**This is an Open Access-journals' PDF published in
Ebensen, T., Guzmán, C.A.
Immune modulators with defined molecular targets: cornerstone to
optimize rational vaccine design.
(2008) Human Vaccines,4 (1), pp. 13-22.**

Review

Immune modulators with defined molecular targets

Cornerstone to optimize rational vaccine design

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Key words: accination, adjuvant, TLR agonist, Malp-2, Cholera toxin, heat labile enterotoxin, AlphaGalCer, Freund adjuvant, Cyclic diGMP

Vaccination remains the most valuable tool for preventing infectious diseases. However, the performance of many existing vaccines should be improved and there are diseases for which vaccines are still not available. The use of well-defined antigens for the generation of subunit vaccines has led to products with an improved safety profile. However, purified antigens are usually poorly immunogenic, making essential the use of adjuvants. Despite the fact that adjuvants have been used to increase the immunogenicity of vaccines for more than 70 years, only a handful has been licensed for human use (e.g., aluminium salts, the micro-fluidized squalene-in-water emulsion MF59 and monophosphoryl lipid A). Thus, the development of new adjuvants which are able to promote broad and sustained immune responses at systemic and mucosal levels still remains as a major challenge in vaccinology. Recent advances in our understanding of the immune system have facilitated the identification of new biological targets for screening programs aimed at the discovery of novel immune stimulators. This resulted in the identification of new candidate adjuvants, which made possible the modulation of the immune responses elicited according to specific needs. A number of promising adjuvants which are currently under preclinical or clinical development will be described in this review.

Introduction

A key requirement for the immune system is the discrimination between self and nonself, particularly in the context of microbial agents able to cause disease. The recognition of pathogenic microorganisms is in part performed by the presence of pattern recognition receptors (PRR), such as the Toll-like receptors (TLR), on cells from the innate immune system. This system has also evolved other means to identify potentially dangerous entities, such as the CD1d-mediated recognition of ceramides; the complement system;

specialized receptors enabling natural killer cells to sense “nonself,” “missing-self” and “induced-self;” and the Nod proteins by which unique microbial motifs are detected (e.g., peptidoglycan), thereby initiating pro-inflammatory signaling cascades.^{1,2-5} On the other hand, the recognition of dangerous entities by the innate immune system is a prerequisite for the stimulation of pathogen-specific adaptive immune responses.^{6,7} It is now known that adaptive immunity dependency on the innate immune system results from the need for antigen processing and presentation. These functions are displayed by professional antigen presenting cells (APC), such as dendritic cells (DC).⁸ The initial uptake and phagocytosis of microbes by APC is facilitated by recognition of pathogen-associated molecular patterns (PAMP) by PRR. This leads to the activation of DC maturation, a process entailing upregulation of major histocompatibility complex (MHC; class I and class II) and costimulatory (e.g., CD40, CD80 and CD86) molecules, together with the production of different cytokines, which in turn results in optimal antigen processing and presentation to naïve CD4+ and CD8+ T-cells. A well orchestrated innate and adaptive immune response usually leads to pathogen eradication and long-lasting immunity against the specific agent (Fig. 1). In contrast, failure to efficiently discriminate between self and nonself can lead to unchecked spread of the pathogen or autoimmunity.

Although highly effective vaccines are currently available for a number of infectious diseases, vaccine formulations can still be improved. The use of well-defined antigens for the formulation has led to less reactogenic subunit vaccines, which unfortunately are less immunogenic than their live or whole cell inactivated counterparts. This has created a major need for efficient adjuvants. On the other hand, conventional vaccines are usually administered by the parenteral route. This mainly promotes the elicitation of systemic immunity. However, most pathogens enter the body through mucosal surfaces. Therefore, parenteral vaccines are suboptimal, since they fail to induce a local mucosal response able to block the early stages of infection. This roadblock can be eliminated by administering antigens by the mucosal route. Furthermore, mucosal vaccination offers several additional benefits, such as easy administration logistics and high acceptance by the public, as a result of the lack of pain.⁹ However, mucosal vaccines have to overcome formidable barriers. Antigens administered by this route can be cleared by non specific mechanisms (e.g., ciliary activity, mucous

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Submitted: 08/08/07; Accepted: 01/10/08

Previously published online as a *Human Vaccines* E-publication: www.landesbioscience.com/journals/vaccines/article/5560

Figure 1. The success or failure of an antigen-specific immune response depends on the interface between innate and adaptive immunity. For a successful removal of a pathogen it is essential that microbes are recognized by components of the innate immune system. The systems for microbial sensing are complementary and they are involved in the development of the ensuing adaptive immune response. Adaptive responses may tend toward tolerance or productive activation of T helper type 1 (Th1) or type 2 (Th2) cells. Different responses are required for the elimination of different microbes (e.g., clearance of intracellular pathogens usually requires robust Th1 and CTL responses).

entrapment, peristaltism), are degraded by enzymes and may be affected by extreme pH, before having the chance of reaching APC. One of the strategies to overcome the poor immunogenicity is the co-administration of antigens with mucosal adjuvants.¹⁰ These compounds generate a local microenvironment conducive towards antigen processing and presentation, thereby promoting the elicitation of robust responses at both local and systemic levels.¹¹

The chemical nature of the adjuvants and their mode of action are extremely variable. Potential mechanisms by which they exert their biological activity are the “depot” effect, antigen targeting to APC, improvement of antigen processing and presentation and immune activation or modulation through the upregulated expression of cellular mediators. However, it is also important to consider that their biological activities may not only favor the elicitation of adaptive responses, but also the appearance of side effects. A strong stimulation may result in local or general inflammation and tissue destruction, with the resulting distress for the vaccinees. On the other hand, the stimulation of vigorous responses is insufficient to protect against a specific pathogen. It is essential to promote an adequate type of response. Otherwise, we may even lead to immune pathological reactions or a more severe course of infection. Thus, the identification of adjuvants able to promote predictable responses is a major issue in vaccinology. The lack of adjuvants which are able to stimulate cell-mediated responses represents also a bottleneck. Only a few molecules have been identified exhibiting this property, being most of them unacceptable for human use due to their reactogenicity. Therefore, the choice of a specific adjuvant for a formulation reflects a compromise between the required immune modulatory effect and an acceptable (i.e., low) level of side effects.

Dozens of adjuvants have demonstrated their efficacy in preclinical and clinical studies. Nevertheless, only a few, such aluminum-based

salts,^{12,13} the squalene-in-water emulsion MF59^{14,15} and mono-phosphoryl lipid A (MPL)¹⁶⁻¹⁹ have been approved for human use (Table 1). In fact, aluminum salts are at the moment the most widely used adjuvants for both human and veterinary vaccines. Although severe side effects are rare, alum has the potential to cause sterile abscesses, eosinophilia and myofascitis.²⁰ There are also concern regarding the potential role of aluminum in neurodegenerative diseases.²⁰ Alum induces strong antibody and Th2 responses. Therefore, adjuvants able to promote Th1 and CTL responses are still needed. Interestingly, the use of alum in combination with IL12 can redirect responses from Th2 towards a Th1-dominant response.²¹ On the other hand, MF59 promotes both humoral and cellular immune responses.^{14,15,22,23} Squalene or squalane emulsions are efficient adjuvants which can be stabilized by micro-fluidization, so that the emulsions can be frozen or kept for years at room temperature, allowing also their sterilization by terminal filtration.¹⁴ Antigens are added after emulsification, so that conformational epitopes are not lost by denaturation, as well as to facilitate manufacturing.^{24,25} Clinical trials of several MF59-adjuvanted vaccines, which were performed in different age groups (from newborns to elderly), have demonstrated their safety and immunogenicity. However, phase IV studies are mandatory, since the use of oil-based adjuvants may be associated with a higher risk for autoimmunity in experimental models.²⁶ This seems to be related with the hydrocarbon's ability to induce IL12, IL6 and TNF α . Whether this is of relevance for human vaccination is matter of discussion, since immunotoxicity depends on many factors, such as the species, genetic makeup, route, dose and duration of the administration. In fact, up to now, clinical studies do not seem to support a high risk for autoimmune disease.

Saponins are a chemically heterogeneous group of sterol glycosides and triterpene glycosides, which are common constituents of plants.

Table 1 **Adjuvants included in human and veterinary vaccines**

Adjuvant	Administration Route	Side Effects
Aluminium based salts	parenteral	Mild systemic reactions (93%), ²⁷ local side effects (e.g., suprapubic pain and vesical tenesmus) ^{20,28}
Squalene oil-in-water emulsion	parenteral	Local side effects ^{29,30}
Monophosphoryl lipid A (MPL)	parenteral and mucosal	Pyrogenic at high doses ³¹
Endogenous human cytokines or hormones, (e.g., INFs, IL12, GM-CSF)	parenteral	May cause adverse effects
Bacterial toxoids (e.g., derivatives of cholera toxin and E. coli heat labile toxin)	parenteral and mucosal	Mild side effects, may result in retrograde homing to neural tissues when administered by nasal route ^{32,33}
Muramyl dipeptide (MDP), lipid A derivatives	parenteral and mucosal	Currently under study
Lipopeptides, MALP-2	parenteral and mucosal	
Saponins (e.g., QS-21)	parenteral and mucosal	May cause allergic reactions when given at high dosages
Immunostimulating complex (ISCOMs)	parenteral and mucosal	Currently under study
CpG-ODN	parenteral and mucosal	Conflicting data on effect following long term administration, may cause mild damage. ³¹

They are known to cause substantial enhancement of immune responses since the 1920s. Naturally occurring saponins from *Quillaja saponaria* stimulate humoral responses against T-dependent and T-independent antigens and CTL responses.³⁴ Despite their use in animal vaccines, the development of saponin-based formulations for humans has been impeded by their complexity and concerns about toxicity.^{35,36} On the other hand, QuilA, which results from partial purification from crude food-grade extracts, is contained in several veterinary vaccines. Further purification provided concentrated saponin fractions, such as QS-21, which is currently under clinical investigation in humans.³⁷⁻³⁹ Interestingly, purified saponins seems to be also effective as adjuvants when delivered by oral route.³⁷

Saponins have been combined with cholesterol and other lipids to generate immunostimulating complexes (ISCOMs), which are open cage-like structures with build-in adjuvant activity that promote antibody, T helper and CTL responses. ISCOMs seem to enhance antigen targeting to APCs, as well as their subsequent uptake, processing and presentation. The use of ISCOMs also result in the production of pro-inflammatory cytokines, such as IL1, IL6 and IL12.⁴⁰ Liposomes represent a related delivery system, which also has build-in adjuvant properties. They are vesicular structures limited by a bilayer membrane composed of phospholipids and cholesterol. Liposomes can carry both membrane associated antigens, as well as water soluble molecules. Their physical properties are highly variable, depending on the composition and the manufacturing method. This allows the optimization of the design for specific tasks (e.g., targeting, co-incorporation of adjuvants). Liposomes have a long history as vehicles for antigen delivery.^{41,42} Recently, the so-called virosomes have been developed, by incorporating the hemagglutinin from

the influenza virus into liposomes.⁴³⁻⁴⁶ This glycoprotein guides virosomes to APC and promote their fusion with the endosomal membrane at low pH. This in turn leads to the cytoplasmic release of the antigens, thereby providing optimal processing and presentation in the context of MHC class I molecules.

There are other candidate adjuvants in advance stages of preclinical and clinical development, such as a new generation of water-in-oil emulsions (e.g., Montanide, CSA 720), which were demonstrated able to trigger more efficient humoral responses than alum in several animal species and humans.⁴⁷⁻⁴⁹ A small immunomodulatory peptide, CEL-1000, also promoted the elicitation of protective Th1 responses against infectious agents and tumors in experimental animal models.^{50,51} Nevertheless, despite the availability of a number of molecules with adjuvant properties, to optimize rational vaccine design a broader palette of adjuvants able to promote different types of immune response would be necessary. In this context, the

availability of immune-modulators with well-defined molecular targets would represent a clear asset. This approach would not only allow fine-tuning and customizing responses according to the specific needs, but would also facilitate the prediction of potential side effects.

Immune Modulators with Defined Molecular Targets

The advent of whole-genome sequencing of bacterial pathogens has revolutionized the field of vaccinology. This approach not only provides the full array of potential candidate antigens for vaccine design, but has also revealed new PAMP, which might exert immunomodulatory activities by acting on PRR.⁵² Adjuvant exhibiting this property deliver a danger signal to the host immune system through the activation of PRR, thereby mimicking infectious agents. This results in the expression of soluble mediators (e.g., cytokines, chemokines) and APC activation. The stimulation of the innate immune system in turn determines and shapes adaptive responses. The exploitation of this knowledge for the identification of new molecular targets is facilitated by recent developments on mass mutagenesis, high throughput screening, gene expression profiling and combinatorial chemistry.⁵³ However, the intrinsic human genetic variation is a major factor which might lead to differential responses after stimulation with PRR agonists. Therefore, immunogenetics is a critical building block of discovery programs.⁵⁴

TLR Agonists

The mammalian TLR family consists of at least 13 members (10 TLRs (TLR1–10) in human and 12 TLRs (TLR1–9 and TLR11–13) in mice) have been found and each TLR is involved in recognizing a variety of microorganism-derived molecular structures

(Table 2).^{55,56} TLR comprise a extracellular domain with dozens of leucine-rich repeat motifs, a trans-membrane domain (with the exception of TLR3) and a cytoplasmic Toll/IL1R (TIR) domain similar to that of the IL1 receptors (IL1Rs).⁵⁷ The TLR1, TLR2, TLR4, TLR5, TLR6 and possibly TLR11 are expressed on the cellular surface, whereas TLR3, TLR7, TLR8 and TLR9 are believed to reside inside the cells (e.g., endoplasmic reticulum and/or endosomes). Each TLR is expressed in a variety of immune cells, including macrophages, DC and B-cells, as well as in other cell types (e.g., endothelial cells, epithelial cells). TLR can recognize molecular patterns conserved among but unique to microbes, which usually do not exist in the host. Pathogen recognition by TLR provokes a rapid activation of the innate immune system by inducing production of pro-inflammatory cytokines and upregulation of costimulatory molecules. The activated innate immune system subsequently leads to effective adaptive immunity. Depending on the TLR triggered, specific signaling pathways are activated (Fig. 2). Different TLR can exert distinct, but to some extent also overlapping sets of biological effects.⁵⁵ Stimulation of TLR2 and TLR4 failed to increase CTL responses, whereas ligands of TLR3, TLR5 and TLR7 exhibited moderate activity. In contrast, stimulation of TLR9 dramatically increases CTL responses.⁵⁸

Agonists able to either control the over-expression of Th2 cytokines or skew the Th1:Th2 balance towards a Th1 profile would be of clinical relevance for the control of allergic disease.⁶⁰ In fact, different TLR ligands were demonstrated to be able to inhibit Th2 cell activation and IgE-dependent release of Th2 cytokines in animal experimental models of allergy.^{61,62} TLR are also important for the stimulation of Th2-type responses, since they augment the overall maturity of DC.^{63,64} Some of the initial studies performed with native ligands gave conflicting results due to contamination with other moieties. However, the availability of well-defined synthetic derivatives made possible to dissect the signal transduction events triggered by the specific activation of a TLR. Thus, the exploitation of well-defined TLR agonists for the establishment of immune prophylactic or therapeutic interventions seems to be an extremely promising field. However, safety aspects need to be carefully addressed during preclinical and clinical development, since TLR seem to be involved in the pathogenesis of human diseases (e.g., autoimmunity, allergies, asthma and cardiovascular diseases).

In this context, bacterial lipoproteins and their synthetic analogues are strong immune modulators during infection, signaling through TLR2/TLR1 heterodimers. Their synthetic derivatives, such as Pam₃Cys-SK₄, have been exploited as adjuvants and modulators of T-cell responses. Previous studies suggested that Pam₃Cys-SK₄ is able to ameliorate established allergic airway

Table 2 **Toll-like receptors (TLRs) and their ligands**⁵⁹

TLR	Interaction with other TLR	Ligand (Exogenous)	Ligand (Endogenous)	Source
TLR1	TLR 2	Peptidoglycan, triacyl lipopeptides, diacyl		Gram-positive bacteria
TLR2 and TLR6	TLR1	lipopeptides, lipoteichoic acid, glycolipids	HSP70	Measles virus
TLR3	-	dsRNA, siRNA	mRNA	West Nile virus, mouse CMV, <i>Schistosoma mansoni</i> , synthetic RNA molecules
TLR4	-	Lipopolysaccharide, taxol, RSV fusion protein, mouse mammary tumor virus envelope protein, phosphorylcholine, glycan	HSP70, β -defensin 2, fibrinogen, fibronectin, hyaluronic acid	Gram-negative bacteria, plants, respiratory syncytial virus, mouse mammary tumor virus, <i>Bacillus anthracis</i> , helminths
TLR5	-	Flagellin	-	Flagellated bacteria
TLR6	TLR2	Lipopeptides (e.g., MALP-2)	-	Bacteria
TLR7	-	ssRNA, Imidazoquinoline, Resiquimod, Imiquimod, CpG DNA, hemozoin	U1snRNP (spliceosomal complex), autoantigens,	synthetic compounds, viruses (e.g., influenza virus, HIV-1)
TLR8	-	-	-	-
TLR9	-	-	Chromatin complex	Bacteria, synthetic ODN, DNA viruses, <i>Plasmodium falciparum</i>
TLR10	-	Not determined	-	-
TLR11	-	Profilin-like molecule	-	<i>Toxoplasma gondii</i> , uropathogenic bacteria
TLR12	-	Not determined	-	-
TLR13	-	Not determined	-	-

Figure 2. The Th1/Th2 network. IL4 and IFN γ do not promote a direct inhibition of Th1 or Th2 cells differentiation, but rather block the differentiation of these subsets from naive precursors.

inflammation by promoting Th1 response rather than by affecting regulatory T-cells.⁶⁵ On the other hand, its co-administration with anti-CD3 antibodies resulted in modulated immune responses, by inducing the proliferation of both regulatory and effector T-cells in the absence of APC. Thus, it seems that Pam₃Cys-SK₄

improves adaptive responses by expansion of effectors and mitigation of suppressive activity of Tregs.^{66,67}

Natural and synthetic derivatives of diacylated lipopeptides from *Mycoplasma* spp. and *Francisella tularensis*, such as the macrophage-activating lipopeptide of 2 kD (MALP-2), induce the maturation and activation of APC with release of soluble mediators able to act on bystander cells via activation of the TLR2/TLR6 heterodimer.^{68,69} Their co-administration with antigens by either parenteral or mucosal route results in the elicitation of strong humoral and cellular responses.^{68,70} Additional studies demonstrated that MALP-2 promotes a T-cell-independent activation and maturation of B-cells, increasing also the frequency of Ig-secreting cells. Activated B-cells exhibited increased expression of both activation markers and ligands which are critical for cross-talk with T-cells (e.g., CD19, CD25, CD80, CD86, MHC I, MHC II and CD40). Immunization of mice lacking T-cells also showed that MALP-2-mediated stimulation of TLR2/TLR6 was unable to circumvent the need of T-cell help for efficient antigen-specific B-cell activation. On the other hand, immunization of mice lacking B-cells demonstrated that B-cells are critical for MALP-2-dependent improvement of T-cell responses.⁷¹⁻⁷³ Thus, B-cell stimulation by PRR seems to be a basic mechanism which can be exploited to improve the immunogenicity of vaccine formulations.

The lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, stimulates adaptive responses. Since the 1970s it was known that the adjuvant effect depends on the integrity of the *lps* locus.⁷⁴⁻⁷⁷ Then, positional cloning showed that the *lps* locus was identical to *tlr4* locus.⁷⁸ However, the toxicity of the LPS prevented its clinical use, making necessary the development of non toxic derivatives. Among them, MPL, a derivative of lipid A from *Salmonella minnesota*, was proven to be safe and effective as a vaccine adjuvant for humans.⁷⁹ A new class of fully synthetic lipid A mimetics (e.g., aminoalkyl glucosaminide 4-phosphates, RC-529) have been engineered to target human TLR4 and are showing promise results as vaccine adjuvants and therapeutics against a wide range of infectious agents.⁸⁰ Thus, it seems that the potent immune modulatory properties of TLR4 agonists allow their exploitation as both vaccine adjuvants and stand-alone therapeutics.

Synthetic double stranded polyribonucleotides act at the level of TLR3, inducing the production IFNs and other cytokines. This activity can be exploited to increase the effect of antiviral drugs. For example, the antiretroviral effect of zidovudine is enhanced by combination with polyribonucleotides.⁸¹ Poly I:C was one of the first therapeutic agents used to treat HIV and leukemia patients, but it was abandoned due to toxicity.⁸² Synthetic polyribonucleotides also induce the activation and maturation of DC. In vitro results demonstrated that a novel non toxic analogue of poly I:C (Ampligen) effectively induce in vitro maturation of monocyte derived human DC with sustained production of IL12. DC primed with tumor lysates and matured with synthetic dsRNA may offer a valid alternative for optimizing Th1 anti-tumor responses in cancer patients.⁸³

The binding of bacterial flagellin to TLR5 results in DC stimulation and promotion of Th1 responses.^{1,84,85} This depends, at least in part, in the upregulated expression of IL12 resulting from phosphorylation of p38 and c-Jun N-terminal kinase 1/2. Monomeric flagellin acts as local and systemic pro-inflammatory factor via activation of the TLR 5-NFκB axis and IL8 production.^{58,86}

Co-administration of tetanus toxoid (TT) with flagellin to mice by intranasal route resulted in significantly enhanced TT-specific humoral responses in the mucosal and systemic compartments. Vaccinated mice were completely protected after challenge with a 200-fold minimum lethal dose of tetanus toxin. FlaB colocalized with CD11c as patches in putative DC and a clear increment in the number of TLR5-expressing cells was also observed in cervical lymph nodes.^{87,88}

Imiquimod, a synthetic imidazoquinoline which was approved in 1997 for the topical treatment of genital warts, exhibits antiviral and anti-tumor activities. Imiquimod binds TLR7 and TLR8 on DC leading to the production of pro-inflammatory cytokines, such as IFNα, which in turn stimulates both the innate immune system and the cellular arm of the acquired immune system (e.g., improvement of CTL responses).^{89,90} This suggests that imiquimod mimics natural ligands of TLR7 and TLR8 (e.g., ssRNA from viruses). However, recent experimental and clinical data indicate that imiquimod also exerts direct pro-apoptotic activity on tumor cells.⁹¹ Imiquimod is insoluble in water, but in most clinical studies its incorporation into an oil-into-water cream emulsion (1–5%) was well-tolerated with mild-to-moderate drug-related side effects, such as itching, burning sensation, pain, erythema, erosion and oedema.⁹² An analogue, the so called resiquimod (R848), is able to promote similar Th1-biased responses, but at 10-fold lower dosages than imiquimod.⁹³ Initial clinical studies suggested that resiquimod reduces the recurrence rate of genital herpes, however, phase III trials were suspended due to lack of efficacy.⁸⁹

In the course of natural microbial infections, TLR9 recognizes double stranded DNA which, in contrast to mammalian DNA, is characterized by the abundance of unmethylated deoxycytidyl-deoxyguanosine dinucleotides (CpG). The stimulatory activity of microbial DNA can be mimicked by synthetic oligodeoxynucleotides (ODN) containing such motifs (CpG-ODN).⁹⁴ The TLR9-mediated stimulation of the vertebrate innate immune system and subsequently, of the adaptive immune system, allows the use of TLR9 agonists as highly effective vaccine adjuvants for infectious diseases, as well as stand-alone or part of combination therapies against cancer.^{95,96} TLR9 shows a restricted cellular and sub-cellular pattern of expression, which changes also between animal species. CpG-ODN acts on human B-cells and plasmacytoid DC increasing the production of pro-inflammatory cytokines and promoting APC maturation and Th1 responses.^{97,98} These biological activities enable CpG-ODN to act as adjuvants when co-administered with a given antigen by systemic or mucosal route, thereby leading to improved antigen-specific responses. These effects are optimized by maintaining close physical contact between the CpG-DNA and the immunogen. Ongoing clinical studies also indicate that CpG-ODN are well-tolerated in humans.^{98,99} During infection, recognition of CpG DNA of intracellular pathogens skews immune response towards a Th1 dominant pattern. This can be also desirable in therapeutic interventions aimed at the prevention of allergic responses.

Bacterial Toxins and Their Derivatives

Cholera toxin (CT) and its close relative the heat-labile enterotoxin of *Escherichia coli* (HLT) are A/B moiety toxins. They are composed by an A subunit, which is responsible for the enzymatic

activity and a pentamer of B subunits, which mediates the binding of the holotoxin to the receptors present in the membrane of the target cells. CT and LT-I bind to the ganglioside GM₁, LT-IIa binds with high affinity to the ganglioside GD_{1b} and with low avidity to the gangliosides GD_{1a} and GM₁ and LT-IIb binds with high affinity to GD_{1a}.^{100,101} These two toxins are powerful adjuvants when co-administered with antigens by either systemic or mucosal route.¹⁰² The molecular mechanisms by which they are able to stimulate the innate and adaptive immune systems are not fully elucidated. However, direct effects on T-cells and APC have been observed, which provide insights into how these molecules may exert their activity. In vitro studies performed with primary B-cells and macrophages showed an increased phosphorylation of different signaling molecules, including Erk1/2 and p38.¹⁰³ In this context, the B subunit of CT induces signaling events resulting in cellular activation, expression of surface molecules and production of cytokines, by promoting a transactivation of two transcriptional elements, the cyclic AMP-responsive element and NFκB.¹⁰³ The observed GM1-dependent nuclear translocation of NFκB in DC demonstrates the critical role played by this receptor in the stimulation of signal transduction cascades.¹⁰⁴ On the other hand, there is a paucity of information regarding the structurally related members of the serogroup II HLT, namely LT-IIa and LT-IIb, which have different binding specificities for ganglioside receptors.

The use of CT and LT in humans was initially hampered by their high toxicity. However, site-directed mutagenesis has permitted the generation of LT and CT derivatives with reduced toxicity, which retain their adjuvanticity.¹⁰⁵ Different mutants, such as LTK63 (LT with a serine-to-lysine mutation at position 63 of the A subunit), have been exploited as systemic or mucosal adjuvants.¹⁰⁶ A preferential induction of Th2-type responses was observed when these molecules were incorporated in the vaccine formulations. Interestingly, their use also prevented or alleviated autoimmune diseases, thereby demonstrating their wide-ranging effects on the immune system. The observation that this improvement is associated with the generation of regulatory T-cells, which in turn inhibit pathogenic Th1 responses, explains in part the two apparently contradictory outcomes after exposure to CT-like enterotoxins. Nevertheless, further investigations are required to unravel the mechanisms leading to either adjuvanticity or tolerance induction to fully exploit their potential in the context of vaccine design and immunotherapies.¹⁰⁷

CD1d Agonists

NKT-cells represent a unique subset of immune regulatory T-cells, which are able to recognize glycolipids presented by the MHC class I-like molecule CD1d. Because of their biological properties, NKT-cells are attractive targets for the development of immunotherapies. The prototypical NKT-cell ligand αgalactosylceramide (αGalCer), originally isolated from a marine sponge, has potent immune modulatory activities. αGalCer is a ligand of invariant Vα14+ NKT-cells, which is presented by CD1d on APC. Administration of αGalCer to mice results in a rapid activation of NKT-cells, which is characterized by cytokine secretion, surface receptor downregulation, expansion and secondary activation of a variety of bystander cells from the innate and adaptive immune systems. Different studies have demonstrated the efficacy of

αGalCer against metastatic tumors, infections and autoimmune diseases.¹⁰⁸⁻¹¹¹ Studies performed with structural analogues of αGalCer also showed that β-anomeric GalCer can induce CD1d-dependent biological activities in mice, albeit at lower potency than α-anomeric GalCer.¹¹² The response of NKT-cells to distinct GalCer analogues seems to differ both at quantitative and qualitative levels. These findings indicate that specific glycolipids could be exploited to fine-tune NKT-cells responses, thereby shaping the elicited immune responses in vivo. This approach will certainly facilitate the development of effective and safe NKT-cell-based immunotherapies.^{24,112-115}

Different studies have also demonstrated that αGalCer could act as an effective adjuvant when co-administered with antigens by either systemic or mucosal route. When αGalCer was administered with the model antigen ovalbumin by intranasal route to C57BL/6 and BALB/c mice, strong ovalbumin-specific humoral and cellular immune responses were stimulated at systemic and mucosal level, characterized by a mixed Th1/Th2 cytokine profile. Vaccinated mice also showed complete protection against challenge with the ovalbumin-expressing tumor cell line EG7. Furthermore, intranasal vaccination with the hemagglutinin from the influenza virus A/PR/8/34 co-administered with αGalCer also conferred significant protection against viral infection. Interestingly, when αGalCer was given with a replication-deficient adenovirus to mice, significantly enhanced immune responses were detected. The adjuvant effect induced by intranasal co-administration with αGalCer was blocked in CD1d^{-/-} mice, indicating that the immune responses were exclusively dependent on the CD1d molecule present on APC. Experiments performed using CFSE-labeled OT-1 cells which were adoptively transferred into syngenic mice showed that naive T-cells were activated and stimulated to differentiate into functional effector T-cells.¹¹⁶ The efficacy of αGalCer as adjuvant was also demonstrated in the context of other vaccine relevant antigens (e.g., malaria, influenza, hemagglutinating virus of Japan).^{113,117,118} Despite the excellent results obtained using αGalCer, there are major drawbacks preventing the efficient transfer into the clinic, such as its poor solubility. To provide soluble formulations, non-organic solvents or detergents are needed, which represent a safety concern and might affect the immunological properties of some antigens. However, a new pegylated derivate, αGalCerMPEG, has been recently described, which is water-soluble and retains both the specificity for the CD1d receptor and the immune stimulatory properties when tested in vitro or in vivo, even at 33-fold lower concentrations of the active moiety.¹¹⁹

Cytokines

The process by which mammals meet the dual need of an immediate response to danger and initiation of long-term protection is regulated by pro-inflammatory cytokines, which are primarily produced by cells from the innate immune system. Therefore, cytokines have been widely exploited as natural adjuvants for the design of immunotherapeutic or prophylactic interventions. Many cytokines were demonstrated to be able to enhance protection against infectious agents and tumors, both in preclinical and clinical studies. One of the most exploited ones is IL12, which plays a key role linking the innate and acquired immune systems. IL12 is critical for activation of NK cells, promotion of CTL development,

T-cell independent induction of IFN γ , activation of differentiated CD4 $^{+}$ and CD8 $^{+}$ T-cells, development of Th1 responses, provision of complementary immune-regulatory signals to IL2 and defense against intracellular pathogens.¹²⁰ Therefore, also other members of the IL12/IL23/IL27 family, which share ligand and receptor subunits and play overlapping roles in innate and adaptive responses are also considered as prime candidates.¹²¹ The use of IL2 also gave promising results. The mechanism of anti-tumor activity of this cytokine is related to its ability to expand and activate the NK and CTL subsets.¹²² There is also increasing evidence that local or systemic effector cell dysfunction, which is characteristic of patients with advanced cancer, can be reverted by IL2. Another important cytokine, IL15, is produced by several leukocytes in response to infections. IL15 exhibits many homologies to IL2 and, like IL2, stimulates NK cells.¹²³ Chemokine-antigen fusions also resulted in enhanced immunogenicity. The rational use of combinations between cytokines and chemokines could promote the targeting of antigens to APC, their subsequent maturation, the attraction of critical bystander cells, the steering of cellular immune responses toward a Th1 response pattern, the improvement of CTL responses and the enhanced production of systemic IgG and secretory IgA. Several review articles have provided additional information about the most commonly used cytokines and chemokines for immunotherapies.¹²⁴⁻¹³⁰

Cell Wall Components

Freund's complete adjuvant (FCA) has been the adjuvant of choice for animal experimentation for decades. A water-in-oil emulsion is generated, in which water droplets containing antigen are emulsified in mineral oil containing killed mycobacteria or their cell walls. However, its high toxicity precludes its use in humans. Nevertheless, subsequent studies allowed the isolation of the active components and a better understanding of the underlying mechanisms of adjuvanticity. This resulted in the identification of molecules exhibiting similar immune modulatory properties and an adequate safety profile. N-acetylmuramyl-L-alanyl-D-isoglutamine (i.e., muramyl dipeptide; MDP) was the first.¹³¹ This is a synthetic derivative of a component present in the bacterial peptidoglycan. The activity of MDP can be attributed to its ability to cause the release of multiple cytokines. Animal studies established that MDP exhibits a broad array of immunological effects, including: (1) enhancement or suppression of antibody levels dependent on the time of administration relative to antigen; (2) improvement of cell-mediated immunity; (3) enhancement of nonspecific immunity to bacteria, viruses, fungi and parasites; (4) stimulation of natural resistance to tumors; (5) promotion of cytokine release and (6) pyrogenicity. MDP is recognized by NOD2, but not by TLR2 or heterodimers formed by TLR2 with TLR1 or TLR6.¹³² In contrast to intact and diacylated MDP, derivatives with a single octanoyl or stearyl fatty acid chain were found to activate TLR2 and TLR4 (see above) and exert their activities through the MyD88-dependent pathway on APC.¹³³ Studies performed using a *Mycobacterium tuberculosis* animal experimental model also showed that NOD2 and TLR are two non redundant recognition mechanisms which synergize for the induction of pro-inflammatory cytokines.¹³⁴ Interestingly, MDP also exerts pronounced neuropharmacological activities, probably through the interaction with 5-hydroxytryptamine receptors.

However, some of these biological activities are undesirable for clinical use.¹³⁵ Thus, structurally well-defined synthetic derivatives from the MDP were generated, which exhibit improved pharmacological properties.^{73,136,137-139} Among these non toxic derivatives can be mentioned the adamantylamide dipeptide (AddP), MDP-Lys (L18) and murabutide. The AddP is a synthetic compound in which the dipeptide was combined with the antiviral compound amantadine. AddP exerts its adjuvant properties when administered by either parenteral or mucosal route, leading to the elicitation of strong humoral and cellular responses at both systemic and mucosal levels.¹⁴⁰⁻¹⁴³

Co-Stimulatory Molecules

Processed antigenic peptides are presented by APC to T-cells in the context of MHC molecules, which are the only physiological ligands for the T-cell receptor (TCR). However, several additional receptors form part of the immunological synapsis. In fact, ligation of the TCR to the MHC II-peptide complexes (signal 1) leads to various T-cell effector functions, depending on the specific nature of the co-stimulatory receptors (signal 2). Thus, surface molecules such as CD40 and CD154 are central to the cross-talk between T-cells and antigen-presenting cells. The study of the immune responses against intracellular pathogens showed the relevance of CD40-CD154 interactions in the regulation of IL12 and IFN γ secretion.¹⁴⁴ Furthermore, anti-CD40 antibodies can be effective adjuvants when administered either separately (high dosages) or conjugated (low dosages) to the antigen. In the former case, it is likely that side effects such as splenomegaly would occur, whereas when conjugates are used, side effects may be avoided.^{145,146} GM-CSF has been shown to be synergistic with IL12 or CD154 for induction of CTL responses and the triple combination of GM-CSF, IL12 and TNF α appears to induce the most effective protection in some experimental models. The combination of co-stimulatory molecules with vaccine antigens has also resulted in a synergistic effect.¹⁴⁷ Another potential target molecule is CD134, a member of the TNFR superfamily. CD134 is transiently expressed on T-cells following ligation of the TCR. The CD134 ligand, known as OX40L, is a TNF family member expressed on APC. The OX40/OX40L pathway offers opportunities for both enhancing responses through use of agonists (adjuvants) and abrogating unwanted responses, such as those generated in autoimmune diseases, through the use of blocking reagents and antagonists.¹⁴⁸ Another widely studied co-stimulatory pathway is the CD28-CD80/CD86. CD28 is constitutively expressed on T-cells and can be seen as analogous in function to CD40 on B-cells. The ligands for CD28 are CD80 and CD86 (also known as B7-1 and B7-2). They are members of the immunoglobulin superfamily, which are transiently expressed on activated APC. Forced expression of B7 on tumor cells, which do not normally express it, resulted in enhanced T-cell responses.¹⁴⁹⁻¹⁵²

Bis-(3',5')-Cyclic Dimeric Guanosine Monophosphate (cdiGMP)

In many bacteria bis-(3',5')-cyclic dimeric guanosine monophosphate (cdiGMP) signaling determines the timing and amplitude of complex biological processes, such as biofilm formation, virulence or photosynthesis. Besides its role as an intracellular and intercellular signaling molecule in prokaryotes, cdiGMP also

affects eukaryotes.^{153,154} This interesting molecule inhibits cancer cells *in vitro*, by affecting basal and growth factor-induced cell proliferation.¹⁵⁵ Recent studies have also demonstrated that cdiGMP exhibits potent adjuvant properties. Subcutaneous co-administration of antigens with cdiGMP promotes the elicitation of vigorous antigen-specific humoral and cellular immune responses, which were characterized by a balanced Th1/Th2 response pattern.¹⁵⁶ Similar results were obtained when cdiGMP was used as mucosal adjuvant. However, the IgG1/IgG2a ratio of antigen-specific antibodies and the profiles of secreted cytokines suggested that a dominant Th1 response pattern is promoted when cdiGMP is administered by mucosal route.¹⁵⁷ Intraperitoneal injection of cdiGMP in mice resulted in the local recruitment and activation of monocytes and granulocytes. Human immature DC cultured in the presence of cdiGMP showed increased expression of the co-stimulatory molecules CD80 and CD86, the maturation marker CD83 and MHC class II molecules. IL12, IFN γ , IL8, MCP-1, IFN γ -inducible protein 10 and RANTES were also produced by cdiGMP-treated DC, which exhibited enhanced T-cell stimulatory activity.¹⁵⁸ It seems that the activation of the p38 MAPK in human DC and an ERK phosphorylation in human macrophages may be involved in cdiGMP signaling. However, the underlying signaling events need to be further elucidated.¹⁵⁸

Conclusions

The advent of subunit vaccines rendered obvious the pressing need for developing efficient and safe adjuvants. This is particularly true when mucosal vaccination strategies are coming into consideration, since only a few moieties have been identified so far exhibiting this property. In particular, there is a high demand for adjuvants able to stimulate cellular immunity, which is essential for combating intracellular pathogens and tumors. In this context, it would be helpful to have well-characterized moieties, which are able to exert their biological activity through stimulation of defined cellular targets and/or signaling cascades. This is now possible due to the identification of agonists of PRR present of APC, which are thought to provide a link between the innate and adaptive immune system. An in depth understanding of the underlying mechanisms of action of these moieties, together with the availability of synthetic derivatives with a well-defined structure will certainly reduce the likelihood of undesired side effects. It would be also feasible to choose the most appropriate entity in order to stimulate predictable immune responses, according to the specific clinical needs. Another aspect which requires attention is the minimization of effector functions in untargeted cells. To this end, efforts are being invested to achieve the specific targeting of adjuvants and/or formulations. The targeting encompasses both organ and cell-specific delivery (e.g., local lymph nodes, solid tumors, APC). This is expected to facilitate optimal stimulation with negligible side-effects. Thus, basic research in immunology will continue being the main driving force for innovation in the vaccinology field. Increase predictability for candidates tested in preclinical and clinical studies will facilitate the cost-efficient development of efficient vaccines with an optimal safety profile.

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