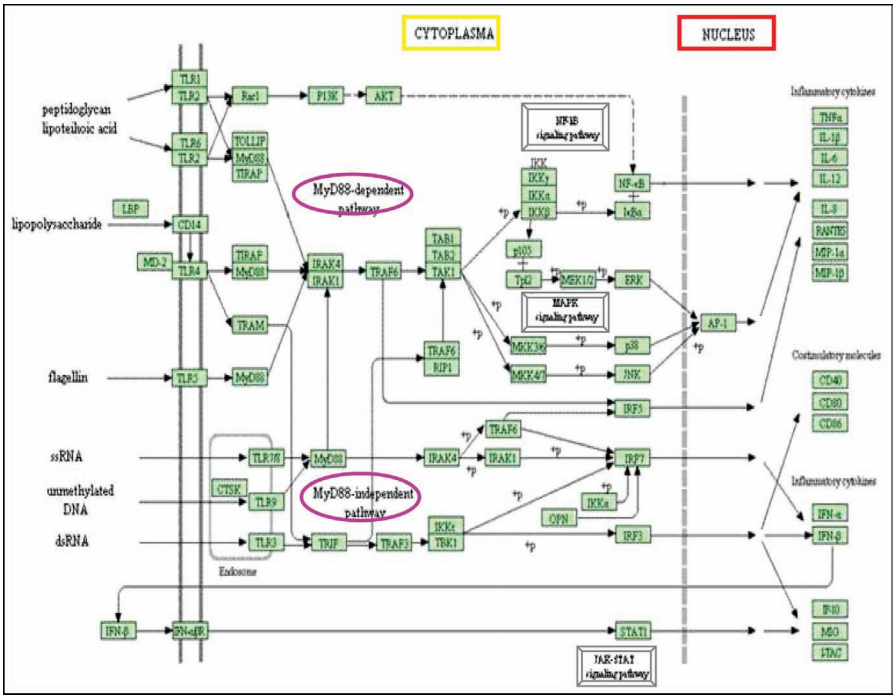

Figure 4. *TLR signaling..*
The toll-like receptors act in response to spectra of molecular patterns from different bacteria and viruses. TLR--specific signaling pathway leads to the recruitment of different adaptors and signaling molecules leading in downstream activation of NF-kB, MAPK and IRF pathways. Those pathways are responsible for innate inflammatory (cytokines and chemokines) and acquired immunity response (co-stimulatory molecule expression).

mechanism involving gene duplication *(24)* and directional allotype selection, possibly in response to pathogen challenge. This extensive genetic selection has resulted in recognition of the chemically similar microbial molecules of different origin: lipoproteins and peptidoglycans by TLR1/2 and TLR2/6; LPS and glycoproteins by TLR4; proteins like bacterial flagellin by TLR5; double--stranded RNA by TLR3 or single-stranded RNA by TLR7 (in humans also TLR8); CpG DNA by TLR9 (Table 1). The crystallographic analysis of TLR-ligand interaction employing 'hybrid LRR method' has demonstrated that hydrophobic ligands specific for TLR1, TLR2, and TLR4 bind within internal protein pockets while hydrophilic ligands, like double-stranded RNA, interact with TLR3 via the surface of LRR-domain. Binding of specific ligands, in a homotypic or heterotypic TLR--dimer format, induces dimerization of the ectodomains forming dimers strikingly similar in shape. These 'm'-shaped complexes, the C-termini of the extracellular domains of the TLRs, converge in the middle. This observation suggests the hypothesis that, upon dimerization, the extracellular domains undergo conformational changes and force allosteric activation and dimerization of the intracellular signaling TIR domains *(21)*.

It is not surprising that TLRs expressed on the cell surface (TLRs 1, 2, 4, 5, 6, 10) primarily recognize bacterial structural components, while those expressed within cellular compartments, like ER, endosomes, and lysosomes (TLRs 3, 7, 8, 9), are critical for the recognition of viral products and nucleic acids *(25, 26, 27)*. Functional activity of these intracellular TLRs requires lower pH for efficient ligand binding and downstream signaling that could be blocked by endosomal acidification inhibitor choroquine *(28)*. Although it was originally considered that single-stranded RNA ligand binding to TLR7 and TLR8 does not require specific nucleotide sequence, Heil *et al. (27)* have shown that GU-rich ssRNA represents physiological ligand for those TLRs as previously shown for TLR9 that recognizes unmethylated linear CpG oligonucleotide sequences *(29)*.

TLR SIGNALING

TLRs represent the key components of both innate and adaptive immunity and allow distinction between self and nonself via specific recognition mechanisms. Despite diverse mechanisms of ligand interaction, the organization of ligand-TLR dimer complexes may apply to all TLRs. The formation of 'm' shaped TLR dimer structure causes dimerization of the intracellular domains for signal initiation *(21)*. The resulting TIR-TIR complex initiates downstream signaling through recruitment of specific adaptor molecules (Figure 4). So far, five adaptors with TIR domain have been described: myeloid differentiation factor 88 (MyD88), MyD88-adaptor like (MAL), TIR domain-containing adaptor inducing IFN--beta (TRIF), TRIF-related adaptor molecule (TRAM), and sterile alpha and heat-Armadillo motifs (SARM) *(30)*. Depending on the adaptors recruited, downstream signaling events could be, in general, split in two pathways: (1) MyD88-mediated pathway resulting in activation of transcription factor NFκB (all TLRs except TLR3), or TRIFF-mediated pathway (MyD88-independent pathway) leading to activation of the interferon-regulated factors (IRF), family of transcription factors *(31, 22)*. MyD88 also contains another protein interaction domain, the death domain (DD) that enables subsequent association with the DD-bearing IL-1 receptor-associated kinases (IRAK) through homophilic DD-DD interac-

TABLE 1

Diversity of TLR ligands.

hTLR	Cell. location	PAMP	Cytokine profile	Immune response
TLRl/2	surface membrane	lipoproteins	IL12p70^{low}	Th2
TLR1/6	surface membrane	peptidoglycans lipoteichoic acid	IL-10^{high}	Treg Thl7
TLR3		dsRNA	IL12p70^{high} IFN-β	Thl
TLR4	surface membrane	LPS HSP60/80 RVS-F protein	IL12p70^{high} IFN-β	Thl
TLR5	surface membrane	flagellin	IL12p70^{high}	Thl
TLR7	endosome membrane	viral ssRNA	IFN-α^{high}	Thl
TLR8	endosome membrane	Virak ssRNA	IL12p70^{high} IFN-α	Thl
TLR9	endosome membrane	unmethylated DNA	IFN-α^{high}	Thl
TLR10	surface membrane	?	?	?

tion, to receptor-adapter complex. MyD88 pathway, besides IRAK-1 and IRAK-4 serin/threonin kinases, involves TNF receptor-associated factor 6 (TRAF-6) and other mitogen-activated kinases (MAPK) that associate with Pellino scaffold protein assembling the signalosome necessary for NFκB activation *(32)*. The Pellino protein enables tethering multiple members of a TLR-signaling pathway that culminate in the NFκB-mediated transcription of pro-inflammatory cytokine genes such as IL-1, IL-6, TNF-α, IL-12, IFNs, chemokines, adhesion molecules, co-stimulatory molecules, growth factors, tissue--degrading enzymes such as metalloproteinases, and enzymes that generate inflammatory mediators such as cyclo-oxygenase 2 and inducible nitric oxide synthetase *(33)*. In contrast, TRIF pathway predominantly results in IRF-mediated synthesis of interferons and potent anti-viral immune response.

Different microbial agents could trigger multiple pathways in different cell types and induce the expression of a distinct subset of genes ultimately shaping innate and adaptive immune responses *(34, 35)*. Interestingly, activation of TLR4 by LPS can consolidate both TRIF and MyD88-dependent pathways, inducing pro-inflammatory and anti-viral execution program.

BRIDGING INNATE AND ADAPTIVE IMMUNITY BY TLR AGONISTS

The sensing of the environment by TLRs and subsequent cellular response to infection via signaling pathways are some of the earliest events of immune response. The most diverse repertoire of TLRs has been detected in hempoietic immune cells *(36)*. In addition to the immune cells, non-immune cells can also respond to TLR stimulation. Most of them express some TLRs, including epithelial and endothelial *(37)* cells of the genital tract *(38)*, intestinal tract *(39),* and respiratory tract *(40)*. As frontline of infection, these cells recognize pathogens and release cytokines and chemokines that could in turn modulate TLR expression *(41)*. They also release cytokines and chemokines to initiate inflammatory response as result of the immune cell recruitment. Among them, dendritic cells (DC), first described by Steinmen RM (Nobel Prize laureate for 2011.), have a central role in the development of adaptive response *(42)*.

DCs are professional antigen (Ag)-presenting cells, comprising a complex subsets of cells with distinct myeloid (myeloid DC) or lymphoid (plasmacytoid DC) origin *(43)*. The immature DCs have a potent inherited capacity to internalize and process antigens, but when stimulated they mature, express high levels of surface co-stimulatory molecules and release cytokines to optimally regulate primary/secondary T cell responses.

DCs constantly patrol in different tissues, uptake pathogens and present Ag to T cells. Critical function of DCs is to sense an invading pathogen by mechanisms of innate immunity and transfer the information about the threat to adaptive immune system. One of the key mechanisms is PRR-PAMP interaction that could sensibilize DCs about possible danger. DCs are armed with a collection of pattern recognition receptors that can specifically interact with pathogen PAMPs, including the mannose receptors, c-type lectins, TLRs and others *(44, 45)*. Among these, TLRs have drawn lot of attention in recent years due their diversity and strong activation potential. In humans, 11 different TLRs have been identified, each reacting with different pathogen patterns (Table 1).

Different subsets of DCs could express different TLRs. For example, human myeloid DCs (mDC) have been shown to express all TLRs except TLR7 and TLR9 whereas plasmacytoid DCs (pDC) have more limited pattern with predominant TLR7 and TLR9 expression *(46, 47)*. It has been proposed that DCs use many different TLRs to detect several features of a pathogen si-

multaneously and transmit 'danger signal' to direct a specific response. Thus, TLR-induced signals could serve as one of the mechanisms of self/non-self discrimination *(48)*. Triggering distinct TLRs on DCs also elicits different cytokine profiles, resulting in specific activation status and capability of DCs to shape adaptive immune response. For example, signals triggered via TLR7 and TLR9 in pDCs can activate downstream IRF7 pathway and predominant interferon (IFN)-α production *(47)*.

The discovery that pDCs are the major source of IFN-α during virus infections established that lymphoid DCs as a key player in the defense against intracellular pathogens. Upon encounter with naïve T cells, pDC force Th1 differentiation via IFN-α-dependent mechanism. In contrast, signals generated by triggering TLRs in the mDCs lead to the production of large quantities of IL-12p70, a cytokine that also favors the differentiation of Th1 cells and IFN-γ production *(49)*.

MyD88-TRAF6 interaction in DCs is followed by the activation of NF-κB, JNK, p38 MAPK and expression of different genes responsible for the generation of Th1, Th2, or other helper T cell subsets and cytotoxic T cells. DCs efficiently upregulate CCR7 and migrate to T cell area in the lymph nodes *(46)* as well co-stimulatory molecules CD40, CD80 and CD86, providing the second activation signal to T cells necessary for their differentiation and proliferation *(49)*.

Once activated by PAMPs, DCs acquire full capability to react but additional studies have demonstrated that micro-environmental factors could influence the development of immunity or tolerance *(50)*. Immature DCs express a low level of co-stimulatory molecules and cytokines, and it was postulated that immaturity tends to induce anergy or tolerance of T cells, whereas maturation of DCs is required to optimally activate T cells *(51)*.

However, reports suggest that DCs are also required to display a mature phenotype to successfully mediate tolerance induction *(52)*. Therefore it is more accurate to define the role of a subset of DCs by the effects they confer on the immune system *(53)*.

Since TLR ligands appear to promote the capacity of DCs in the induction of T cell responses, reasonably many have focused on the application of TLR ligands as a component of the next vaccine generations against infectious diseases and cancer. But before TLR ligands are about to be used as adjunctive agent in human vaccines, better understanding of the effect of TLR agonist on the activation of DCs and the regulation of immune responses by different DCs must be achieved.

USE OF TLR-AGONISTS IN PROPHYLACTIC AND THERAPEUTIC VACCINES

The era of immunization started when the first vaccine against rabies was made by Louis Pasteur and Pierre Paul Émile Roux in 1885. Later, at the beginning of the 20th century, many bacterial and viral vaccines entered immunization protocols that consisted of whole pathogens able to trigger many PRR-ligands even before relevant innate recognition receptors have been discovered.

Although mechanisms of action were not characterized at the time, it was observed that the addition of bacterial extracts exhibited adjuvant potency and would increase antibody production to the given antigen. A number of preparations were developed, like Freund adjuvant and Coley adjuvant. These formulations were enriched with different bacterial products but, due to exaggerated adverse effects, were used only experimentally. Adjuvant characteristic to stimulate the immune system without specific antigenic effect was used to increase the response to a vaccine, and generally adjuvants represent the essential part of an effective vaccine. Biological characteristics of potent adjuvants could be described as rendering them capable to: (1) accelerate the generation of robust, long lasting immune responses; (2) generate antibodies with increased avidity and neutralization capacity; (3) enhance immune responses in individuals with weakened immune systems; (4) reduce the amount of antigen and number of doses needed while reducing the cost of vaccination programs; (5) activate the cellular arm of adaptive response.

TLR-AGONISTS IN INFECTIOUS DISEASES

A large number of microbial components exhibit potent adjuvant properties and represent novel tools in creating vaccines. The older generations of vaccines for infectious diseases used attenuated or inactivated whole-pathogen formulations, incorporating all or most of pathogen derived antigens. These systems included a number of different antigens capable to stimulate innate and adaptive immunity, and sufficient to establish the productive immune response. But balance between immunogenicity and occurrence of adverse effects was jeopardized by overwhelming inflammatory reaction driven by activation of many PRR-mediated mechanisms. Clear example is pertussis vaccine where use of cellular pertussis vaccine has led to development of the protective immunity in vaccinated children and a decrease in disease prevalence during the last century. But in a number of vaccinated children, adverse effects were identified like serious irreversible brain damage that concomitantly influenced the development of acellular pertussis vaccine *(54)*. The acellular vaccine contains purified and detoxified pertussis toxin as a major bacterial antigen responsible for disease protrusion plus filamentous hemagglutinin and pertactin adsorbed on aluminum salts. Exclusion of a whole bacterial cell from vaccine formulation resulted in a removal of many different molecules with PRR-agonistic activity that could be responsible for the observed adverse post-vaccinated symptoms. Acellular formulation contains aluminum salts whose adjuvant activity seems to be related to inflammasome activation although there are conflicting findings *(55)*. Aluminum salts (Alum) have been in use for more than 80 years in vaccine formulations and showed strong sa-

fety profile and effectiveness, even before the mechanism of action has been determined. In this case, selection of an appropriate adjuvant with exact dose contributed in protective immune response and decrease of adverse symptoms in vaccinated children.

Another good example is whole virus vaccine against influenza virus that was introduced in 1940 in inactivated form. Although highly immunogenic, this vaccine form was accompanied with more frequent adverse effects than inactivated split or subunit vaccine *(56)*. Despite that, inactivated whole virus influenza vaccine still comprises about 30% of all influenza vaccine production; the rationale for the development of split influenza virus vaccine could be the ability of viral surface proteins to solely activate TLR4 *(57)* and initiate immune response. On the other hand, whole virus vaccine has additional pathogen associated molecules that could trigger other PRRs. Related to intracellular viral life cycle, host cells have developed intracellular receptors that sense viral nucleic acid during replication phase. Intracellular PRRs that recognize influenza virus are TLR3, TLR7, TLR8 (in humans) and later discovered RIG-I and MDA-5 receptors *(58)*. Depending on a manufacturing protocol, innate recognition of split and inactivated influenza virus relies probably on TLR4, TLR3, TLR7 and TLR8 but not RIG-I pathway since it could be exclusively triggered only by the live virus. As in a case of acellular pertussis vaccine, immunogenicity of split influenza vaccine is reduced and therefore different adjuvants are included. One of them is squalene (MF59) that exhibits potent adjuvant MyD88-dependent activity, independent of TLRs and inflammasome *(55)*.

Discovery of PRRs and their specific ligands as potent adjuvants accelerated the research of natural and synthetized compounds for possible clinical application. Currently only monophosphoryl lipid A (MPL) FDA licensed adjuvant (TLR4 ligand) is used in Cervarix®, a prophylactic vaccine against HPV types 16 and 18, and Fendrix® a hepatitis B vaccine both manufactured by *GlaxoSmithKline*. MPL is a nontoxic derivate of LPS discovered in 1979 by Ribi *et al. (59),* specifically recognized by the TLR4 but, unlike LPS, it triggers complete TRIF and incomplete MyD88 signaling without activation of caspase-1 required in the processing of biologically active IL-1β directly related to reduced toxicity *(60)*.

Immunostimulatory activity of imiquimod (TLR7 agonist) which has not yet been used as an adjuvant in vaccine preparations, is the first TLR-based medication FDA-approved in 1997 to treat actinic keratosis, superficial basal cell carcinoma, and external genital warts. As immune response modifier, imiquimod acts on innate and specific immunity to eradicate premaligant and human papillomavirus infected epithelial cells.

TLR-AGONISTS IN CANCER

Discovery that TLR7 agonist imiquimod can act upon immune system eradicate to malignant disease has renewed interest in the concept of immunotherapy as an approach to cancer treatment. The role of adjuvants in anti-cancer treatment had been studied in different systems employing mycobacterial and bacterial structural elements before information about PPRs has emerged. Detailed insight in the mechanisms involved and discovery of a spectrum of different PRRs that can be triggered by microbial products led to numerous preclinical and clinical studies to evaluate single or multiple agonists that can elicit effective anti-tumor adaptive immune response and possibly eradicate disease. The most studied are agonists recognized by intracellular TLRs that once triggered by a specific ligand, stimulate type I IFN production known to have a beneficial role in anti--cancer immune response. Moreover, stimulation of intracellular TLRs in Treg cells can overcome their regulatory function, allowing the reactivation of anergic tumor specific effector T cells and lysis of tumor cells *(61)*. It is not surprising that adjuvants play an essential and central role in the next generation of cancer vaccines *(62)*. An example is anti-cancer vaccine that besides antigen, utilizes three different adjuvants (squalene, MPL and CpG), two of them being TLR4 and TLR9 agonists with potent immunomodulatory activity.

STRATEGIES FOR NEXT GENERATION ADJUVANTS

Targeting intracellular TLRs

Several companies are developing promising new adjuvant candidates based on triggering intracellular TLRs as a potent immunostimulatory platform for infectious diseases and cancer.

3M Drug Delivery Systems has a portfolio of patent--protected TLR agonists that have shown promise as vaccine adjuvants. The lead candidate, resiquimod (TLR7/8 agonist) has shown promising results in a number of animal models and has an extensive toxicology and clinical data package to support further development as a cancer vaccine adjuvant *(63)*.

Celldex Therapeutics, Inc. entered a non-exclusive clinical research collaboration with 3M Drug Delivery Systems to access resiquimod for clinical study with the company's Antigen Presenting Cell (APC) Targeting Technology™ based on CDX-1401, a fusion protein consisting of a fully human monoclonal antibody with specificity for the dendritic cell receptor DEC-205 linked to the NY-ESO-1 tumor antigen, which is currently in a Phase I/II trial in combination with immune stimulating agents from 3M Drug Delivery Systems for advanced cancers of the bladder, breast, ovary, non-small cell lung cancer, myeloma, sarcoma and melanoma.

Juvaris BioTherapeutics, Inc. entered into an exclusive license agreement with Colby Pharmaceutical Company for the worldwide development and commercialization of Juvaris' Cationic Lipid-DNA Complex technology and related JVRS-100 product candidate. JVRS-100 contains un-methylated DNA that serves as a potent TLR9 ligand. When combined with a split influenza vaccine antigen,

JVRS-100 stimulates the adaptive immune response including specific antibodies and T-cell responses *(64)*.

Idera Pharmaceuticals have developed numerous compounds that act as agonists for TLR3, TLR7, TLR8, or TLR9, which the company believes have the potential to be used as adjuvants in vaccines. In preclinical animal models, Idera's TLR agonists have shown adjuvant activity when combined with various types of antigens *(65)*.

VentiRx Pharmaceuticals, Inc. has several TLR8 agonist candidates that have entered clinical trials in allergic and cancer diseases, as single or adjunctive agents.

GlaxoSmithKline, Inc. have launched a clinical trial with GSK2245035 compound that is a highly selective TLR7 agonist. Intranasal administration of GSK2245035 causes changes in the upper airway microenviroment driven by IFN-α that could alter a bystander's immune responsiveness to aeroallergens and contribute to reduction of allergic reactivity in subjects with respiratory allergies *(66)*.

Targeting surface TLRs

Encouraged by the MPL clinical achievements, many clinical trials related to TLR4 ligands have been introduced. Moreover, other surface TLRs have been explored for their specificity and potency as possible vaccine adjuvants.

Allergy Therapeutics, Inc. have conducted clinical phase II evaluation of gradually increasing quantities of an allergen with MPL in patients allergic to birch, hazel, alder rye and grass pollen. Allergen-specific immunotherapy combined with MPL, a TLR4 agonist, represents a curative approach which directly treats the underlying allergic disease by possibly changing specific immune response profile *(67)*.

Immune Design Corp. is developing its proprietary adjuvant known as glucopyranosyl lipid A (GLA) based on successful implementation of the first TLR4-specific agonist MPL in several vaccine formulations. GLA is a novel generation of human TLR4 agonists. GLA is a pure synthetic small molecule, manufacture with excellent stability and rationally designed to optimally activate human TLR4 receptors, that elicits broad humoral and cellular immunity *(68)*. GLA was also shown to be safe and well-tolerated in human in Phase I clinical study in combination with the influenza virus vaccine Fluzone® manufactured by *Sanofi Pasteur*.

CONCLUSIONS

Natural TLR ligands represent pathogen derived molecules that immune systems recognize as potential threat and act upon in order to sustain microbial spread and harm they might cause. Details about the exact mechanisms of action and key pathways involved enable the design of preferable immune responses by formulated next generation vaccines with selected TLR-ligands, individually or in combination with other PRR-ligands.

Proper formulation and exact molecular composition lead to the development of TLR-ligands capable of inducing robust directed CD4 or CD8 T cell responses, as well as affecting the quality and quantity of humoral responses. Employment of proprietary TLR ligands would enable the fine tuning of adaptive immune responses in specific target groups, like infants and the elderly.

At the same time, advanced vaccine formulations would exclude over-activation of inflammation with the establishment of active balance between immune-reactivity and immune-regulatory mechanisms. This is extremely important in the case of intracellular TLR ligand formulations since the development of some autoimmune diseases has been related to deregulated TLR7 and TLR9 activation.

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