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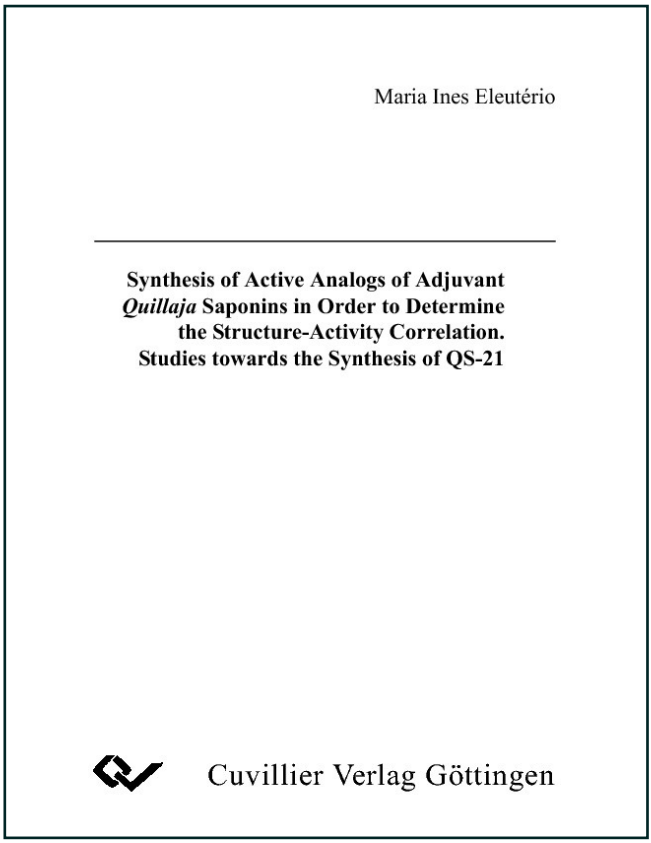
Synthesis of Active Analogs of Adjuvant Quillaja Saponins in Order to Determine the Structure-Activity Correlation. Studies towards the Synthesis QS-21



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1 Introduction and Objectives

1.1 Immune Response to Vaccine Antigens

The immune system is a remarkably adaptive defense system that has evolved in vertebrates to protect them from invading pathogenic microorganisms and cancer. A productive immune response is defined by the generation of clonally expanded antigen-specific T and or B cells, capable of specifically recognizing and eliminating a variety of foreign invaders. This initially requires presentation of the foreign antigen to specific T-cell receptors (TCRs) on naive T cells or to cell membrane bound immunoglobulins (Ig) on B cells.[1,2] Thus, following injection, vaccine antigens may take three different pathways[3] (see Fig. 1.1), which are dependent on the characteristics of the antigen, but may also be influenced by the presence of adjuvants. The antigen taken up by antigen presenting cells (APCs), namely macrophages, monocytes or dendritic cells[5] is processed into peptide epitopes and directed through two pathways to major histocompatibility complex (MHC)[1,2] molecules class I and II, which present the peptide for interaction with either $CD8^+$ or $CD4^+$ T cells, respectively.[1,3] The stimulated T cells then secrete cytokines or costimulatory molecules to upregulate the immune response ($CD4^+$ T cells), or lyse target cells ($CD8^+$ T cells) and hence are termed cytotoxic T lymphocytes (CTLs).[2,5] The latter are required to combat malignant cells or particular intracellular agents.[1,6a] Antibodies, on the other hand, capture and neutralize extracellular pathogens and are produced by B cells with help provided by the cytokines produced by $CD4^+$ T cells.[1,2,3] The latter have two major subsets Th1 and Th2 that can be identified based on their secretion of different cytokine patterns.[2,6a] A Th1 immune response, which is mediated by Th1 helper cells, is characterized by the induction of delayed-type hypersensitivity responses and the secretion of interferon-γ (IFN-γ)[4], interleukin-2 (IL-2), IL-12, tumour necrosis factor-β (TNF-β) and an enhanced production of IgG_{2a}, IgG_{2b} and IgG_3 in mice.[6a]

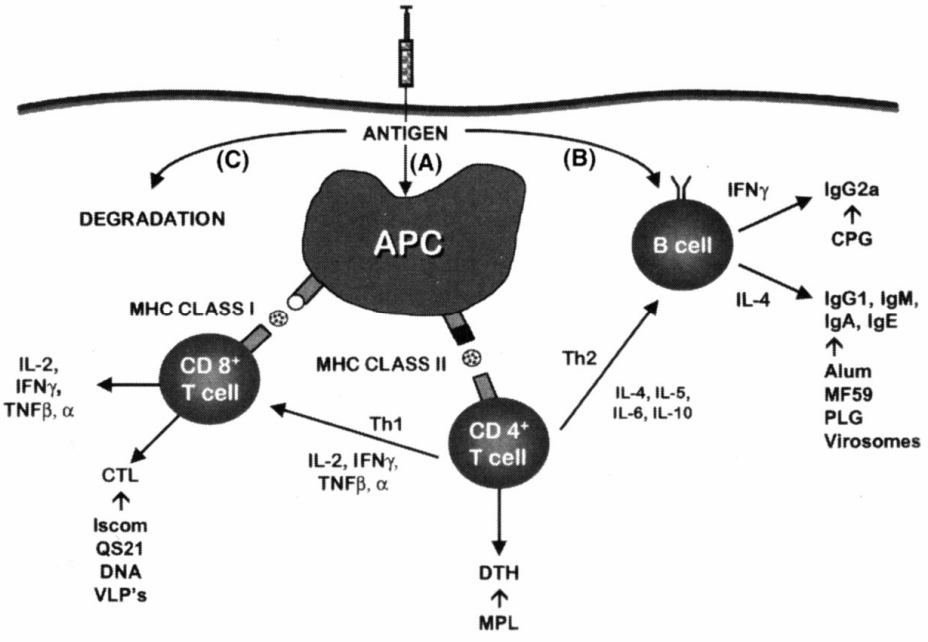

Fig. 1.1: *Simplified representation of the immune response to an injected vaccine antigen. (A) antigen directly taken up by antigen presenting cells (APC), (B) bind to the surface antibody on B cells, (C) undergo degradation.*[3]

A Th2 immune response, mediated by Th2 helper cells, is characterized by the induction of circulating or secretor antibodies and the secretion of IL-4, IL-5, IL-6 and IL-10, and an enhanced production of IgG_1 and secretor IgA.[6a] A Th1 response is a requisite for CTL production, and hence required for protective immunity against intracellular infectious agents, such as certain viruses, bacteria and protozoa, and presumably against cancer cells.[2] On the other hand, Th2 immunity is effective for protection against most bacteria as well as certain viral infections, but ineffective in antibody mediated cell killing.[2,6a]

Experimental vaccines containing antigens produced by recombinant DNA technology[7a,b] and derived from either infectious agents or cancer cells have shown that an effective immune response depends on both the type and the strength of the response. This requires an adequate antigen choice together with an adjuvant capable of optimizing **humoral** and **cell mediated immune responses**.[6a] In general, the humoral immune response acts against extracellular pathogenic microorganisms and toxins, whereas the control of the infections provoked by intracellular pathogenic agents is performed by cell immune responses.[1,6a] The latter type of immune response is therefore crucial for vaccines directed against intracellular pathogens as well as for therapeutic vaccines. Adjuvants have a strong effect on the nature of the immune response, and can bias the immune system toward either Th1 or a Th2 type response.[6a] Different kinds of adjuvants are associated with the main element of the immune response they induce (see Fig 1.1).

1.1.1 T-Dependent Antigens (Humoral Immune Response)

The immunogen in a subunit vaccine is typically a T-dependent antigen (TD), which is a purified protein or peptide containing T cytotoxic (Tc) cell or T helper (Th) cell epitopes that stimulates cytolytic and helper T cell responses when presented on class I or II MHC antigen of APCs, thus expressing either $CD4^+$ or $CD8^+$ markers.[1,8] Although both types of T cells recognize processed protein antigen (Ag), exogenous proteins are usually presented in association with class II MHC Ag, whereas proteins produced by endogenous gene expression, such as viral proteins, are presented with class I MHC Ag.[8]

TD antigens are poorly immunogenic and require a potent adjuvant for maximization of immune responses. *Quillaja* saponins have been found to be potent stimulators of antibody response to TD antigens.[9a] Examples of subunit antigens for which saponin adjuvant augment IgG response in mice include keyhole limpet hemocyanin (KLH), human immunodeficiency virus (HIV)-1 gp120[11a,], and influenza[9b] nucleoprotein.[10b]

1.1.2 T-Independent Antigens (Humoral Immune Response)

T-independent antigens (TI) are non-protein antigens such as nucleic acids, polysaccharides and lipids that lack the peptide moieties required for binding to T cell receptors.[10b] These T independent antigens fail to bind to T lymphocytes but can bind to B cells. They stimulate antibody production in the absence of class II MHC-restricted T cell help[10b] and can be classified into two classes: TI-1 and TI-2. TI-1 antigens (include lipopolysaccharides) are mitogenic for B cells and induce an IgG_{2a} response in mice. TI-2 antigens (include large molecular weight polysaccharides that contain

repeating units) are unable to stimulate class II MHC-dependent T cell help, although T cells may still be involved in stimulating antibody production to these antigens. Generally, non-saponin adjuvants able to stimulate TD antigens failed to stimulate TI antigens, whereas *Quillaja* saponins were able for both TD and TI antigens.[10a,10b]

1.1.3 Cytotoxic T Lymphocyte Response

Protection against viruses and other intracellular pathogens and clearance of infections are associated with induction of antigen specific $CD8^+$ CTLs.[11b,8] These are cells with an effector function for lysis of antigen presenting class I MHC expressing cells.[10b] Endogenous antigen such as a viral antigen synthesized and processed in the cytoplasm (see Fig. 1.2b),[1] induces antigen specific $CD8^+$ CTL after presentation of processed antigen with nascent class I MHC molecules, via a classical class I presentation pathway.[8] Exogenous antigen can also induce $CD8^+$ CTL, although with varying efficiency depending on whether the antigen is soluble or particulate. The latter are known to induce CTL after being phagocytosed by macrophages[10b] or dendritic cells[5,11c]. These phagocytosed particulate antigens may either enter the cytoplasm of the APC cells (see Fig. 1.2a)[1] or undergo a non-classical class I presentation pathway (cross-presentation).[10b,11c] On the other hand, exogenous soluble antigens do not efficiently induce $CD8^+$ CTL, although they induce the alternative response class II MHC Ag-restricted response.[1,8] Hence, subunit vaccines of soluble proteins require novel strategies in order to induce a CTL response, which includes antigen encapsulation into fusogenic proteoliposomes and incorporation of synthetic lipopeptides.[10b] However a highly effective strategy is the use of saponins,[9a] either mixed with the antigen or used as Immunostimulating Complexes (ISCOMs) (see section 1.4).

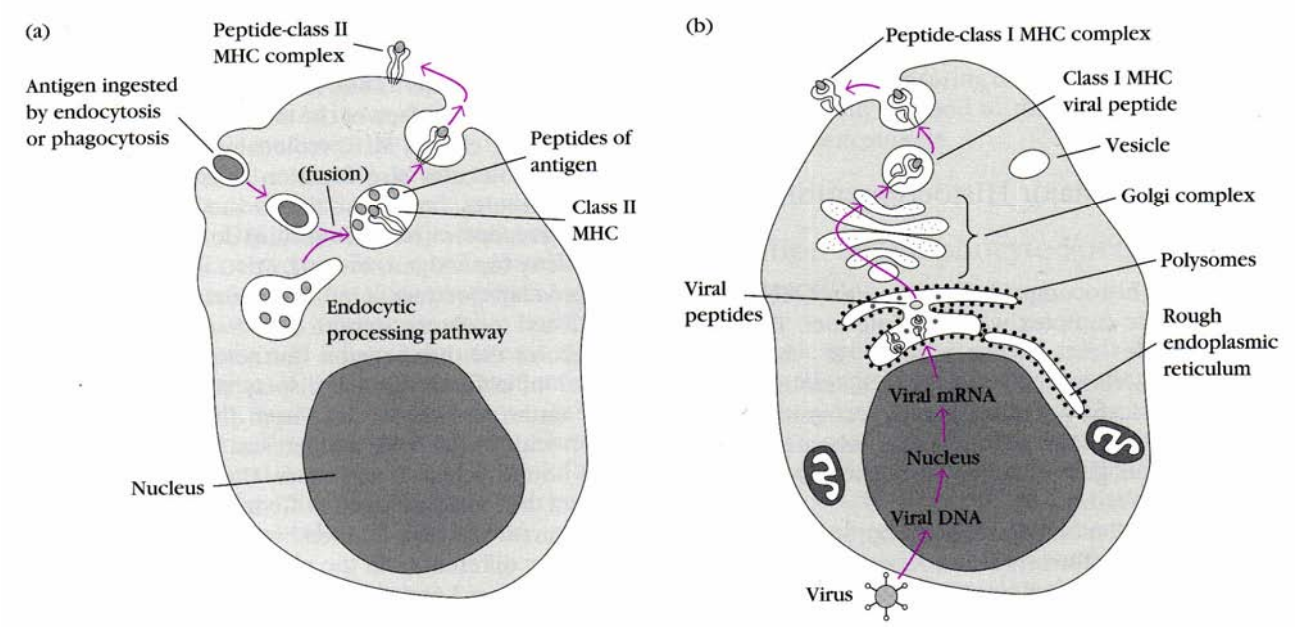

Fig. 1.2: *Processing and presentation of (a) exogenous and (b) endogenous antigens.*[1]

1.2 Vaccine Adjuvants

The successful elimination of pathogens following prophylactic immunization depends to a large extent on the ability of the host's immune system to recognize when it is necessary to become activated and how to respond most effectively, with minimal injury to healthy tissue. The purpose of vaccination is protection of the individual against pathogens, which may require cell-mediated, antibody-mediated, or both responses, depending on the nature of the pathogen.[3] The rational design of vaccines involves identification of the immune effectors mechanisms responsible for protection against disease and the subsequent selection of an antigen able to elicit the desired adaptive response and its delivery to the host's immune system.[2,6a] Vaccines have traditionally consisted of live attenuated, replicating pathogens and non-replicating, inactivated pathogens or their subunits.[3] The live attenuated vaccines, although having proven successful in the past, present several limitations that prevent their use against diseases such as hepatitis C or AIDS:[9a]

- Some live attenuated vaccines can cause disease in immunosupressed individuals by reverting to a more virulent phenotype.
- Whole inactivated vaccines contain reactogenic components that can cause undesirable side effects.
- Some pathogens are difficult or even impossible to grow in culture (e.g. hepatitis B[12a], hepatitis C and human papillomavirus).

On the other hand, non-replicating vaccines, such as subunit of recombinant proteins, synthetic peptides and plasmid DNA offer the advantage of lower toxicity, but are poorly immunogenic when administered alone.[7,9a] Therefore they require immunological adjuvants that are potent, safe and compatible with new generation vaccines, including DNA vaccines, to elicit an adequate immune response.

1.2.1 Classification of Adjuvant Types

Immunological adjuvants (*adjuvare* is Latin for "to help") are a group of structurally heterogeneous compounds that are not immunogenic when administered alone, but induce a stronger immune response, when used in combination with a specific antigen, than the antigen alone would do.[3,9a] In absence of adjuvant a lack of responsiveness may occur and naive antigen-specific T cells may recognize the antigen but become tolerant. The most important issue in adjuvant development is safety, which has restricted their development since aluminium-based mineral salts (alum)[12b] were first introduced more than 50 years ago. The latter are the only adjuvants currently approved by the US Food & Drug Administration.[3] Many other experimental adjuvants have advanced to clinical trials and some have demonstrated high potency, but most have proved too toxic for routine clinical use (complete and incomplete Freund's adjuvants CFA and IFA respectively).[3,9a] Alum, although it has a good safety record, is a weak adjuvant for both antibody induction to protein subunits and cell mediated immunity.[3,9a] A notable limitation is their incapacity to evoke IL-2 and IFN-γ-producing T-helper cells.[13a] Moreover, alum adjuvants can induce immunoglobulin E (IgE) antibody responses[9a] but are ineffective for vaccines against intracellular pathogens, parasites, or cancer cells.[6b]

Additional issues that are important for adjuvant development include biodegradability, stability, ease of manufacture, cost, and applicability to a wide range of vaccines. Ideally, for ease of administration and patient compliance, an adjuvant should allow a vaccine to be given by a mucosal route.[9a] The following adjuvants have been evaluated in clinical trials[3,14] (see Table 1.1).

	Adjuvants	**Mode of action** (see section 1.2.2)
Mineral salts	Aluminum hydroxide	(a), (d)
	Aluminum phosphate	(a), (d)
	Calcium phosphate	(d)
Immunostimulatory adjuvants	Cytokines (IL-2,IL-12, GM-CSF)	(e)
	Saponins (**QS-21**)	(a)
	Muramyl dipeptide (MDP)	(c)
	CpG motifs	(c)
	Lipopolysaccharide (LPS)	(c)
	Monophosphoryl Lipid A (MPL)	(c)
Lipid particles	Emulsions (Freund's, SAF, MF59)	(a)
	Liposomes	(a)
	Virosomes	(a)
	ISCOMs	(a), (c)?
	Cochleates	(a)
Microparticulate adjuvants	PLG microparticles	(b)
	Poloxamer particles	(b)
	Virus-like particles (VPL)	(b)
	Polyphosphazenes	(b)
Mucosal adjuvants	Heat-labile enterotoxin (LT)	
	Cholera toxin (CT)	
	Mutant toxins (LTK63 and LTR72)	
	Microparticles	
	Polymerized liposomes	

Table 1.1: *Examples of vaccine adjuvants.*

With the exception of CpGs, cochleates, and polymerized liposomes, all of the above mentioned adjuvants have been evaluated in clinical trials.[3] However, only the mineral salts and the virosomes are currently included as adjuvants in approved vaccine products for human use.

Although the mechanisms of action of adjuvants are poorly understood, they can be classified according to their principal mode of action.[3,9a,14]

- ***Immunostimulatory adjuvants*** exert their effects predominantly at the cytokine level, either through activation of MHC molecules, through costimulatory signals, or through related intracellular signalling pathways. They can also be included to enhance the level of immune activation or to focus the response through a desired pathway (Th1 or Th2).

- ***Particulate adjuvants*** have comparable dimensions to the pathogen to be combated by the immune system, and are therefore targeted for uptake by APCs to facilitate the induction of potent immune responses.

- ***Microparticle adjuvants*** have the ability to control the rate of release of entrapped antigens to APCs, which may allow the development of single-dose vaccines, thus resulting in improved vaccine compliance.

- ***Mucosal adjuvants*** induce immunity by local immunization at the sites where the majority of pathogens initially establish infection of hosts. Some advantages of vaccines' mucosal administration relatively to intramuscular injection include easier administration, reduced side effects and the potential for frequent boosting.

1.2.2 Mode of Action of Adjuvants

The *in vivo* molecular and cellular mechanisms required for the generation of an effective immune response, which depends critically on co-injection of adjuvant, are still poorly understood. Immunization often activates several complex cascades of immune effectors, only some of which are relevant to the induction of an antigen-specific response. Ascertaining the exact effectors enhanced by a particular adjuvant is often difficult to clearly define *in vivo*. Some concepts of immunogenicity, by which adjuvants can mediate their stimulating effects, were proposed[14] (see Fig. 1.3).

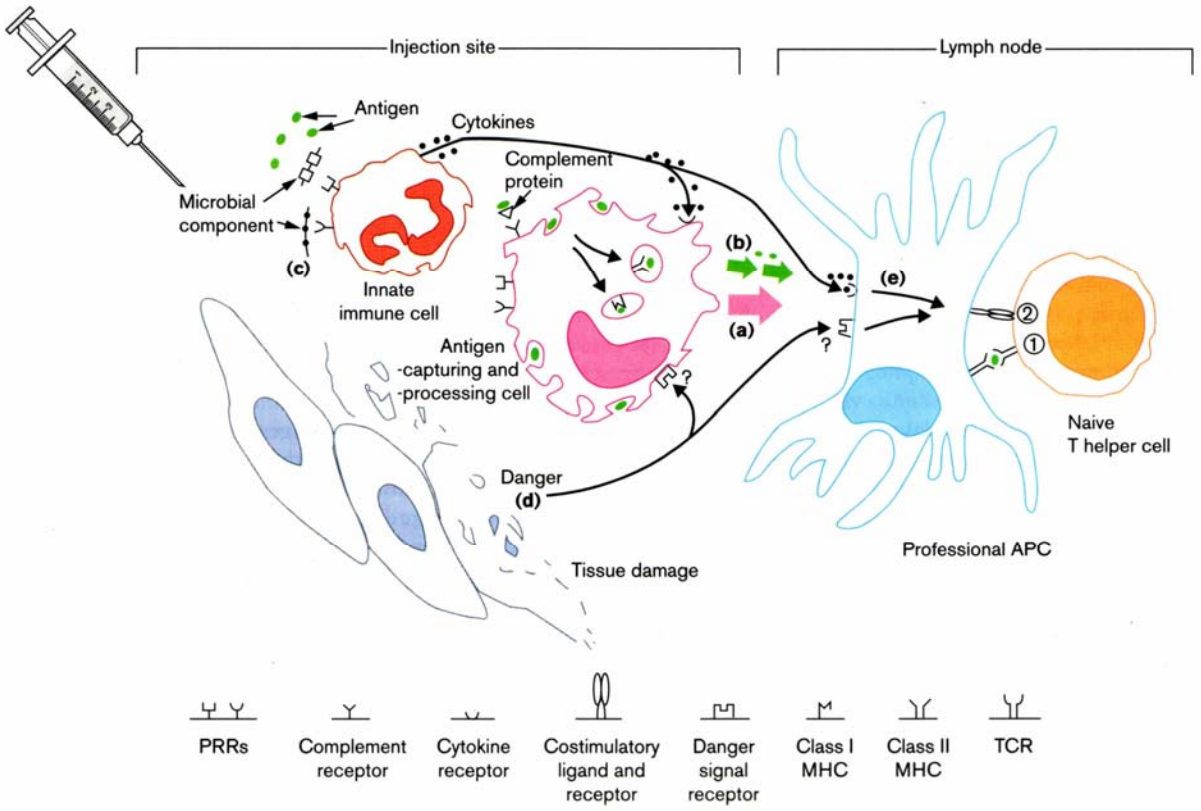

Fig. 1.3: *Essential steps of different concepts of adjuvanticity.*[14]

(a) Uptake and distribution: facilitation of antigen transport, uptake and presentation by antigen-capturing and processing cells in the lymph node draining the vaccine injection site

The induction of an immune response depends on the antigen reaching and being available in lymphoid organs (signal 1),[14] where the initiation of immune responses takes place by interactions between the antigen-loaded APCs and T cells, allowing the completion of T cell dependent immune responses.[14] Naive cells are not effective in entering non-lymphoid areas of the body,[11c] thus the antigen transport, uptake, and presentation by APC in the lymph node is done by antigen-capturing dendritic cells (DCs),[11c,14] which capture antigen either by receptor-mediated endocytosis or fluid-phase pinocytosis.[2,11c] Upon activation they move from the periphery towards the nearest draining lymph node. DC activation occurs by microbial infections, microbial products, stress and vaccine adjuvants.[14] The latter may act preferentially involving the appropriate type of competent DC (professional APC)[11c], able to internalize the antigen, to migrate towards the T cell zone and present antigen to naive T cells (see Fig. 1.2).[1] Thus immunostimulation by adjuvants may result from: increased attraction of DCs towards the injection site; increased loading of APCs; or increased transport of antigen-loaded APCs towards the lymph node. Continued adjuvanticity provided by replicating micro-organisms/vectors is based on provision of sufficient amounts of antigen to the lymphoid tissue, as long as the replication proceeds.[14]

(b) Depot Effect: Repeated or prolonged release of antigen to lymphoid tissues

The prolonged delivery of antigens is important for costimulation-independent immune response. Thus the antigen should trigger T cells in the lymph nodes for a sufficient period of time.[9a] In particular, antigen retention on follicular DCs within the draining lymph node is responsible for stimulating continued antibody production.[9a,14] Thus, as soon as antigen-specific antibodies have been formed as a result of a primary immune response, the persistence of antigen in the lymph node or infection site is an important factor for the duration of the response.[14] Antigen maintenance at the injection site is effectively established by oil-based adjuvants that form a deposit of antigen.[9a] However, after a while the release rate of antigen from the oil-induced injection granuloma declines and becomes insufficient to maintain optimal responses, resulting in excision of the walled-off injection site and ceasing of antibody response formation in the draining lymph node.[14] Aluminium salts, for instance, were demonstrated to dissolve within one hour after injection,[14] leading to resolution of antigen depot, whereas PLG microparticles are effective controlled-release delivery systems.[3,9a]

(c) Signal 0: Signalling of PRRs activates innate immune cells to release cytokines necessary for upregulation of costimulatory molecules

A signal 0 is defined as the recognition of conserved microbial structures, so-called pathogen-associated microbial patterns (PAMPs)[15a]. These PAMPs, representing the signature of potentially noxious substances,[14] are defined by pathogen-recognition receptors (PRRs), which are constitutively expressed on cells from the innate system. They are essential for immunogenicity by generating signal 2 on APCs, which becomes activated and provides the delivery of costimulatory molecules or cytokines to the priming of T helper cells, and their subsequent delivery to antigen-specific help for B cells and CTL effectors. PRRs have been selected over evolutionary time

to recognize microbial structures that are essential for survival of the pathogen but distinct from eukaryotic cell surface structures of the host. Consequently, the immunostimulatory activity of vaccine adjuvants may result from the induction of secondary signals of receptors such as B-7 and DC40 on APCs via direct or indirect steps. Adjuvants based on microbial components such as pertussis toxin[15b], mycobacterium-derived MPD, LPS, lipid A, CPG-rich motifs (see table 1.1), mimicking microbial structures, can be recognized by the phylogenetically ancient PRRs present on accessory cells,[14] and thus stimulate macrophages to produce cytokines. However, this concept is not verified for aluminium, oil or saponin-based adjuvants.

Accordingly, non-infectious virus-like particles or inactivated pathogens can be recognized by PRRs on innate immune cells since they strongly resemble PAMPs. However, in contrast to live virus, they fail to elicit an effective antibody response when administered without adjuvant.[14] The cage-like structures of ISCOMs may also mimic PAMPs and stimulate immune responses efficiently.

(d) Induction or representation of danger molecules: danger signals from stressed or damaged tissues alert the antigen-presenting cells to upregulate costimulatory molecules.

Antigen-capturing APCs become activated when signals from damaged or stressed cells start an immune response, and directly evoke expression of costimulatory molecules on the APC. Danger signals from damaged or infected cells include tissue destruction, necrosis, infection, cell stress, temperature shifts, hypoxia, trauma, mitochondria and heat-shock proteins. Most common adjuvants are recognized as having the ability to cause danger by inducing local reactions at the injection site, thus mimicking danger signals. Accordingly, necrosis of individual muscle fibres and intramuscular oedema in interstitial connective tissue has been observed within hours after vaccination for a number of adjuvants including aluminium hydroxide, calcium phosphate and mineral oils surface active agents.[14] Also, co-administration of ovalbumin with necrotic cells or with freshly isolated and damaged blood vessels increased primary anti-ovalbumin delayed-type-hypersensitivity reactions almost as efficient as complete Freund's adjuvant.

(e) Signal 2: cytokine induction

Inflammatory cytokines produced at the injection site may be essential communicators of adjuvant activity.[14] Their production by activated macrophages or regulatory Th cells is essential for the development of antibody and CTL responses to foreign antigen (see section 1.1). Different profiles of cytokines have been detected in the lymph or lymph nodes from sheep with immunized adjuvants. Crucial cytokines for immunogenicity include the pro-inflammatory IFN-α/β, TNF-α, IL-1, IL-6, IL-12, IL-15, IL-18,[2,14] which influence antigen presentation. Others may act more downstream during clonal expansion and differentiation of T and B cells, with IL-2, IL-4, IFN-γ[3], as prototypes.[14] IL-1 and IL-12 were identified as essential and sufficient signals, together with the TCR (signal 1) and IL-2 (signal 2) to optimally activate naive $CD4^+$ and $CD8^+$ TCR-transgenic T cells, respectively.[14] IL-1 constitutes a second signal for T cell activation provided by APC carrying the first signal, i.e. class II MHC molecules,[1,13b] and upregulates *in vitro* the activity of T helper cells, possibly influencing the balance between different subsets.[13b] Adjuvants known to stimulate *in vitro* IL-1 production are LPS, MPD, CT and ISCOMs.[13b] IL-6 on the other hand, promotes the

differentiation of B lymphocytes into antibody-secreting cells and increases the production of IgG, IgA and IgM,[13c] whereas synergized with IL-1 promotes T cell proliferation and differentiation of T helper cells as well as the development of CTL by $CD8^+$ cells.[13c] *In vitro* studies showed that IL-6 and IL-2 together with the costimulatory molecule B7.1, constitute the minimal requirement for the generation of primary CTL.[13c] Adjuvants may induce the production of the prototypic initiating molecules, IL-12 or IL-6/IL-4, through activation of particular types of innate immune cells or activation of particular receptors on one innate immune cell type.[14]

Efficient vaccination against certain infectious diseases depends on the means to induce T cell responses with desired properties.[9a] Different adjuvants may induce comparable levels of functional antibodies, although the respective cytokine profiles and antibody isotypes may differ.[14] The most appropriate adjuvant for a given vaccine will depend on the type of immune response (Th1 or Th2) that is required for protective immunity. The currently available adjuvants chiefly stimulate a Th2 type immune response, which is frequently ineffective against intracellular pathogens and malignant cells.[6a] The most used adjuvants such as water/oil emulsions and alum elicit only a Th2 immune response, whereas adjuvants such as lipid A and its derivatives (MPL, LPS) are capable of modulating cytokine and IgG isotype profiles characteristic of a Th1 immune response, but are unable to stimulate the production of CTL against soluble or exogenous antigens,[6a] which is essential for the development of effective subunit vaccines against malignant cells or particular infectious agents (see section 1.1). Adjuvants capable of stimulating a Th1 type response[14] and the production of antigen specific CTL are the immunostimulatory triterpenoid glycosides derived from *Quillaja saponaria*.

1.3 *Quillaja* Saponins as Immunoadjuvants

The saponins from *Quillaja saponaria* are derived by aqueous extraction from the cortex of the South American tree *Quillaja saponaria* Molina, a member of the family of Rosaceae.[16] This tree, found in Chile, Peru and Bolivia, was first described in 1782 by *Juan Ignatius Molina*[10b,16] and has remained of special interest because of its bark (soap bark, Panama wood). The generic name is from Chilean word *quillean*, to wash. *Quillaja* bark is known as a saponin crude drug and has been commercially used in the food industry[17] and as a foaming agent ("sapon" = soap) namely as detergent, dentifrice and expectorant[18]. Moreover, the saponin mixture possesses antibiotic, anti-inflammatory and immunoadjuvant activities as well as a plasma cholesterol lowering effect[19a]. The adjuvant activity of saponins has been known at first from the *Espinet's*[19b] inclusion of saponins into veterinary vaccine in 1951. Later *Dalsgaard* analysed saponins from several plant species for adjuvant activity and found that the most potent adjuvants were derived from *Quillaja saponaria.*[20] A comparison of adjuvanticities by *Bomford et al.*[21a], among several saponins extracts from different plant species showed that optimum primary antibody responses were most marked with the *Quillaja* saponins, whereas secondary responses were nearly equivalent for saponins from *Quillaja saponaria* and *Gypsophilla paniculata*, lower but detectable with *Saponaria officialis*, and absent with the other saponins' extracts (soyasaponin, alfalfa, *Chenopodium quinoa*, *Glycyrrhiza radix*).[21a] Because the strongest adjuvant activity was noted with saponins from *Quillaja saponaria*, a considerably higher number of purification methods has been applied to these crude saponins in an attempt to separate the adjuvant active components from the toxic ones. Another approach to overcoming the