Development of nanoparticulate adjuvants based on aluminium salts

Vrieling, H.

Chapter 1

GENERAL INTRODUCTION

INTRODUCTION

Vaccination is one of the most powerful interventions to protect humans against infectious diseases. Administration of a vaccine triggers the immune system aiming to develop a protecting immune response. Next to the development of individual protection as a result of vaccination, herd immunity may protect both non-vaccinated humans and humans with a weak immune system from being infected. This is achieved when a high percentage of the population is immune, either through vaccination or infection. The major success story of vaccination is the worldwide eradication of smallpox in the 1980s [1]. In addition, the number of people infected with numerous diseases, such as diphtheria, pertussis, poliomyelitis and tuberculosis, has been reduced dramatically since the introduction of matching vaccines. To illustrate, vaccination has led to a decrease in disease cases by at least 90%, and in some cases even more than 99% in the US [2].

The first attempts to vaccinate consisted of small amounts of active virus material that was inoculated to humans [3]. The risk of becoming ill because of this inoculation has been reduced by developing vaccines that contain live attenuated or inactivated pathogens. Live attenuated vaccines contain whole bacteria or viruses that have been weakened or altered such that they induce a mild infection without inducing illness or severe complications. These vaccines induce a strong and long-lasting immune response. Inactivated vaccines contain either whole bacteria or viruses that have been killed, or parts of the pathogen, *i.e.* subunits of viral or bacterial material, such as polysaccharides or proteins. Subunit vaccines, sometimes referred to as 'acellular', may contain an inactivated bacterial toxin, i.e. a toxoid, or conjugates of bacterial polysaccharides. Recombinant vaccines are produced by inserting a selection of bacterial or viral DNA into host cells. This DNA may encode a surface protein, which can be expressed in the human body after vaccination to subsequently elicit an immune response. Alternatively, recombinant vaccines may be produced by expressing proteins such as viral surface antigens in host cells, which are extremely immunogenic and result in a very efficacious vaccine.

ADJUVANTS

Purified antigens are often not as effective as inoculation with live attenuated viruses or bacteria. Therefore, additional vaccine components are needed in order to achieve effective vaccines. The term adjuvant is derived from the Latin 'adjuvare', which means 'to help'. Adjuvants help the immune system to evoke a proper immune response against an antigen. Most human licenced subunit vaccines contain adjuvants, *e.g.* aluminium salts, Toll-like receptor (TLR) agonists such as monophosphoryl lipid A (MPL), and oil-in-water emulsions such as MF59 (Table 1). Aluminium salt-based adjuvants were the only licenced adjuvants until the 1980s and are still present in the majority of adjuvanted vaccines (see Table 2).

***Table 1**. Adjuvants in vaccines licenced in the EU. Adapted from [4] and [5].*

Adjuvant	Description	Mechanism of action	References
Aluminium salts	Insoluble salts, *e.g.* phosphates and hydroxides, having an extensive safety record. Being used to develop a second generation adjuvants, *i.e.* nanoparticulate aluminium salts. Antigens are typically adsorbed to the particle surface.	Antigen delivery system, inducer of DAMPs[a], potent inducer of cytokines, chemokines, antibodies and Th2 response.	6-9
MF59	Squalene oil-in-water emulsion adjuvant that has been part of a licensed flu vaccine since 1997. Antigens are not associated with emulsion droplets. Extensive safety record.	Antigen delivery system, enhanced antigen uptake by APCs[b], inducer of Th1/Th2-type immune response.	10-12
AS03	Squalene oil-in-water emulsion adjuvant with the added immune potentiator alpha tocopherol, used in flu vaccines during 2009 pandemic. May be associated with narcolepsy.	Transient induction of cytokines at the site of injection, recruitment of granulocytes and monocytes, increased influx of antigen-loaded monocytes in draining lymph nodes.	13-16
AS04	Combination of aluminium adjuvant with the TLR4[c] agonist MPL[d] co-adsorbed. Approved as a licensed HPV[e] vaccine (Cervarix).	Transient induction of cytokines at the site of injection recruitment of granulocytes and monocytes, increased influx of antigen-loaded monocytes in draining lymph nodes, stimulate APCs[b]	17
Virosomes	Influenza virus envelopes reconstituted in phoshatidylcholine bilayers (virosomes).	Antigen delivery system, depot effect, strong inducer of antigen-specific antibody and Th1/Th2-type immune response.	18,19

[a] *Damage-associated molecular patterns*
[b] *Antigen-presenting cells*
[c] *Toll-like receptor 4*
[d] *Monophosphoryl lipid A*
[e] *Human papillomavirus*

Table 2. Overview of currently licenced adjuvanted vaccines in the US and Canada. Extracted from VIOLIN [20, 21].

	Trade name	Composition	Used in	Target disease(s)	Type of vaccine
Aluminium salts	Alhydrogel®	Aluminium oxyhydroxide	Avaxim®	Hepatitis A	Inactivated or "killed"
	Alhydrogel®	Aluminium oxyhydroxide	Bexsero®	Meningitis	Subunit
	Alhydrogel®	Aluminium oxyhydroxide	Biothrax®	Antrax	Subunit
	Alhydrogel®	Aluminium oxyhydroxide	Certiva®	Diphtheria, tetanus, whooping cough	Toxoid
	Alhydrogel®	Aluminium oxyhydroxide	Comvax®	Meningitis	Conjugate
	Alhydrogel®	Aluminium oxyhydroxide	Engerix-B®	Hepatitis B	Subunit
	Alhydrogel®	Aluminium oxyhydroxide	Fsme-immun®	Tick-borne Encephalitis	Inactivated or "killed"
	Alhydrogel®	Aluminium oxyhydroxide	Havrix®	Hepatitis A	Inactivated or "killed"
	Alhydrogel®	Aluminium oxyhydroxide	Infanrix®	Diphtheria, meningitis, tetanus	Toxoid
	Alhydrogel®	Aluminium oxyhydroxide	Infanrix/Hib®	Diphtheria, meningitis, tetanus, whooping cough	Subunit
	Alhydrogel®	Aluminium oxyhydroxide	Infanrix-IPV®	Diphtheria, polio, tetanus, whooping cough	Subunit + inactivated or "killed"
	Alhydrogel®	Aluminium oxyhydroxide	Infanrix-IPV/Hib®	Diphtheria, meningitis, polio, tetanus, whooping cough	Subunit + inactivated or "killed"
	Alhydrogel®	Aluminium oxyhydroxide	Ixiaro®	Japanese encephalitis	Inactivated or "killed"
	Alhydrogel®	Aluminium oxyhydroxide	Kinrix®	Diphtheria, polio, tetanus, whooping cough	Inactivated or "killed"
	Alhydrogel®	Aluminium oxyhydroxide	Menjugate®	Meningitis	Conjugate
	Alhydrogel®	Aluminium oxyhydroxide	Neisvac-C®	Meningitis	Conjugate

	Trade name	Composition	Used in	Target disease(s)	Type of vaccine
Aluminium salts	Alhydrogel®	Aluminium oxyhydroxide	Pediarix®	Diphtheria, hepatitis B, polio, tetanus, whooping cough	Toxoid
	Alhydrogel®	Aluminium oxyhydroxide	PedvaxHIB®	Meningitis	Conjugate
	Alhydrogel®	Aluminium oxyhydroxide	Recombivax HB®	Hepatitis B	Subunit
	Alhydrogel®	Aluminium oxyhydroxide	ViVaxim®	Hepatitis A, salmonellosis	Subunit + inactivated or "killed"
	Alhydrogel® + AdjuPhos®	Aluminium oxyhydroxide and aluminium phosphate	Boostrix-Polio®	Diphtheria, polio, tetanus, whooping cough	Toxoid + subunit + inactivated or "killed"
	Alhydrogel® + AdjuPhos®	Aluminium oxyhydroxide and aluminium phosphate	PedvaxHIB®	Meningitis	Conjugate
	Alhydrogel® + AdjuPhos®	Aluminium oxyhydroxide and aluminium phosphate	Infanrix-hexa®	Diphtheria, hepatitis B, meningitis, polio, tetanus, whooping cough	Subunit + inactivated or "killed"
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Actacel®	Diphtheria, meningitis, tetanus, whooping cough	Subunit
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Adacel®	Diphtheria, tetanus, whooping cough	Toxoid
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Adacel-Polio®	Diphtheria, polio, tetanus, whooping cough	Toxoid
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Boostrix®	Diphtheria, tetanus, whooping cough	Toxoid + subunit + inactivated or "killed"
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Daptacel®	Diphtheria, tetanus, whooping cough	Toxoid
	AdjuPhos®	Aluminium oxyhydroxyphosphate	DT Polio Adsorbed®	Diphtheria, polio, tetanus	Subunit + inactivated or "killed"

	Trade name	Composition	Used in	Target disease(s)	Type of vaccine
Aluminium salts	AdjuPhos®	Aluminium oxyhydroxyphosphate	Intanza®	Influenza	Inactivated or "killed"
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Meningitec®	Meningitis	Subunit
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Pediacel®	Diphtheria, meningitis, polio, tetanus, whooping cough	Subunit + inactivated or "killed"
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Pentacel®	Diphtheria, meningitis, polio, tetanus, whooping cough	Subunit + inactivated or "killed"
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Prevnar 13®	Pneumonia	Conjugate
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Quadracel®	Diphtheria, polio, tetanus, whooping cough	Subunit + inactivated or "killed"
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Rabies Vaccine Adsorbed (RVA)®	Rabies	Inactivated or "killed"
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Synflorix®	Pneumonia	Conjugate
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Td Adsorbed®	Diphtheria, tetanus	Toxoid
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Td Polio Adsorbed®	Diphtheria, polio, tetanus	Subunit + inactivated or "killed"
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Tenivac®	Diphtheria, tetanus	Toxoid

	Trade name	Composition	Used in	Target disease(s)	Type of vaccine
Aluminium salts	AdjuPhos®	Aluminium oxyhydroxyphosphate	Tetanus and Diphtheria Toxoids Adsorbed®	Diphtheria, tetanus	Toxoid
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Tetanus Toxoid Adsorbed®	Tetanus	Toxoid
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Tripacel®	Diphtheria, tetanus, whooping cough	Subunit
	AdjuPhos®	Aluminium oxyhydroxyphosphate	Trumenba®	Meningitis	Subunit
	Aluminium potassium sulfate adjuvant (alum)	Aluminium potassium sulfate	Decavac®	Diphtheria, tetanus	Toxoid
	Aluminium potassium sulfate adjuvant (alum)	Aluminium potassium sulfate	Diphtheria & Tetanus Toxoids Adsorbed®	Diphtheria, tetanus	Toxoid
	Aluminium potassium sulfate adjuvant (alum)	Aluminium potassium sulfate	Tripedia®	Diphtheria, tetanus, whooping cough	Toxoid
	AAHS	Amorphous aluminium hydroxyphosphate sulfate	Gardasil®	HPV infection	Subunit
	AAHS	Amorphous aluminium hydroxyphosphate sulfate	Vaqta®	Hepatitis A	Inactivated or "killed"

	Trade name	Composition	Used in	Target disease(s)	Type of vaccine
Emulsions	AS03®	Squalene-based oil-in-water emulsion	Arepanrix H1N1®	Influenza	Inactivated or "killed"
	AS03®	Squalene-based oil-in-water emulsion	Influenza A (H5N1) virus monovalent vaccine, adjuvanted®	Influenza	Inactivated or "killed"
	MF59®	Squalene-based oil-in-water emulsion	Aflunov®	Influenza	Inactivated or "killed"
	MF59®	Squalene-based oil-in-water emulsion	Fluad®	Influenza	Inactivated or "killed"
	MF59®	Squalene-based oil-in-water emulsion	Focetria®	Influenza	Inactivated or "killed"
	-	Virosomes	Epaxal®	Hepatitis A	Inactivated or "killed"
LPS derivative	AS04®	Monophosphoryl Lipid A adsorbed to aluminium salt	Cervarix®, Fendrix®	HPV infection	Inactivated or "killed"

IMMUNE RESPONSE UPON VACCINATION

The onset of an immune response starts with recognition of molecular patterns that are present in pathogens (pathogen-associated molecular patterns (PAMPs)) or induced by tissue damage (damage-associated molecular patterns (DAMPs)). The NOD-like receptor (NLR)-family member NLRP3 (also known as NALP3) is a cytoplasmic pattern recognition receptor that recognises PAMPs and DAMPs, such as components of a bacterial cell membrane, bacterial toxins, ATP, uric acid crystals, silica, asbestos, and aluminium adjuvants [22]. NLRP3 is present on the surface of innate immune cells. Upon recognition of PAMPs or DAMPs, NLRs get activated and induce the cleavage of pro-IL-1ß into active IL-1ß. Secretion of this cytokine triggers the recruitment and activation of other immune cells, amplifying the inflammatory response [23].

Immune responses are generally categorised into non-specific innate and specific adaptive immune responses. Although cellular and humoral immunity are often associated with adaptive immunity, also the innate immune response can be subdivided into innate and adaptive immunity [24-26] (Figure 1). Cellular immunity comprises an immune response mediated by immune cells, such as antigen-presenting cells (APCs), B cells and T cells. Humoral immunity is provided by proteins and enzymes, such as cytokines (secreted by APCs and T cells from the cellular immune system) and antibodies (secreted by B cells in the humoral immune response). As the name suggests, the innate immune system consists of cells and proteins that are already present in the body before an infection occurs and are ready to fight pathogens. Its main components are physical epithelial barriers, phagocytic leukocytes, dendritic cells, natural killer cells, and circulating plasma proteins, *i.e.* the complement system. Innate immune responses form the first line of general defence and can respond quickly to an invading pathogen. The adaptive immune system is activated later and responds more specifically to the pathogen. Cells involved in the adaptive immune response include B and T lymphocytes, amongst others. Antibodies produced by B lymphocytes mediate the specific humoral immunity, while the cells themselves mediate cellular immunity. The innate immune system plays a critical role in initiating and directing an adaptive immune response. Both the innate and adaptive immune system are required for an effective immune response to an infection or immunisation.

Vaccine adjuvants enhance the adaptive immune response after immunisation by the activation of innate immune cells that in turn provide signals for activation of lymphocytes [27]. Upon vaccination, APCs, such as monocytes and dendritic cells, that are present at the site of injection internalise the antigen, whether or not adsorbed to an adjuvant, and present the processed antigen to T cells. This leads to the induction of a cellular and/or a humoral immune response [28]. All licenced adjuvants augment the immune response by activating APCs and inducing pro-inflammatory cytokines, albeit via different mechanisms. LPS derivatives activate the immune system via

TLR4, which is a transmembrane pattern recognition TLR that recognises PAMPs. After a signalling cascade via adaptor molecules including TIR-containing adaptor protein (TIRAP), myeloid differentiation primary response gene 88 (MyD88), TRIF-related adaptor molecule (TRAM) and Toll/IL-1 receptor (TIR)-domain-containing adapter protein inducing IFN-ß (TRIF), proinflammatory cytokines such as IL-1ß are secreted [29, 30]. The oil-in-water emulsion MF59 triggers activation of cells at the site of injection, inducing a local chemokine-driven gradient that recruits immune cells [31]. In addition, MF59 induces the release of extracellular ATP from the muscle that may serve as endogenous danger signal 10. The traditional pathogen detection systems (TLRs that signal through the MyD88 or TRIF adaptor pathways) are not required for the adjuvant effect of aluminium salt-based adjuvants [32]. Instead, in the case of aluminium salt-based adjuvants, NLRs are able to recognise PAMPs and other endogenous danger signals [33].

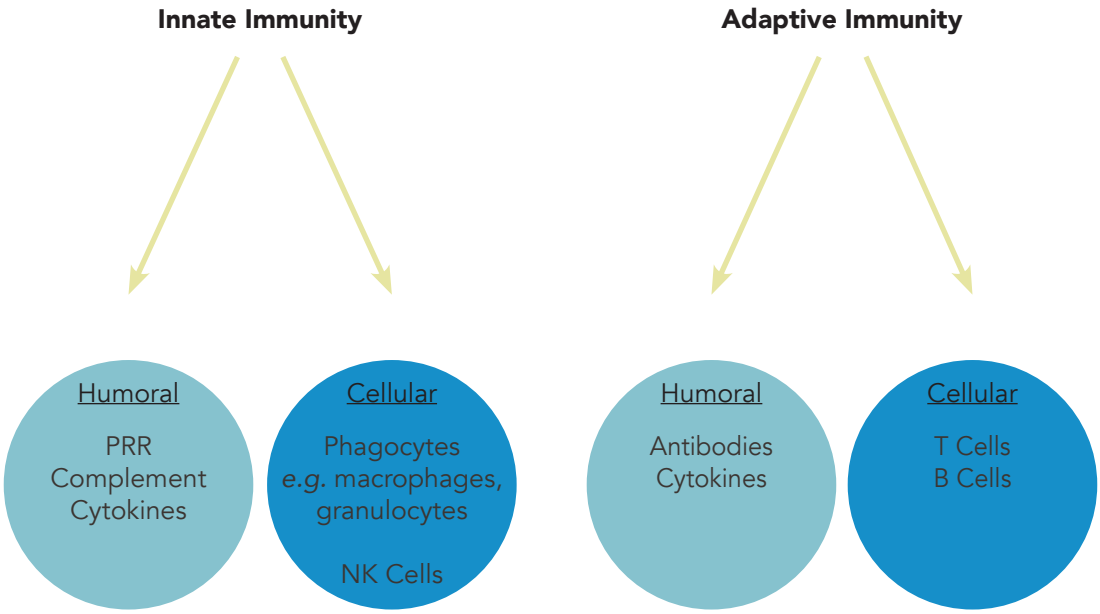


Figure 1. *Both the innate and adaptive immunity consist of a cellular as well as a humoral immune response.*

DEVELOPMENT AND MECHANISM OF ACTION OF ALUMINIUM SALT-BASED ADJUVANTS

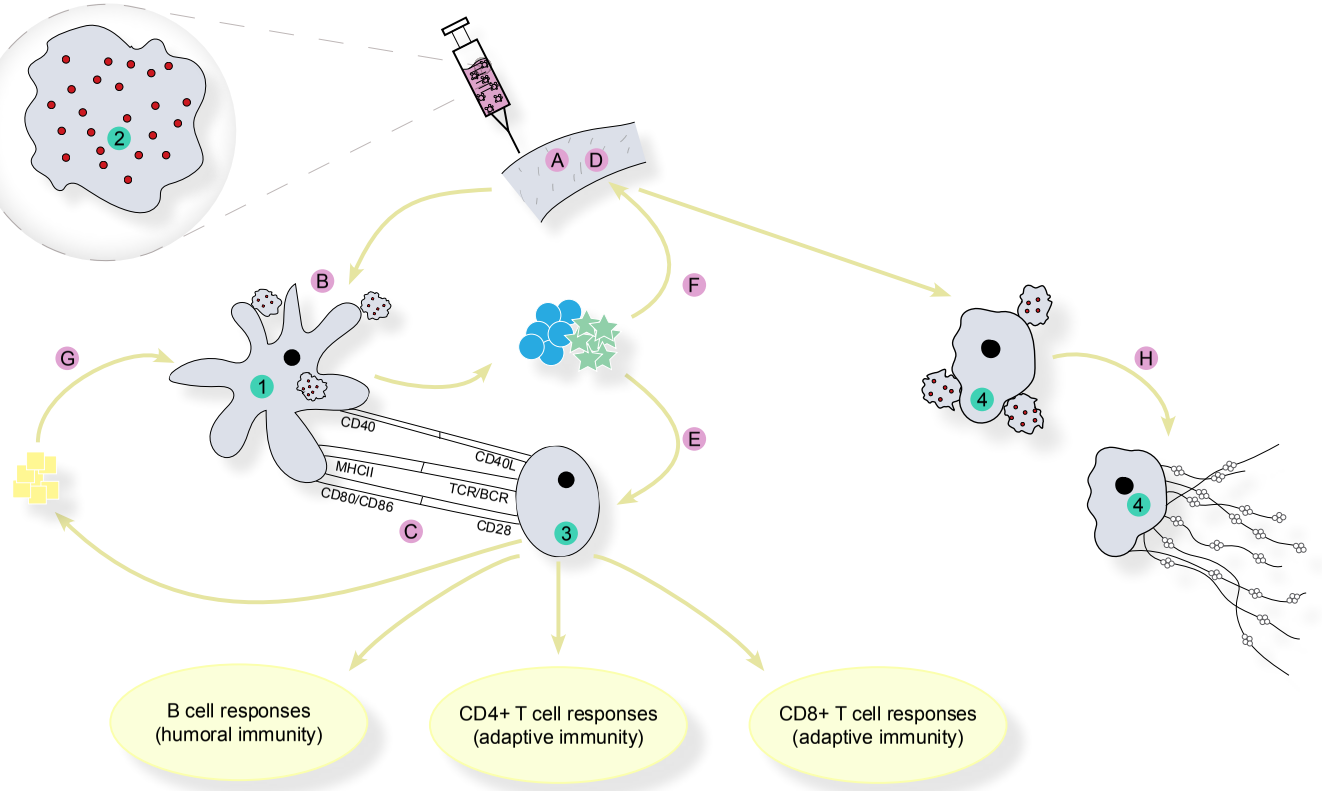
The use of an aluminium salt-based adjuvant was first reported in 1926 by Glenny *et al.*, who described that antibody titres of guinea pigs that were immunised with diphtheria toxoid were increased upon precipitation of the toxoid with 'potassium alum' [34], which is in fact a hydrated form of potassium aluminium sulphate. Since then, the use of aluminium salts as vaccine adjuvant has been further explored, ultimately leading to insoluble aluminium salts being the most commonly used

vaccine adjuvant to date [35]. Currently, two types of aluminium salts are being used as adjuvant in human vaccines: aluminium hydroxide and aluminium phosphate. Aluminium hydroxide originally exists of poorly crystalline aluminium oxyhydroxide particles with average dimensions of 4.5 x 2.2 x 10 nm [36], while aluminium phosphate exists as amorphous aluminium oxyhydroxyphosphate particles of 10-50 nm [37]. These insoluble aluminium salts form aggregates of up to a few micrometres upon dispersion in water [38]. The physical and chemical characteristics of these complex aggregates are not well defined.

Upon their discovery, it was suggested that colloidal aluminium salts might enhance immune response due to a depot effect, causing a slow release of the antigen which is adsorbed to the adjuvant [34] (Figure 2). However, Mendez *et al*. reported that antigen adsorption to an adjuvant did not increase antibody titres, compared to vaccines where no adsorption had occurred in mice [7]. Likewise, HogenEsch *et al*. suggested that sustained release of antigen from a depot is not beneficial for the adjuvant activity of aluminium salts. Instead, they suggested that antigen adsorption to an adjuvant is related to a high local concentration of antigen at the injection site, which may improve antigen uptake by APCs [39]. Other scientists suggested that aluminium salt-based adjuvants activate the complement system [40], enhance cellular uptake and antigen presentation [41, 42], and promote the secretion of the pro-inflammatory cytokine IL-1ß by monocytes and dendritic cells via activation of the NLRP3 inflammasome [8]. In addition, inflammasome activation induces the secretion of the pro-inflammatory cytokine IL-18. IL-1ß and IL-18 trigger the recruitment of APCs, which ultimately contributes to an increased immune response. In addition, IL-1ß and IL-18 activate Th2 cells. These cells release IL-4, which induces an increase in the expression of MHC class II molecules on APCs [9, 39, 43]. The presence of aluminium salts not only triggers the recruitment of APCs to the site of injection, but also that of neutrophils. The latter form neutrophil extracellular traps, which play a significant role in the adjuvant activity of aluminium salt-based adjuvants by providing cellular DNA [44]. This host DNA drives antigen-specific T cell responses and B cell responses and enhances antigen presentation by dendritic cells by prolonging the interactions between T cells and dendritic cells [45, 46].

In addition to the direct adjuvant effect of aluminium salts themselves, the salts also trigger the activation of immune cells indirectly (Figure 2). Aluminium salt-based adjuvants induce necrotic cell death of macrophages and neutrophils *in vitro* [47] and *in vivo* [48, 49], which leads to the release of DAMPs such as DNA [45, 46], uric acid [50] and ATP [51]. In addition, aluminium-containing adjuvants induce oxidative stress, which is also a trigger for the production of uric acid. When co-administrated to protein antigens, crystals of uric acid show adjuvant properties via activation of the NLRP3 inflammasome in a phagocytosis-dependent manner through destabilisation of the phagosome and/or reactive oxygen species (ROS) generation [52].

Figure 2. *Mechanism of action of aluminium salt-based adjuvants.*



A - Depot effect
B - Enhanced antigen uptake
C - Enhanced antigen presentation
D - Cellular damage and cell death inducing DAMPs (DNA, uric acid, ATP)
E - Activation of Th2 cells
F - Activation and recruitment of APCs
G - Increased MHCII expression
H - Formulation of neutrophil extracellular traps, providing host cell DNA

1 - APC (e.g. macrophage, monocyte, dendritic cell)
2 - Colloidal aluminium salt
3 - Lymphocyte
4 - Neutrophil

- IL-4
- IL-1β
- IL-18

ALUMINIUM SALT-BASED ADJUVANTS IN VACCINE FORMULATIONS

The interactions between aluminium adjuvants and antigens are critical for antigen adsorption (Figure 3). Aluminium salt-based adjuvants interact with antigens via hydrophobic and van der Waals forces, via electrostatic attraction and by ligand exchange. Hydrophobic and van der Waals forces are too weak to allow for antigen adsorption. Electrostatic interactions are strong enough to facilitate antigen adsorption in the formulation. As a general guideline, optimal antigen adsorption is obtained in the pH interval between the isoelectric point (IEP) of the antigen, *i.e.* the pH of a solution at which the net surface charge of a protein is zero as obtained by electrokinetic measurements, and the point of zero charge (PZC) of the adjuvant, *i.e.* the pH of a solution at which the net charge of a particle is zero as obtained by acid-base titration. Within this interval, the adjuvant and the antigen will have opposite electrical charges, facilitating adsorption. Ligand exchange, such as the exchange of functional chemical groups, is the strongest adsorption force between adjuvant and antigen. For example, phosphorylated antigens adsorb very firmly to aluminium hydroxide via ligand exchange: the phosphate groups on the surface of phosphorylated antigens displace surface hydroxy groups on aluminium hydroxide. The more phosphate groups on a molecule, the higher the affinity for aluminium hydroxide and the slower the release of the antigen. However, it is possible that tight binding of antigens onto aluminium-containing adjuvants significantly reduces the amount of antigen that can elute from the aluminium salts *in vitro*, resulting in a weak antibody response *in vivo* [53-55].

Factors that influence antigen adsorption, and potentially vaccine efficacy, include solution pH, presence of salt, presence of phosphate in solution, and consistency in aluminium adjuvant quality during the manufacturing process [56]. The characteristics of adsorption of proteins to surfaces can be described by the Langmuir isotherm, which is based on 1) monolayer adsorption, 2) no interactions between the adsorbed molecules, and 3) identical adsorption sites. The isotherm can be used to determine the adsorption capacity, which is the amount of protein adsorbed at a monolayer coverage, and the adsorption coefficient, which is a measure of the affinity of the adsorbing solute for the surface. Competing proteins that have opposite charge can also bind to the adsorbed proteins, so that two or more layers are adsorbed onto the surface of the adjuvant. This alters the surface charge of the adjuvant-antigen complex, which may affect its efficacy. Noteworthy, it may not be necessary to adsorb an antigen to the adjuvant: aluminium adjuvants can also stimulate the immune response to non-adsorbed antigens [7, 57]. However, even a small adsorbed amount of antigen may affect vaccine efficacy. The effect of antigen adsorption to an adjuvant on the efficacy of a formulation is thus still debatable.

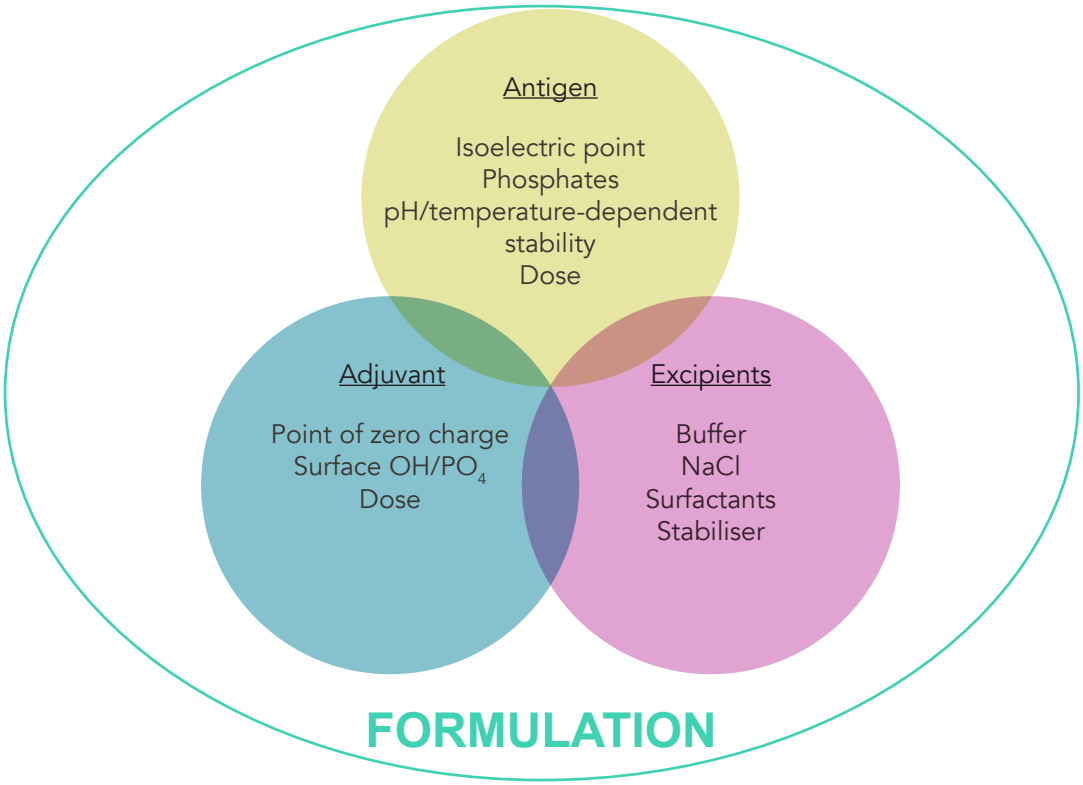


Figure 3. *Formulation of a vaccine with aluminium adjuvants requires knowledge of the chemical and physical properties of both the adjuvant and the antigen, and rigorous characterisation of the antigen and antigen–adjuvant formulation.*

NEW GENERATION ALUMINIUM-SALT BASED ADJUVANTS

Current aluminium-salt based adjuvants induce (minor) side effects at the site of injection, such as redness and swelling. Also, immunisation with vaccines containing aluminium salt-based adjuvants is related to higher IgE levels, which is correlated with local reactions and allergy [58]. Ironically, allergy vaccines are typically adjuvanted with a colloidal aluminium salt [59]. Moreover, aluminium salt-based adjuvants generally stimulate a Th2-related immune response, while a more balanced Th1/Th2 response may be more advantageous [60]. In addition, aggregates of aluminium salt-based adjuvants are too big to be sterilised by filtration and therefore require autoclavation or irradiation. Current aluminium salt-based adjuvants are sterilised by autoclavation, which affects the adjuvant characteristics by reducing their surface area [61]. Improvement of the traditional aluminium salt-containing adjuvants is thus desired.

A new generation of aluminium salt-based adjuvants may improve vaccine efficacy and reduce encountered side effects, such as redness and swelling. The physicochemical properties of nanoparticles modulate their cellular uptake and the immune response they may (help to) elicit. These properties include size [62-64], shape [65], surface charge [66] and agglomeration state [67, 68]. In this regard, nanoparticles are emerging as promising adjuvant candidates. Nanoparticles are of particular interest because they may facilitate manufacturing of vaccines, as these particles can be homogeneously produced. However, the substitution of current aluminium salt-based adjuvants, which form aggregates of a few micrometres upon dispersion, with its nanoparticulate form may entail several formulation challenges.

IMMUNE RESPONSE UPON VACCINATION

Classical aluminium salt-based adjuvants are obtained via bottom-up precipitation of aluminium salts that form aggregated microparticles, referred to as hydrated gels [69]. Aggregate formation in these hydrated gels is difficult to control, resulting in batch-to-batch variation [70, 71]. Other synthesis methods, such as hydrothermal treatment (treatment of a solution containing aluminium salts at high pressure and high temperature), after which stable nanoparticles can be obtained, may not encounter this disadvantage [72]. Besides these bottom-up approach, *i.e.* synthesis, also a 'top-down approach' (i.e. reducing the size of bigger aggregates) can be used to obtain nanoparticles of mineral salts. However, this entails the risk of re-aggregation towards the size of the original aggregates. The advantages and disadvantages of the top-down and bottom-up approaches are summarised in Table 3.

Table 3. *Comparison of top-down and bottom-up synthesis of nanoparticles.*

	Top-down approach	Bottom-up approach
Definition	Particle size reduction of large particles into smaller particles	Start with a homogenous solution in which nanostructures are synthesised by stacking molecules onto each other, creating crystal planes; these crystal planes also stack onto each other, resulting in nanostructures
Techniques	Various wet milling techniques such as media milling, microfluidisation, high pressure homogenisation, ultrasonication	Supercritical fluid processes, spray drying, emulsion-solvent evaporation, hydrothermal treatment, precipitation
Advantage	• Relatively easy to produce	• Growth can be controlled and tailored towards a preferred size and/or shape • Cost-effective
Disadvantage	• High energy input required • Highly inefficient • Considerable amount of heat generated, thus difficult to process thermolabile materials • Mechanic stress may induce crystal defects, generation of amorphous regions • Poor control of particle size and shape	• Often involves harsh solvents • Residual solvent is hard to remove and can cause physical and chemical instability of the formulation

The physicochemical properties of nanoparticles, such as point of zero charge (PZC), surface molecules and specific surface area, may differ from those of microparticles composed of the same chemical substances. These properties influence, amongst others, the extent and strength of antigen adsorption, residence time at the injection site and biodistribution. Consequently, the immunogenicity of vaccines containing nanoparticles may be altered from that of vaccines containing microparticles.

THESIS SCOPE AND OUTLINE

The aim of this thesis was to develop aluminium salt-based nanoparticles that may ultimately be used as adjuvant in human vaccines. Compared to traditional aluminium salt-based adjuvants, nanoparticles may improve vaccine efficacy and reduce encountered side effects. Nanoparticles were synthesised and their physicochemical properties were investigated. Their immunostimulating properties were investigated *in vitro* and *in vivo* and compared to traditional aluminium salt-based adjuvants.

In ***Chapter 2***, the effects of two commercially available aluminium salt-based adjuvants on the innate immune response were compared *in vitro* and *in vivo*. The proteome of human primary monocytes that were incubated with either aluminium hydroxide or aluminium phosphate was analysed to determine the immunological pathways activated by these adjuvants. The site of injection was investigated *in vivo* by analysis of immune cells that were present at the site of injection and proteome analysis of the injected muscle tissue.

In ***Chapter 3***, aluminium phosphate nanoparticles were made by using a top-down approach. A commercially available micro-sized aluminium phosphate adjuvant was sonicated and the effect of several potential stabilisers on the colloidal stability of the nanoparticles was studied. Particular amino acids, *i.e.* arginine, asparagine, aspartic acid, threonine and L-alanyl-L-1-aminoethylphosphonic acid, were selected to further investigate their effect on the stability of aluminium phosphate nanoparticles. In addition, the immunogenicity of diphtheria toxoid adjuvanted with a combination of one of the above-mentioned amino acids and aluminium phosphate nanoparticles was assessed in mice.

Two aluminium (oxy)hydroxide nanoparticles with different shapes, *i.e.* hexagonal-shaped gibbsite and rod-shaped boehmite, were made by using a bottom-up approach in ***Chapter 4***, the activation of human monocytes by gibbsite and boehmite was investigated by analysing the transcriptome and proteome of the monocytes. In addition, the effects of gibbsite and boehmite on the cellular maturation, differentiation, activation and cytokine secretion of human monocytes was investigated. The effects of the nanoparticles were compared to that of classical aluminium oxyhydroxide adjuvant.

Aluminium salts are used in combination with LPS derivatives as adjuvant system in human vaccines (*e.g.* Cervarix). A novel combination of LPS derivatives and aluminium salts as potential adjuvant is described in ***Chapter 5***. A commercially available aluminium hydroxide adjuvant and the two synthesised nanoparticulate aluminium salts, *i.e.* gibbsite and boehmite, were combined with either ΔLpxL1 or ΔLpxL1-PagL and investigated for their adjuvant effect. The immunogenicity of diphtheria toxoid adjuvanted with one of the novel combinations was investigated

in vivo. The activation of TLR4 was investigated *in vitro*.

The results and conclusions or this thesis are summarised in ***Chapter 6***. In addition, the opportunities for using aluminium salt-based nanoparticles as vaccine adjuvants are discussed.

REFERENCES

1. (WHO), W. H. O. The global eradication of smallpox. (1979).
2. Orenstein, W. A. & Ahmed, R. Simply put: Vaccination saves lives. *Proceedings of the National Academy of Sciences of the United States of America* **114**, 4031-4033, doi:10.1073/pnas.1704507114 (2017).
3. Plotkin, S. A. *History of vaccine development.* (Springer Science & Business Media, 2011).
4. Brito, L. A. & O'Hagan, D. T. Designing and building the next generation of improved vaccine adjuvants. *J Control Release* **190**, 563-579, doi:10.1016/j.jconrel.2014.06.027 (2014).
5. Sarkar, I., Garg, R. & van Drunen Littel-van den Hurk, S. Selection of adjuvants for vaccines targeting specific pathogens. *Expert review of vaccines* **18**, 505-521, doi:10.1080/14760584.2019.1604231 (2019).
6. Riteau, N. *et al.* ATP release and purinergic signaling: a common pathway for particle-mediated inflammasome activation. *Cell Death Dis* **3**, e403, doi:10.1038/cddis.2012.144 (2012).
7. Romero Mendez, I. Z., Shi, Y., HogenEsch, H. & Hem, S. L. Potentiation of the immune response to non-adsorbed antigens by aluminum-containing adjuvants. *Vaccine* **25**, 825-833, doi:10.1016/j.vaccine.2006.09.039 (2007).
8. Kool, M. *et al.* Cutting Edge: Alum Adjuvant Stimulates Inflammatory Dendritic Cells through Activation of the NALP3 Inflammasome. *The Journal of Immunology* **181**, 3755-3759, doi:10.4049/jimmunol.181.6.3755 (2008).
9. Ulanova, M., Tarkowski, A., Hahn-Zoric, M. & Hanson, L. A. The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. *Infect Immun* **69**, 1151-1159, doi:10.1128/IAI.69.2.1151-1159.2001 (2001).
10. Vono, M. *et al.* The adjuvant MF59 induces ATP release from muscle that potentiates response to vaccination. *Proc Natl Acad Sci U S A* **110**, 21095-21100, doi:10.1073/pnas.1319784110 (2013).
11. Calabro, S. *et al.* Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. *Vaccine* **29**, 1812-1823, doi:10.1016/j.vaccine.2010.12.090 (2011).
12. Ciabattini, A. *et al.* Modulation of Primary Immune Response by Different Vaccine Adjuvants. *Front Immunol* **7**, 427, doi:10.3389/fimmu.2016.00427 (2016).
13. Szakács, A., Darin, N. & Hallböök, T. Increased childhood incidence of narcolepsy in western Sweden after H1N1 influenza vaccination. *Neurology* **80**, 1315-1321, doi:10.1212/WNL.0b013e31828ab26f (2013).
14. Nohynek, H. *et al.* AS03 Adjuvanted AH1N1 Vaccine Associated with an Abrupt Increase in the Incidence of Childhood Narcolepsy in Finland. *PLOS ONE* **7**, e33536, doi:10.1371/journal.pone.0033536 (2012).
15. Persson, I. *et al.* Risks of neurological and immune-related diseases, including narcolepsy, after vaccination with Pandemrix: a population- and registry-based cohort study with over 2 years of follow-up. *Journal of internal medicine* **275**, 172-190, doi:10.1111/joim.12150 (2014).
16. Morel, S. *et al.* Adjuvant System AS03 containing alpha-tocopherol modulates innate

immune response and leads to improved adaptive immunity. *Vaccine* **29**, 2461-2473, doi:10.1016/j.vaccine.2011.01.011 (2011).

17. Didierlaurent, A. M. *et al.* AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. *J Immunol* **183**, 6186-6197, doi:10.4049/jimmunol.0901474 (2009).
18. Moser, C., Muller, M., Kaeser, M. D., Weydemann, U. & Amacker, M. Influenza virosomes as vaccine adjuvant and carrier system. *Expert Rev Vaccines* **12**, 779-791, doi:10.1586/14760584.2013.811195 (2013).
19. Wilschut, J. Influenza vaccines: the virosome concept. *Immunol Lett* **122**, 118-121, doi:10.1016/j.imlet.2008.11.006 (2009).
20. Xiang, Z. *et al.* VIOLIN: vaccine investigation and online information network. *Nucleic acids research* **36**, D923-928, doi:10.1093/nar/gkm1039 (2008).
21. He, Y. *et al.* Updates on the web-based VIOLIN vaccine database and analysis system. *Nucleic acids research* **42**, D1124-1132, doi:10.1093/nar/gkt1133 (2014).
22. Martinon, F., Mayor, A. & Tschopp, J. The inflammasomes: guardians of the body. *Annual review of immunology* **27**, 229-265, doi:10.1146/annurev.immunol.021908.132715 (2009).
23. Baroja-Mazo, A. *et al.* The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response. *Nat Immunol* **15**, 738-748, doi:10.1038/ni.2919 (2014).
24. Sebina, I. & Pepper, M. Humoral immune responses to infection: common mechanisms and unique strategies to combat pathogen immune evasion tactics. *Curr Opin Immunol* **51**, 46-54, doi:10.1016/j.coi.2018.02.001 (2018).
25. Rettig, T. A., Harbin, J. N., Harrington, A., Dohmen, L. & Fleming, S. D. Evasion and interactions of the humoral innate immune response in pathogen invasion, autoimmune disease, and cancer. *Clin Immunol* **160**, 244-254, doi:10.1016/j.clim.2015.06.012 (2015).
26. Shishido, S. N., Varahan, S., Yuan, K., Li, X. & Fleming, S. D. Humoral innate immune response and disease. *Clin Immunol* **144**, 142-158, doi:10.1016/j.clim.2012.06.002 (2012).
27. Hogenesch, H. Mechanism of immunopotentiation and safety of aluminum adjuvants. *Front Immunol* **3**, 406, doi:10.3389/fimmu.2012.00406 (2012).
28. Amanna, I. J. & Slifka, M. K. Contributions of humoral and cellular immunity to vaccine-induced protection in humans. *Virology* **411**, 206-215, doi:10.1016/j.virol.2010.12.016 (2011).
29. Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* **11**, 373-384, doi:10.1038/ni.1863 (2010).
30. Kawai, T. & Akira, S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* **34**, 637-650, doi:10.1016/j.immuni.2011.05.006 (2011).
31. Del Giudice, G., Rappuoli, R. & Didierlaurent, A. M. Correlates of adjuvanticity: A review on adjuvants in licensed vaccines. *Seminars in Immunology* **39**, 14-21, doi:https://doi.org/10.1016/j.smim.2018.05.001 (2018).
32. Gavin, A. L. *et al.* Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. *Science* **314**, 1936-1938, doi:10.1126/science.1135299 (2006).
33. Fritz, J. H., Ferrero, R. L., Philpott, D. J. & Girardin, S. E. Nod-like proteins in immunity, inflammation and disease. *Nat Immunol* **7**, 1250-1257, doi:10.1038/ni1412 (2006).
34. Glenny, A. T., Pope, C. G., Waddingtom, H. & Wallace, H. Immunological notes XVII-XXIV.

Immunological notes **XVII** (1926).

35. Marrack, P., S., M. A. & W., M. M. Towards an understanding of the adjuvant action of aluminium. *Nat. Rev. Imm.* **9**, 7 (2009).

36. Johnston, C. T., Wang, S. L. & Hem, S. L. Measuring the surface area of aluminum hydroxide adjuvant. *J Pharm Sci* **91**, 1702-1706, doi:10.1002/jps.10166 (2002).

37. Shirodkar, S., Hutchinson, R. L., Perry, D. L., White, J. L. & Hem, S. L. Aluminum compounds used as adjuvants in vaccines. *Parmaceutical Research* **7**, 7 (1990).

38. Hem, J. D. & Roberson, C. E. Form and stability of aluminum hydroxide complexes in dilute solution. *Geological Survey Water-supply Paper 1827-A*, 60 (1967).

39. HogenEsch, H. Mechanisms of stimulation of the immune response by aluminum adjuvants. *Vaccine* **20 Suppl 3**, S34-39, doi:10.1016/s0264-410x(02)00169-x (2002).

40. Güven, E., Duus, K., Laursen, I., Højrup, P. & Houen, G. Aluminum hydroxide adjuvant differentially activates the three complement pathways with major involvement of the alternative pathway. *PLoS One* **8**, e74445, doi:10.1371/journal.pone.0074445 (2013).

41. Morefield, G. L. *et al.* Role of aluminum-containing adjuvants in antigen internalization by dendritic cells *in vitro*. *Vaccine* **23**, 1588-1595, doi:10.1016/j.vaccine.2004.07.050 (2005).

42. Ghimire, T. R., Benson, R. A., Garside, P. & Brewer, J. M. Alum increases antigen uptake, reduces antigen degradation and sustains antigen presentation by DCs *in vitro*. *Immunol Lett* **147**, 55-62, doi:10.1016/j.imlet.2012.06.002 (2012).

43. Eisenbarth, S. C., Colegio, O. R., O'Connor, W., Sutterwala, F. S. & Flavell, R. A. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* **453**, 1122-1126, doi:10.1038/nature06939 (2008).

44. Stephen, J. *et al.* Neutrophil swarming and extracellular trap formation play a significant role in Alum adjuvant activity. *npj Vaccines* **2**, doi:10.1038/s41541-016-0001-5 (2017).

45. Marichal, T. *et al.* DNA released from dying host cells mediates aluminum adjuvant activity. *Nat Med* **17**, 996-1002, doi:10.1038/nm.2403 (2011).

46. McKee, A. S. *et al.* Host DNA released in response to aluminum adjuvant enhances MHC class II-mediated antigen presentation and prolongs CD4 T-cell interactions with dendritic cells. *Proceedings of the National Academy of Sciences* **110**, E1122, doi:10.1073/pnas.1300392110 (2013).

47. Goto, N., Kato, H., Maeyama, J., Eto, K. & Yoshihara, S. Studies on the toxicities of aluminium hydroxide and calcium phosphate as immunological adjuvants for vaccines. *Vaccine* **11**, 914-918, doi:10.1016/0264-410x(93)90377-a (1993).

48. Goto, N. & Akama, K. Histopathological studies of reactions in mice injected with aluminum-adsorbed tetanus toxoid. *Microbiology and immunology* **26**, 1121-1132, doi:10.1111/j.1348-0421.1982.tb00261.x (1982).

49. Goto, N. *et al.* Local tissue irritating effects and adjuvant activities of calcium phosphate and aluminium hydroxide with different physical properties. *Vaccine* **15**, 1364-1371, doi:10.1016/s0264-410x(97)00054-6 (1997).

50. Kool, M. *et al.* Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J Exp Med* **205**, 869-882, doi:10.1084/jem.20071087 (2008).

51. Riteau, N. *et al.* ATP release and purinergic signaling: a common pathway for particle-mediated inflammasome activation. *Cell Death Dis* **3**, e403-e403, doi:10.1038/cddis.2012.144 (2012).

52. Shi, Y., Evans, J. E. & Rock, K. L. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* **425**, 516-521, doi:10.1038/nature01991 (2003).

53. Hansen, B., Sokolovska, A., HogenEsch, H. & Hem, S. L. Relationship between the strength of antigen adsorption to an aluminum-containing adjuvant and the immune response. *Vaccine* **25**, 6618-6624, doi:10.1016/j.vaccine.2007.06.049 (2007).

54. Hansen, B. *et al.* Effect of the strength of adsorption of hepatitis B surface antigen to aluminum hydroxide adjuvant on the immune response. *Vaccine* **27**, 888-892, doi:10.1016/j.vaccine.2008.11.078 (2009).

55. Clapp, T., Siebert, P., Chen, D. & Jones Braun, L. Vaccines with aluminum-containing adjuvants: optimizing vaccine efficacy and thermal stability. *J Pharm Sci* **100**, 388-401, doi:10.1002/jps.22284 (2011).

56. Huang, M. & Wang, W. Factors affecting alum-protein interactions. *Int J Pharm* **466**, 139-146, doi:10.1016/j.ijpharm.2014.03.015 (2014).

57. Watkinson, A. *et al.* Increasing the potency of an alhydrogel-formulated anthrax vaccine by minimizing antigen-adjuvant interactions. *Clin. Vacc. Imm.* **20**, 10 (2013).

58. Mark, A., Björkstén, B. & Granström, M. Immunoglobulin E responses to diphtheria and tetanus toxoids after booster with aluminium-adsorbed and fluid DT-vaccines. *Vaccine* **13**, 669-673, doi:10.1016/0264-410x(94)00017-h (1995).

59. Wheeler, A. W. & Woroniecki, S. R. Immunological adjuvants in allergy vaccines: Past, present future. *Allergology International* **50**, 295-301, doi:10.1046/j.1440-1592.2001.00230.x (2001).

60. Brewer, J. M. *et al.* Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4 or IL-13 mediated signaling. *J Immunol* **163**, 7 (1999).

61. Burrell, L. S., Lindblad, E. B., White, J. L. & Hem, S. L. Stability of aluminium-containing adjuvants to autoclaving. *Vaccine* **17**, 2599-2603, doi:10.1016/s0264-410x(99)00051-1 (1999).

62. Li, X., Aldayel, A. M. & Cui, Z. Aluminum hydroxide nanoparticles show a stronger vaccine adjuvant activity than traditional aluminum hydroxide microparticles. *J Control Release* **173**, 148-157, doi:10.1016/j.jconrel.2013.10.032 (2014).

63. Shang, L., Nienhaus, K. & Nienhaus, G. U. Engineered nanoparticles interacting with cells: size matters. *J Nanobiotechnology* **12**, 5, doi:10.1186/1477-3155-12-5 (2014).

64. Sun, B. *et al.* Engineering an effective immune adjuvant by designed control of shape and crystallinity of aluminum oxyhydroxide nanoparticles. *ACS Nano* **7**, 15 (2013).

65. Xu, Z. P. *et al.* Subcellular compartment targeting of layered double hydroxide nanoparticles. *Journal of Controlled Release* **130**, 86-94, doi:https://doi.org/10.1016/j.jconrel.2008.05.021 (2008).

66. Neumann, S. *et al.* Activation of the NLRP3 inflammasome is not a feature of all particulate vaccine adjuvants. *Immunol Cell Biol* **92**, 535-542, doi:10.1038/icb.2014.21 (2014).

67. Forest, V. *et al.* Toxicity of boehmite nanoparticles: impact of the ultrafine fraction and of the agglomerates size on cytotoxicity and pro-inflammatory response. *Inhal Toxicol* **26**, 545-553, doi:10.3109/08958378.2014.925993 (2014).

68. Benne, N., van Duijn, J., Kuiper, J., Jiskoot, W. & Slütter, B. Orchestrating immune responses: How size, shape and rigidity affect the immunogenicity of particulate vaccines. *J Control Release* **234**, 124-134, doi:10.1016/j.jconrel.2016.05.033 (2016).

69. Orr, M. T. *et al.* Reprogramming the adjuvant properties of aluminum oxyhydroxide with nanoparticle technology. *NPJ Vaccines* **4**, 1, doi:10.1038/s41541-018-0094-0 (2019).

70. Gupta, R. K. Aluminum compounds as vaccine adjuvants. *Adv. Drug Del. Rev.* **32**, 18 (1998).

71. Shardlow, E., Mold, M. & Exley, C. Unraveling the enigma: elucidating the relationship between the physicochemical properties of aluminium-based adjuvants and their immunological mechanisms of action. *Allergy Asthma Clin Immunol* **14**, 80, doi:10.1186/s13223-018-0305-2 (2018).

72. Yau, K. P., Schulze, D. G., Johnston, C. T. & Hem, S. L. Aluminum hydroxide adjuvant produced under constant reactant concentration. *Journal of Pharmaceutical Sciences* **95**, 1822-1833, doi:https://doi.org/10.1002/jps.20692 (2006).