Particulate inorganic adjuvants: recent developments and future outlook

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Abstract

An adjuvant is an entity added to a vaccine formulation to ensure that robust immunity to the antigen is inculcated. The inclusion of an adjuvant is typically vital for the efficacy of vaccines using inactivated organisms, sub-unit and DNA antigens, although they are not generally needed for live attenuated systems. With increasing research efforts being focused on sub-unit and DNA antigens because of their improved safety profile, the development of appropriate adjuvants is becoming ever more crucial. Despite this, very few adjuvants are licensed for use in humans (four by the FDA, and four by the EMEA). The most widely used adjuvant, alum, has been used for nearly 90 years, yet its mechanism of action remains poorly understood. In addition, while alum produces a powerful antibody Th2 response, it does not provoke the cellular immune response required for the elimination of intracellular infections or cancers. New adjuvants are therefore needed, and inorganic systems have attracted much attention in this regard. In this review, the inorganic adjuvants currently in use are reviewed and the efforts made to date to understand their mechanisms of action summarised. We then move on to survey the recent literature on inorganic particulate adjuvants, focusing on the most interesting recent developments in this area and their future potential.

1. Introduction

1.1 Preamble

Vaccination is perhaps the most effective public health intervention of all time. It is estimated that some 3 million lives per year are saved as a result of vaccines. They allow infectious diseases to be controlled or even completely purged from countries: for instance, 10 Asian nations including India, Bangladesh and Sri Lanka have very recently been declared free of polio.[1] In favourable cases, diseases may be eradicated, as in the case of smallpox. There exist a range of other disease prevention schemes including screen and treat (which screens an individual for a certain condition and if a positive result is obtained provides medication immediately), aggressive treatment regimens (which target diseases through intense courses of medication) and widespread anti-viral distribution (such as the stockpiling of oseltamivir for the swine-flu epidemic in 2009), all of which can be effective: for instance, screen and treat has successfully been used against cervical cancer.[2] However, vaccination is in general a more cost-effective and safer route for controlling the spread of disease.[3] In vaccination, we aim to expose the immune system to the infectious agent (the "antigen"; a bacterial or viral component) in a safe environment where there is minimal risk of an infection ensuing. This is akin to footballers training before a match.

Vaccination programmes can only achieve their maximum protection potential if the majority of the population are inoculated against a particular infectious disease. This concept is commonly referred to as "herd immunity". There is a threshold percentage of the population that must be inoculated in order to contain the spread of infections; this target vaccination level is dependent on factors including infectivity of the disease and the length of the infectious period.[4] With effective herd immunity, infection rates even amongst unprotected individuals are extremely low, due to the reduced pool of susceptible hosts available for pathogen reproduction and dissemination.

1.2 The human immune system

The human immune system consists of two major arms: the innate and adaptive immune systems. The former responds to commonly-encountered components of infectious agents (pattern-associated molecular pathways, PAMPs) and/or other forms of danger, including cell stress and damage (damage-associated molecular pathways, DAMPs). The immune response is rapid but restricted to a relatively confined array of fixed (germline encoded) specificities. The adaptive immune response is slower to develop, but is capable of producing an extraordinary number of distinct specificities, allowing the response to be tightly targeted at a particular antigen. The key cells involved in the innate and adaptive immune response are detailed in Table 1. Dendritic cells (DC) are particularly important, because they offer a bridge between innate an adaptive immunity and direct the response of other cell types to an encountered pathogen.

Table 1: A brief summary of the key cells of immunity.

Immune cell	Description
Dendritic cell (DC)	Key antigen-presenting cell. DC are stationed throughout the body and constantly sample their local environment. If a foreign body is detected, then DC can migrate to the lymph nodes for presentation of antigen to naïve T cell populations.
Macrophage (MØ)	An antigen presenting cell whose main purpose is engulf pathogens through phagocytosis.
Monocytes	A type of leukocyte which are a precursor to macrophages and certain dendritic cells and can differentiate into them at the site of infection or inflammation
T cell	There are two types of T cell of importance in this paper: i) T-helper (Th) cells (typically CD4+), which assist other cells in an immune response, including accelerating the maturation of B cells and activating macrophages and cytotoxic T cells; ii) Cytotoxic T lymphocytes (CTL), (typically CD8+), which destroy virally infected cells and cancerous cells.
B cell	Antibody producing cells. The antibodies produced are specific to a particular pathogen, and decorate its surface through a process known as opsonisation. This flags the pathogens for destruction by macrophages.

The adaptive immune system is highly specific, but the first time B cells and T cells encounter a pathogen they are "naïve"; this is, not well-equipped to respond to the infection. The resultant primary response is slow and of relatively low magnitude. Naïve cells of a given specificity are extremely rare, and multiple rounds of proliferation are required to produce sufficient effector cells to combat an infection. These effector cells might produce response-orchestrating cytokines (soluble protein mediators of intracellular communication) [e.g. Th cells], possess cytotoxic activity [e.g. cytotoxic T lymphocytes], or, in the case of B cells, differentiate into plasma cells specialised for antibody secretion. Once the infection has passed some of these matured cells become "memory cells", which are typically present at greater frequency than naïve cells of a given specificity, and have a lower activation threshold. These two factors enable adaptive immunity to rapidly and effectively deal with a repeat infection with the same pathogen. The goal of vaccination is thus to generate a pool of memory B and T cells without exposing the body to the risks of a real infection.

An ideal vaccine would stimulate either strong humoral (Th2) or cell-mediated (Th1) immune responses, or both: the precise response required will depend on the nature of the pathogen. Induction of humoral immunity results in the production of antibodies in response to a pathogen and is ideal for extracellular, bacterial infections.[5] However, cell-mediated immunity, with the activation of cytotoxic T cells, and cytokine-induced macrophage activation, promotes the elimination of intracellular viruses and bacterium.[5] The class of response initiated is controlled by

the T-helper cells, which can be divided into Th1, 2, 9 and 17 subsets based on their cytokine stimulation and transcription factor expression patterns.[6] Th1 cells are linked to cell-mediated immune responses whilst Th2 co-ordinate humoral immunity (and, in particular, IgE secretion). Th9 cells have been linked to allergic and autoimmune inflammations as well as having anti-tumour immunity.[7] Th17 leukocytes have been shown to play roles in chronic inflammatory diseases such as Crohn's Disease,[8] and are important in combatting extracellular bacterial infections.[9]

B cells may produce several classes of antibody, with secretion of immunoglobulin M (IgM) indicative of a primary immune response (*i.e.* a response from naïve B cells).[5] If an individual is exposed to the same pathogen subsequently then if a robust immunological memory has been inculcated, rapid synthesis of pathogen-specific IgG will occur, perhaps leading to more effective clearance of the infection.[5]

It should be noted that the human immune system is enormously complicated, and this short section cannot attempt to deal with all its intricacies. Here, we aim solely to give a brief overview sufficient to permit the reader to understand the key concepts discussed below. For more details on the immune system, the interested reader is directed to any recently published immunology textbook.[10]

1.3 Antigen processing

DCs and macrophages are antigen presenting cells (APCs), meaning that they can take up antigen from their environment and process it for presentation to T cells. Antigens taken up by DCs are partially degraded in endosomes (internal membrane-bound compartments) into fragments which are then expressed on the cell surface on major histocompatibility complex class-II (MHC-II) receptors. The resultant MHC-II/peptide complex presents antigen to CD4+ T cells (this is the antigen-specific component of T cell activation, and is known as Signal 1; see Figure 1).

Immature DCs that internalise antigen at the non-lymphatic exposure sites are only weakly capable of stimulating T cell activation; they first must undergo a maturation process which results in elevated surface expression of the co-stimulatory molecules CD80 and CD86. These act as Signal 2 for T cell activation (Figure 1). When they mature, DCs also secrete greater amounts of cytokines, providing a key component of "Signal 3" for T cell activation. Depending on the composition of Signal 3, a Th1 or Th2 (or indeed a Th9 or Th17) response can be initiated, a process known as T cell polarisation. The activation of CD8+ T cells (CTL) occurs similarly, but with Signal 1 being provided by peptide presented on the MHC-I receptor, and a requirement (for naïve CTLs) for "help" from Th cells.

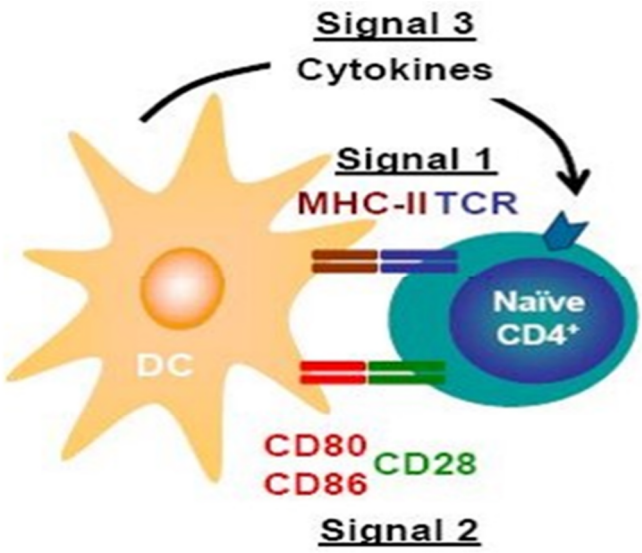

Figure 1: The three activation signals required from antigen-presenting cells to stimulate naïve T cells to differentiate into mature Th-cells. Signals 1 and 2 serve to activate T cells, and polarisation of the immune response is driven by the cytokines and co-stimulatory molecules which comprise Signal 3. Reproduced with permission from ref [11].

1.4 Vaccine types

Vaccines fall into three categories: prophylactic vaccines are given before an individual becomes infected to provide protection, whereas therapeutic vaccines are given when an individual has a condition, and exploit the immune system to remediate the situation.[12] The latter have been explored for the treatment of cancer. Tolerogenic vaccines aim to resolve autoimmune diseases or allergies through restoring tolerance to autoantigens/allergens.[13] A range of vaccine types exists, depending on how the antigen is derived. It is generally not advisable to use a disease-causing bacterium or virus as the antigen, because this would risk the individual getting infected and thus would entirely negate the purpose of the exercise. Instead, the disease causing organism is rendered safe in some way.

1.4.1 Live attenuated vaccines

Live, attenuated vaccines contain a live form of the microbe which has been rendered non-virulent. This means it has lost its capacity to cause disease following vaccination, but it is usually able to multiply to some extent in the body. The main advantage of this class of vaccine is the induction of strong cellular and antibody-mediated immune responses, which can result in the inculcation of lifelong immunity to the pathogen of interest in one or two doses.[14] For instance, this is true for the viral vaccine against measles, mumps and rubella.[15] However, a major problem with these vaccines is the risk of the attenuated strain of the pathogen reverting to its virulent state which could then cause disease upon administration.

1.4.2 Inactivated vaccines

Inactivated vaccines involve the use of chemicals, heat or radiation to inactivate the disease-causing pathogen for use as an antigen. The pathogen in such vaccines is thus dead, and cannot reproduce in the body. Hence, inactivated vaccines – such as the Salk polio vaccine – are safer than live, attenuated vaccines: there is no risk of the pathogen regaining virulence. That said, great care must be taken when inactivating to ensure that no live organisms remain. Inactivated vaccines tend to stimulate a weaker immune response than live, attenuated vaccines, so that sustained protective immunity usually requires multiple doses of vaccine[16] and/or the use of adjuvants.

1.4.3 Subunit vaccines

Subunit vaccines, instead of including a complete microbe, use a protective component from the disease-causing agent as an antigen. Such components might be for instance a part of the sugar capsid of a virus against which the immune system can respond, and may be generated by genetic engineering or by disrupting the cellular structure of the antigen through *e.g.* chemical or mechanical techniques, and subsequent isolation of the desired cellular fraction. As a result, inoculation sees the body exposed to an antigen but there is no associated risk of infection by the pathogen because it has no genomic material and hence no virulence.[17] Therefore, subunit vaccines can be administered to immunocompromised patients such as those with HIV/AIDS or individuals receiving chemotherapy (as can inactivated vaccines). The vaccine is also better defined with a sub-unit system than when working with whole organisms, and immunity is provoked against the minimum necessary set of components. This potentially permits the rational design of advanced vaccines.

1.4.4. Other vaccine types

A range of other vaccine types exist; they largely fall outwith the scope of this review but include toxoid vaccines, appropriate if a neutralising antibody response to a microbial toxin is required (*e.g.* diphtheria and tetanus).[18] Conjugate vaccines associate the antigen or toxoid of a pathogen to a polysaccharide with the purpose of enabling infantile immune systems to recognise the pathogen and provide long-term immunity from infections such as *Haemophilus influenzae B* through stimulation of both cell-mediated and humoral immune responses.[19]

DNA vaccines incorporate DNA encoding one or more protein antigens, under promoters which drive expression in mammalian cells. Following administration, these genes are taken up by cells and the DNA is transcribed and translated for synthesis of the encoded antigen in the host cell, converting these cells into “vaccine-making factories.” The antigen can either be secreted from the cell for

induction of a strong antibody response, or the pathogenic antigen can be expressed either on the host cell surface or intracellularly to stimulate strong cellular responses; so that a well-defined immune response is generated.[20] A range of conditions, including HIV/AIDS and cancers, are being targeted by DNA vaccines.[21-23]

Recombinant vector vaccines use attenuated viral or bacterial strains as the vector in order to introduce microbial DNA to human cells. Scientists have exploited the natural infection cycle of a virus in such vaccines (*e.g.* the Herpes simplex virus[24] and Hepatitis B virus).[25] Viruses tend to attach themselves onto the host cell surface in order to inject their genome into the host, and vaccines can be prepared by extracting the genome from the delivery vector virus and replacing it with genes from another pathogen. For bacterial vectors, the attenuated bacterium has antigen-encoding genes inserted for subsequent expression of the antigen on the vector cell surfaces.[26] Therefore, the harmless bacterium now drives an immune response against antigens originally derived from a harmful pathogen. The main advantage of this class of vaccine is that they mimic the natural infection cycle for reliable induction of an immune response.[16]

1.5 The adjuvant

If subunit antigens are used alone in a vaccine formulation, then their low immunogenicity means that it is likely that they will simply be excreted from the body, with no immune response inculcated. In order to ensure robust immunity, a second component known as an "adjuvant" needs to be added to the formulation. This helps the immune system to recognise the presence of a foreign body, and acts to stimulate the immune response to it. Adjuvants are generally needed for inactivated vaccines as well, but attenuated vaccines usually do not need to be adjuvanted.

The role of the adjuvant is to increase the immune response induced by the administration of the antigen. An ideal adjuvant candidate should be cheap to manufacture, safe to administer, provide life-long immunity and elicit the desired immune response (either humoral, cell-mediated, or both).[27, 28] At the present time, very few adjuvants are licensed for use in humans: see Table 2.

Table 2: Vaccine adjuvants currently licensed for use in humans.[29]

EMA-approved vaccine adjuvants	FDA-approved vaccine adjuvants
MF59	AS04
AS03	AS03
AS04	Virosomes
Aluminium-containing compounds	Aluminium-containing compounds

By far the most widely used adjuvant is "alum", an inorganic salt first identified as potent by Glenny in 1926.[30, 31] However, other metal salts such as calcium phosphate are also used – for instance in use in diphtheria-pertussis-tetanus vaccines in France[32] – and there is a significant research effort underway to look at other inorganic systems. MF59 and AS03 are oil-in-water based adjuvants.

Virusomes are nanoparticles designed to mimic the outer surface of a virus, yet not containing any RNA or DNA and hence having no virulence. As organic adjuvants, they fall outwith the scope of this review. AS04 is a mixture of alum and monophosphoryl lipid A, an attenuated form of lipopolysaccharide (LPS), a component of bacterial cell walls: we will thus touch on AS04 in the discussion that follows.

1.6 Aims and scope

In this review, a brief survey of the different types of inorganic particulate adjuvant will be presented, covering both those currently in use in the clinic and those under research. Attention will be paid to the mechanism by which these materials act to stimulate the immune system, where this is known, and how particle engineering approaches may be used to control and tune the type of immunity induced.

2. Inorganic adjuvant systems

2.1. Alum

"Alum" is used by chemists to refer to materials with the formula $KAl(SO_4)_2{\cdot}12H_2O$ or $[NH_4^+][Al(SO_4)_2]{\cdot}12H_2O$. Immunologists, in contrast, use the term "alum" to refer to all aluminium-containing adjuvants. The alum of immunologists is typically poorly defined in terms of its chemical and physical properties – it is not unusual to receive a sample from a supplier labelled "$Al(OH)_3$ solution" where a cursory visual inspection reveals it to be a suspension rather than a solution, and where a minimal characterisation suggests that there is in fact very little $Al(OH)_3$ present at all. For these reasons, alum is often referred to as "immunologist's dirty little secret".[33]

In fact, most commonly "alum" comprises either aluminium oxyhydroxide [AlO(OH)] or aluminium hydroxyphosphate $[Al(OH)_x(PO_4)_y]$. AlO(OH) is a crystalline layered material, the surface of which comprises only hydroxyl groups. Its structure is depicted in Figure 2. In contrast, $Al(OH)_x(PO_4)_y$ is an amorphous material with both hydroxyl and phosphate groups on the particles surfaces.[34]

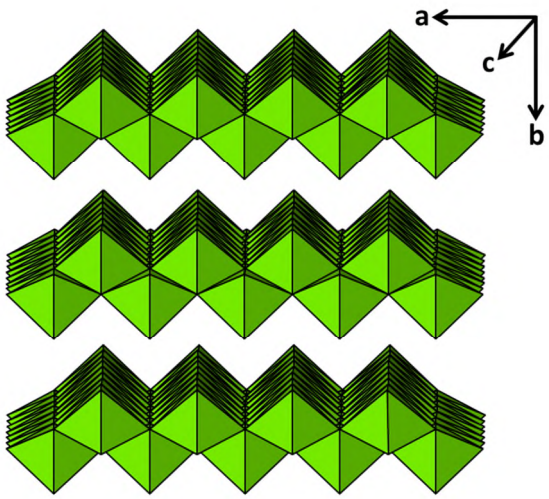

Figure 2: The structure of AlOOH. AlO_6 octahedra are shown in green. H atoms are omitted for clarity.

For most of the time which has elapsed since their first use nearly 90 years ago, aluminium-containing adjuvants have been the only licensed adjuvants for use in human vaccines: only in 1997 was the first alternative adjuvant licensed [in Italy].[35] Aluminium-containing adjuvants are used in a wide variety of vaccines such as those for diphtheria, tetanus and pneumococcal infections.[36] They promote a Th2 response, and so can be effective for targeting extracellular pathogens, but are much less useful for vaccination against intracellular pathogens and tumours.

It should be noted that the use of "Imject Alum" is frequently reported in the literature. This is chemically quite distinct from the aluminium-containing adjuvants used in human and veterinary medicine. It consists of 40mg/ml crystalline magnesium hydroxide and 40mg/ml amorphous aluminium hydroxide. Magnesium ions exert an effect on immune and inflammatory responses,[37] and have also been shown selectively to block calcium-ion channels resulting in the inhibition of macrophage activation and an anti-inflammatory response.[38] The composition of Imject Alum is such that it is not recommended for use in research aimed at deducing the mode of action of adjuvants in licenced human vaccines,[39] nor should it be used for research into the effects of aluminium oxyhydroxide or aluminium hydroxyphosphate.[40]

2.1.1 Adsorption of antigen to alum

Alum and antigen are typically admixed in vaccine formulations, and it is thus vital to consider how the two components might interact to understand formulation efficacy. Antigen adsorption refers to the adhesion of antigen to the surface of an adjuvant via interactions between the two entities. In vaccine prophylaxis, antigen adsorption provides a means of delivering the antigen to the recipient for induction of an immune response. There are two main antigen adsorption mechanisms: electrostatic interactions and ligand exchange, both of which are discussed further below.

Crystalline AlO(OH) has a point of zero charge ranging between pH 7.7 and 9.4.[41] Therefore, at physiological pH it carries a positive charge. In contrast, amorphous aluminium hydroxyphosphate has a point of zero charge at around pH 4 and thus at physiological pH carries a negative charge. Seeber provided experimental evidence for electrostatic interactions being involved in protein adsorption to alum by studying AlO(OH), $Al(OH)_x(PO_4)_y$, lysozyme and albumin.[42] Lysozyme has an isoelectric point (pI) of 11[43] while the equivalent value for albumin is 4.8.[44] The two proteins thus at physiological pH carry positive and negative charges, respectively. Positively charged AlO(OH) strongly adsorbed the negatively charged albumin, but no lysozyme adsorption was recorded. The inverse was shown to be true for the negatively charged $Al(OH)_x(PO_4)_y$. These results were ascribed to the different electrostatic interactions in the systems, as depicted in Figure 3.

Another mechanism of antigen adsorption is ligand exchange; this provides direct covalent bonding between the antigen and adjuvant, and thus leads to stronger antigen binding than electrostatic bonding. Ligand exchange arises if the antigen is phosphorylated, because these phosphate groups can displace hydroxides bound to aluminium.[45, 46] It has been reported that antigen adsorption through either ligand exchange of electrostatic interactions can be sufficient to induce an immune response.[47]

Thirty-four years ago, Edelman postulated that adjuvanticity might be dependent on complete adsorption of antigen to the adjuvant surface.[27] Taken with the more recent results of Seeber (discussed above),[42] this suggests that adjuvant selection should be dependent on the physiological behaviour of both the adjuvant and the antigen. Upon administration of the vaccine, it may be necessary for the adsorbed antigen to be eluted from the surface of the adjuvant in order to induce an immune response. However, it has also been shown that particulate adjuvants can be internalised by DC,[48] and hence elution may not be an absolute requirement, or may occur intracellularly. Antigen elution certainly can occur, however, as demonstrated by Iyer and colleagues, who adsorbed ovalbumin and dephosphorylated α-casein antigens to aluminium hydroxide and injected the admixtures subcutaneously to BALB/c mice.[49] Their results demonstrated that both ovalbumin and dephosphorylated α-casein eluted completely upon exposure to the interstitial fluid. Seeber has additionally proposed that citrate ions in interstitial fluids cause dissolution of the adjuvant, subsequently freeing the antigen into solution.[50]

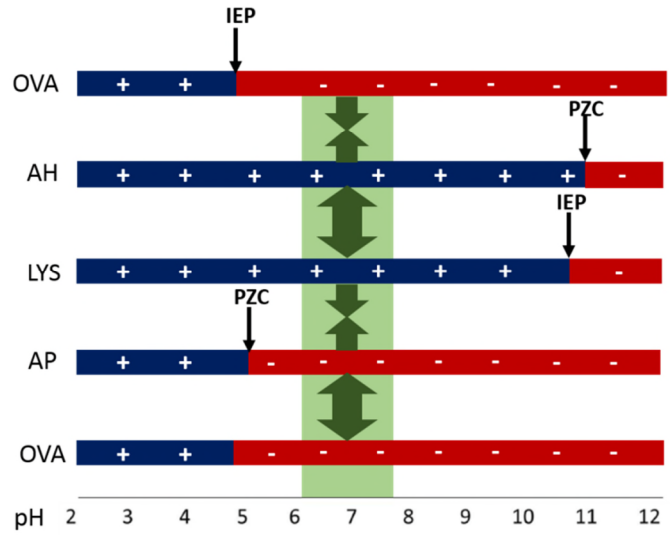

Figure 3: A schematic illustrating possible electrostatic interactions between protein antigens and Al-based adjuvants. The isoelectric point (IEP) describes proteins whilst the point of zero charge (PZC) refers to mineral adjuvants. If the antigen and adjuvant carry opposite charges at physiological pH then there will be electrostatic attractions and binding between them. OVA = ovalbumin, AH = aluminium hydroxide, LYS = lysozyme, AP = aluminium phosphate.

Recent studies have cast doubt on to what degree of antigen adsorption is desirable, however. Hansen and co-workers demonstrated that if the antigen is too strongly adsorbed to the adjuvant surface, the immune response can be impaired.[51] In this study, α-casein and dephosphorylated α-casein were adsorbed to Al hydroxide based adjuvants with different surface OH concentrations, and antibody titres in mice found to be inversely correlated with the strength of adsorption. In addition, non-adsorbed antigen has been shown to elicit an immune response *in vivo*. Romero-Mendez tested three antigen-alum formulations containing a commercial Al hydroxyphosphate based adjuvant (Adju-Phos) in mice.[52] They found that the antibody titres were similar regardless of whether the antigen was adsorbed to the Adju-Phos or not. Results from electron microscopy highlighted that the adjuvants create porous aggregates with the non-adsorbed antigen entrapped in the pores. This was found in *in vitro* studies to allow effective antigen uptake by DC. A range of other studies have cast doubt on the need for antigen absorption onto alum, with similar antibody titres observed both with and without adsorption.[48, 53, 54]

In an extreme example, Braley-Mullen *et al.* have given antigen and alum in separate injections and found that separate injections induced higher antibody titres than the antigen administered alone.[55] However, it should be noted that other studies reported that no immune response was initiated following separate antigen/alum injections.[48, 56] More recently, some authors have suggested that it is in fact favourable to have minimal interactions between alum and the antigen for anthrax vaccination,[57] and also in the hepatitis B setting.[58] One reason for this might be that antigens are thought to be less stable when adsorbed to alum than when in solution,[59] because in the former setting there is an increased susceptibility to physical and chemical degradation.[60, 61] Two recent reviews discuss these challenges in detail, and posit future formulation strategies to maximise vaccine efficacy.[62, 63]

2.1.2 Activation of the immune system by alum-adsorbed antigen

Vaccines are injected at non-lymphatic sites, where antigen desorption from the surface of aluminium-containing adjuvants is thought to occur in the interstitial fluid.[42, 48, 49, 52, 64, 65] Whether desorbed or not, antigen must enter the lymphatic system in order to reach the lymph node and then stimulate naïve B and T cells.[66] Antigenic particles less than 30 nm in size can enter into the lymph node as free antigen; however, larger antigenic species (*e.g.* antigen adsorbed to a particulate adjuvant) must be internalised by resident DCs in order to be delivered to the lymph node and be presented to naïve B- and T cells for induction of a primary immune response.[67]

Particle size thus significantly impacts how an antigen enters the lymphatic system: this is illustrated in Figure 4. Particles of sizes 20 – 1000nm are taken up by an apoptotic cell for internalisation by DCs, or alternatively can be directly taken up by DCs. Antigens ranging between 1 – 30nm can either pass through between the cellular wall of the lymphatic system for DC-internalisation or pass straight into the lymph node as free antigen. All these routes ultimately deliver antigen to naïve B- and T cell populations in the thymus for induction of an immune response.

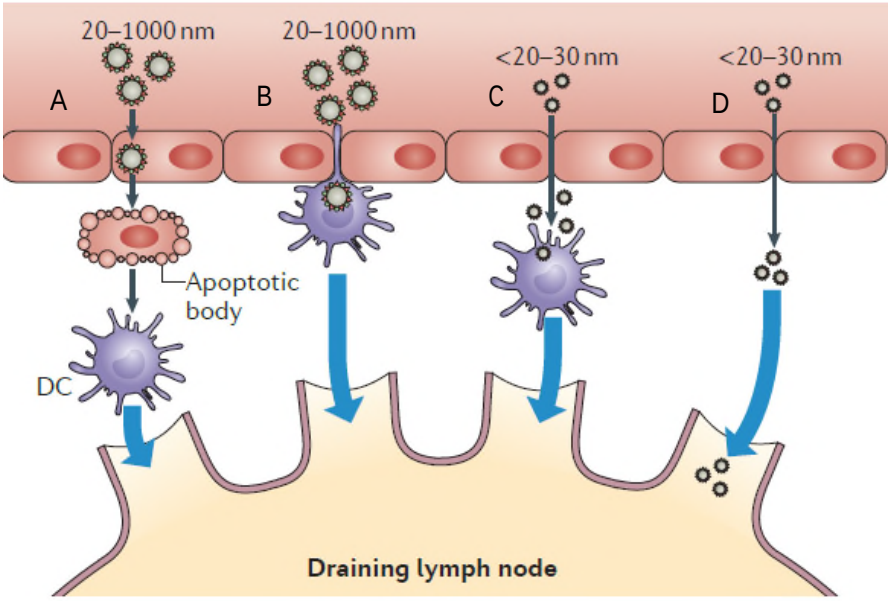

Figure 4: A schematic representation of antigen delivery to the lymphatic system depending on the size of the antigen. Reproduced with permission from ref [68]. Copyright Macmillan Publishers Ltd 2013.

There are three key pathways in which DC may take up antigen from their local environment: macropinocytosis, receptor-mediated endocytosis and phagocytosis.[69] A schematic representation of these pathways is given in Figure 5.

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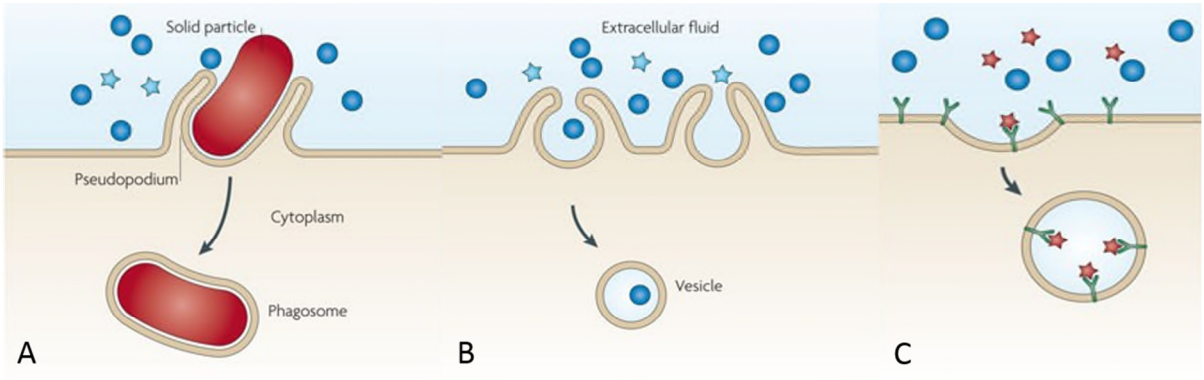


Figure 5: A schematic representation of the main pathways in which antigen may be taken up by dendritic cells. (a) Phagocytosis, in which a DC engulfs solid particles and entraps them in an intracellular vesicle; (b) Macropinocytosis, in which particles of size > 1µm are internalised by invaginations of the cell membrane for entrapment in a vesicle within the DC; (c) Endocytosis, a process by which cells internalise particles of size 60 - 120nm from the extracellular environment and encapsulate them in a vesicle. Reproduced with permission from ref [70]. Copyright Macmillan Publishers Ltd 2010.

Experimental evidence supports the idea that the DC-uptake route of the antigen can affect its presentation. Burgdorf and colleagues showed that endocytosis of the model antigen ovalbumin (OVA) by DCs results in the antigen being presented on the surface by MHC-I co-stimulatory molecules. This leads to the activation of CD8+ cytotoxic T cells.[71] In contrast, if the DC internalises OVA *via* macropinocytosis then the antigen will be presented on MHC-II, causing the activation of Th cells.[72]

It has been suggested that antigen-alum complexes can be more readily internalised by antigen-presenting cells than soluble antigen alone. This might cause increased interaction between the dosed antigen and the immune system,[73, 74] indicating that stronger absorption may be preferred. However, as described in Section 2.1.2, the experimental data is mixed, and it is likely that more than one mode of immune system activation is involved.

2.1.3 Hypothesised modes of action for alum

2.1.3.1 The depot effect

"Alum" was first described in 1926 when Glenny[30, 31] used it as an adjuvant for diphtheria vaccines and hypothesised a "depot-effect" for the mode of action. In this, he proposed that the adjuvant essentially act as a reservoir, slowly releasing the antigen over a prolonged period of time and thus inculcating robust immunity. This idea went largely unchallenged for almost 70 years. Only in the last 20 years have further investigations into the detailed mechanism of action taken place. Since then, the way in which aluminium-containing adjuvants function has been much debated, with evidence supporting a range of hypotheses.

Glenny[30] used a precipitation of the diphtheria toxoid antigen with "potash alum" in order to slow the rate of antigen elimination from the injection site. Guinea pigs were injected with the alum-

precipitated toxoid and three days after vaccination the injection site was recovered. The tissue thereby collected was macerated and then re-injected into naïve guinea pigs. The latter animals developed an anti-toxoid response, indicating the antigen was still present in the tissue. The same reaction not observed when injection sites were recovered from a control group of guinea pigs inoculated with soluble diphtheria toxoid and transferred to a population of naïve animals. The authors concluded that there was sustained release of antigen from the alum adjuvant particles, providing prolonged exposure to the antigen and eliciting a greater immune response. This hypothesis is known as the "depot effect". However, given that the macerated tissue must have contained alum as well as antigen, it is unclear whether this is a true depot effect, or whether the adjuvant performed some other role.

Another early group of researchers also proposed a depot effect to explain the adjuvant effect of alum.[75] These authors suggested that the antigen could potentially remain at the site of injection for up to 7 weeks. Further evidence to support the depot hypothesis comes from the fact that the $Al(OH)_x(PO_4)_y$ has been found to result in less efficacious immune responses than AlO(OH).[76] The phosphate content of $Al(OH)_x(PO_4)_y$ results in this salt having higher solubility, and in it being eliminated more readily from the body, reducing the exposure time of naïve B and T cells to the antigen and thus leading to reduced immunogenicity.

However, a more recent study provides evidence that aluminium-containing adjuvants at the injection site are dispensable for adjuvanticity just two hours after the initial administration.[77] BALB/c mice were injected with alum/OVA (alum provided by Brenntag Biosector, Frederikssund, Denmark) and the injection sites subsequently ablated. The OVA-specific antibody responses were the same in mice which underwent this ablation process as in those which did not.[77] In summary therefore, although the depot effect may have some role to play in the adjuvanticity of alum, it appears it is not the only factor of importance.

2.1.3.2 Molecular mechanisms of action

In recent years, a number of authors have undertaken more detailed mechanistic studies to probe the molecular immunological pathways which underlie alum adjuvanticity. Unfortunately, a range of different protocols have been employed, with a number of different types of "alum". This has resulted in inconsistent findings, and so although steps have been taken toward unravelling alum's mechanism of action, our understanding remains incomplete.

In one of the first such studies, intraperitoneal injection of the model antigen OVA with Imject Alum was found to induce chemoattraction of key immune cells including monocytes to the site of injection.[78] The recruited monocytes internalised the desorbed OVA and migrated to the draining lymph node, where they differented into DCs. These monocyte-derived DCs were "mature" in the sense that they had high levels of MHC-II and CD86 expression, and they induced the proliferation of antigen-specific T cells.[78-81] Depletion of DCs (though diphtheria toxin administration) abolished

OVA/Imject Alum adjuvanticity;[78] thus DC activation *in vivo* is key to the induction of the humoral immune response by aluminium-containing adjuvants. Alum has also been found to enhance macrophages' abilities to drive T cell proliferation and CD83 expression.[81] In addition to monocyte and macrophage recruitment, alum can also accelerate differentiation of monocytes to DCs.[82] The latter have a greater capacity for antigen uptake and subsequent migration in the lymph node to prime naïve B- and T cell populations, leading to an improved immune response.

Alum administration has also been shown to stimulate the release of danger-associated molecular patterns (DAMPs) such as DNA, ATP and uric acid from necrotic (dying) cells at the site of alum injection.[83-86] Such release has been proposed to be crucial for adjuvanticity since the DAMPs produced can aid the recruitment of immature DCs and subsequent T cell activation following alum administration.[78]

Flach and co-workers explored the binding of aluminum cesium sulfate dodecahydrate ("cesium alum") and Imject to the membranes of antigen-presenting cells.[87] Cesium alum was found to bind very strongly to DC membranes, triggering abortive phagocytosis and the internalisation of antigen alone. This resulted in antibody production and humoral immunity only (*i.e.* no cellular immunity) because the antigen was presented on MHC-II rather than MHC-I. Interestingly, strong alum binding was exhibited only by DC – and not by B cell- or macrophage- like cell lines – indicating that DC may be the primary target for alum.

A further hypothesis for alum's mode of action concerns the activation of the NLRP inflammasome. This is a protein complex whose assembly is induced during the innate immune response following microbial or viral recognition; it is depicted in Figure 6. Its activation may occur in response to cellular stress such as the detection of reactive oxygen species (ROS), or in response to microbial components (e.g. flagellin). Inflammasome complex formation begins with the oligomerisation of NLRP-3 protein units.[88, 89] Recruitment and activation of the caspase 1 enzyme then ensues. The latter can stimulate proteolytic cleavage of precursor molecules to proinflammatory cytokines including IL-1β and IL-18, leading to secretion of the mature cytokines. These then induce an inflammatory response, leading to immunity.

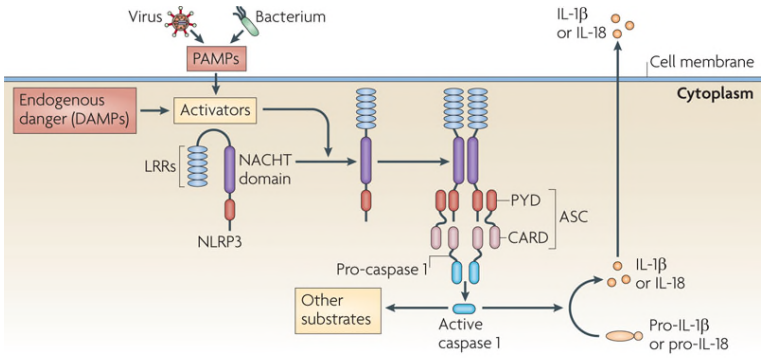


Figure 6: The mechanism of NLRP-3 inflammasome activation, in which pathogen-associated or danger-associated molecular patterns (PAMPs or DAMPs) stimulate the activation and subsequent opening-up of NLRP-3 proteins to expose their PYD domains. Oligomerisation then occurs, leading to activation of the enzyme pro-caspase 1; this in turn results in secretion of mature pro-inflammatory cytokines. Reproduced with permission from ref [88]. Copyright Macmillan Publishers Ltd 2010.

Alum has been proposed to activate NLRP-3, but exactly how it does so remains unclear. Zhang recently demonstrated a positive linear relationship between the surface hydroxyl content of alum and the induction of ROS synthesis,[90] indicating that ROS stress signals may be responsible. Experimental evidence also suggests that the administration of alum can induce inflammasome activation by release of uric acid from necrotic cells.[78] Uric acid is an endogenous danger signal which causes the maturation of caspase-1 leading to the release of IL-1β and IL-18.[91, 92]

A role for the NLRP-3 inflammasome in alum adjuvanticity has not been suggested by all studies, however. While some groups[92-96] have found that aluminium-containing adjuvants act via inflammasome activation for induction of an immune response, others concluded experimentally that the inflammasome is in fact dispensable.[78, 87, 97, 98] A summary of the findings to date can be found in Table 3. De Gregorio *et al.*[97] concluded that the conflicting evidence for aluminium-containing adjuvants' mode of action could arise due to study-to-study variation in formulation route, antigen purity, assay and other variables. This seems a reasonable deduction, given that the aluminium adjuvants often used in research (*e.g.* Imject Alum) are not the same as those used in the clinic, and thus are not recommended for mode of action studies.[39, 64, 99, 100] It seems entirely probably that this difference, coupled with differences in experimental protocol, account for the differences in mechanism observed experimentally.

Table 3: A summary of experimental findings as to whether alum's mode of action involves the activation of the NLRP-3 inflammasome.

Authors and reference	Alum used	Vital role for NLRP-3 inflammasome?
Eisenbarth et al. [93]	Imject-Alum	Yes
Li et al. [92]	Imject-Alum Alhydrogel Adju-Phos	Yes
Li et al. [94]	Alhydrogel	Yes
Hornung et al. [96]	Imject Alum	Yes
Flach et al. [87]	Cesium alum Imject alum "Clinical AlOH"	Dispensible
Franchi and Nunez [101]	"Mixture of alum"	Dispensable
Kool et al. [95]	Imject-Alum	Dispensable
Mckee et al. [98]	Imject Alum Alhydrogel	Dispensable
Kool et al. [78]	Imject Alum	Indirect

Several recent reviews have discussed the mechanism of action of alum in more detail than can be presented here, and the interested reader is directed to these for further information.[102, 103] A summary of the various proposed routes of action of alum is given in Figure 7.

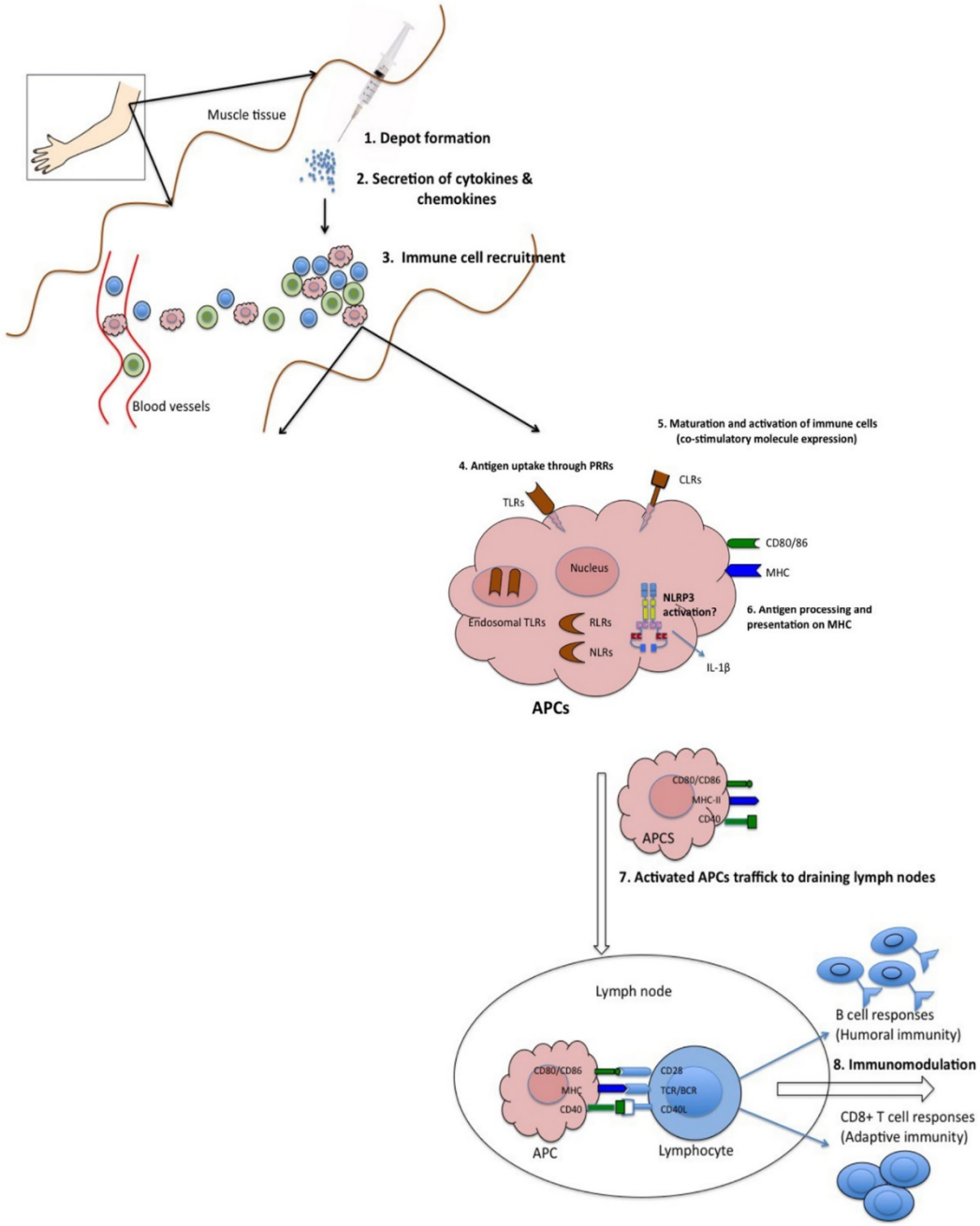


Figure 7: Possible mechanisms of action for alum adjuvants *in vivo*, following intramuscular injection. Abbreviations: - TLR = Toll-like receptor, CLR = C-type lectin receptor, RLR = RIG-I like receptor, NLR = NOD-like receptor, MHC = major histocompatibility, CD = Cluster of Differentiation, TCR = T cell receptor, BCR = B cell receptor, PRR = pattern recognition receptor. These receptors are located at different places on cell surface with the purpose of providing a docking site for their cognate ligand on another cell, in order to induce intracellular signalling cascades. Reproduced with permission from ref [103]. Copyright Frontiers Media S.A 2013.

2.1.4 AS04

AS04 is a new adjuvant developed by GlaxoSmithKline. It comprises a mixture of aluminium hydroxide and monophosphoryl lipid A (MPL-a).[104] The latter is an attenuated version of

lipopolysaccharide (LPS), which is an abundant component of Gram-negative bacteria. LPS is a powerful PAMP, but is considered too dangerous to use in vaccines, whereas MPL-a is safer. Since its regulatory approval, AS04 has been used in a number of vaccines, for instance the Ceravix human papilloma virus (HPV) formulation.[105] AS04 has been reported to perform better than alum alone in a vaccine against Herpes simplex virus.[106] Similarly to alum, AS04 stimulates cytokine production at the injection site; this results in recruitment of dendritic cells and monocytes and ultimately increased numbers of antigen-presenting cells in the lymph nodes.[107] A detailed discussion of AS04 lies outside the scope of this review, but the AS04-adjuvanted HPV vaccine has been found to be highly effective, and an AS04 vaccine for Hepatitis B was determined to be more efficacious than one adjuvanted with alum alone.[108] AS04 is not a panacea however: a vaccine against genital herpes was found to be ineffective.[108]

2.1.5 Effect of size and shape of alum adjuvant particles on the immune response

The possible influence of surface charge on adjuvant/antigen interactions is discussed above. Very recently, some authors have sought to control the particle size and shape of alum and explored how this influences its immunogenicity. Sun and co-workers synthesised a library of AlO(OH) particles of different sizes and shapes.[109] It proved possible systematically to vary the morphology and size of the particles by varying the synthesis pH: see Figure 8.

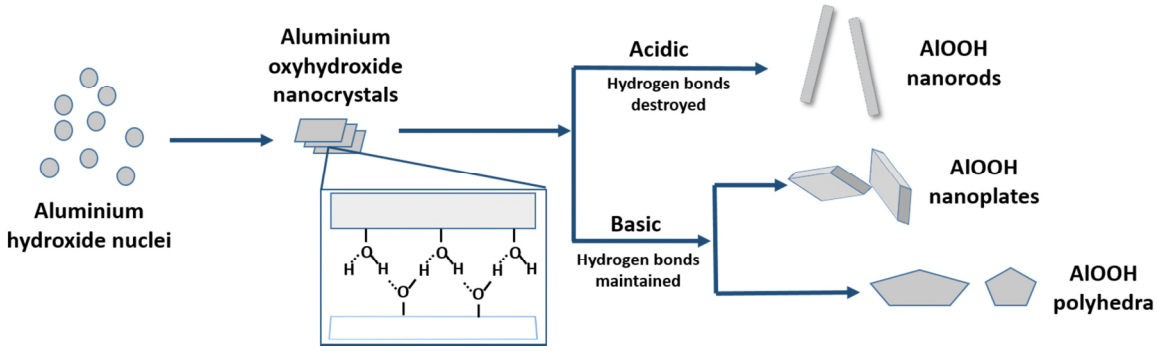


Figure 8: The variation of AlO(OH) particle morphology through synthesis pH. Adapted from ref [109].

At pH 5, rod shaped particles were obtained, while at pH 9 polyhedra-shaped particles were collected and at pH 10, platelets. The samples were found to stimulate ROS production, a property which was ascribed to their high hydroxyl contents. They were also tested for their ability to stimulate macrophages *in vitro*: macrophage-like cells were derived from phorbol 12-myristate 13-acetate (PMA)-treatment of the THP-1 monocytic leukaemia cell line[110] and treated with different AlO(OH) materials in the presence of LPS, to induce the production of pro-IL-1β.[109] The results are presented in Figure 9 and show that three of the synthesised nanorods were capable of significant inflammasome activation and subsequent IL-1β production. There was a clear influence of particle size and shape on cytokine production, with an increase in rod size causing increased secretion. While certain of the rods were effective, the platelet and polyedra shaped particles had very poor

capacity to stimulate IL-1β synthesis. With murine bone-marrow derived DC the rods were found to stimulate the production of IL-6 (a Th2-polarising cytokine), IL-12 (a Th1-polarising cytokine) and IL-1β. The largest rods (rods 1 and 2) were again most effective here. The production of MHC-II, CD86 and CD80 was also increased by the AlO(OH) rods, and again the larger rods are more potent (although the trend is not completely straightforward).

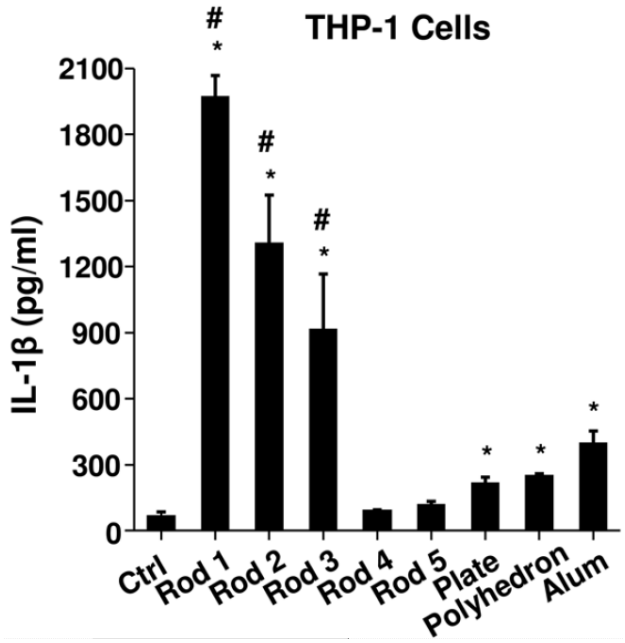


Figure 9: The production of IL-1β in response to treatment with Sun's particle-engineered alum samples and LPS. AlO(OH) samples were administered to THP-1 macrophages at 500μg/ml for 6 hours. 500μg/ml alum (Thermo Scientific, Pittsburgh, USA) was also dosed as a control. In water, the hydrodynamic sizes of the AlO(OH) samples are approx. as follows. Rod 1: 810 nm; rod 2: 592 nm; rod 3; 451 nm; rod 4: 434 nm; rod 5: 244 nm; plate: 93 nm; polyhdrdon: 333 nm; alum: 452 nm. Reproduced with permission from ref [109]. Copyright American Chemical Society 2013.

In vivo studies were also undertaken using OVA in C57Bl/6 mice.[109] The largest rods (1 and 2, 600 – 800 nm) were compared with rod 5, with the smallest particles, and alum. The *ca.* 600 and 800 nm rods were found to stimulate higher OVA-specific IgG1 and IgE titres than 244 nm rods or alum. The 244 nm rods did not significantly boost IgG1 production over the level induced by alum, nor did it increase IgE. Control of adjuvant particle size and shape therefore clearly has great promise for the design of vaccines with maximal efficacy.

A second and very recent study seems to confirm the importance of alum particle size and shape control. Li and co-workers explored the influence of alum particle size on the immune response inculcated *in vivo*.[111] AlO(OH) nanoparticles of size 112 nm (AH-NPs) and microparticles of size 9.3 μm (AH-MPs) were prepared. It should be noted that the AH-NPs were found to be completely amorphous by X-ray diffraction, meaning it is not possible from these data to confirm the chemical composition of the materials, and the AH-MPs appear to comprise a mixture of poorly-crystalline AlO(OH) and $Al(OH)_3$. Whether the two samples are chemically identical is thus unknown.

Li *et al.* observed increased OVA antigen adsorption to the AH-NPs (*cf.* the AH-MPs), as a result of the much greater surface area of the former. This concept is supported by work undertaken by Lundqvist looking at protein interactions with polystyrene nanoparticles.[112] The AH-NPs led to a stronger anti-OVA IgG response in mice than the AH-MPs.[111] Experimental data showing this are presented in Figure 10.

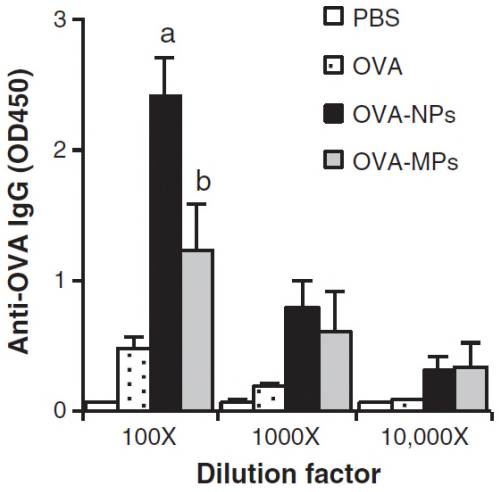


Figure 10: Total serum anti-OVA IgG levels measured on day 27 following BALB/c mice inoculation with OVA/AH-NPs or OVA/AH-MPs (a) p=0.001, OVA *vs.* OVA/AH-NPs and (b) p=0.02, OVA/AH-NPs *vs.* OVA-MPs at 100x dilution factor. Reproduced with permission from ref [111]. Copyright Elsevier 2014.

Similar results were obtained using the anthrax AP antigen:[111] although both the AH-MPs and NPs induced similar antibody production after one week, after 4 weeks the titres were much higher with the NPs. Internalisation of the adjuvant particles by antigen-presenting cells was also demonstrated to be much greater with the AH-NPs than the MPs, and the amount of local inflammation observed to be lower with the former.

2.1.6 Potential disadvantages of aluminium-containing adjuvants

A number of possible side effects from the use of alum in vaccines have been proposed. Aluminium is classed as a neurotoxin[113] and has been shown to accumulate in regions of the brain commonly affected by Alzheimer's' Disease (AD).[114] However, the evidence is non-conclusive: other research groups[115, 116] found no evidence of aluminium accumulation in AD brains. Two groups have also linked elevated levels of aluminium in the brain to autoimmune conditions[117, 118] such as macrophagic myofasciitis syndrome[119, 120] and siliconosis.[121] Their evidence suggests that two or three consecutively dosed alum-containing vaccines could provide a sufficient accumulation of aluminium to overcome genetic resistance to autoimmunity.[122] Aluminium administration has also been linked to *in vivo* effects including erythema (reddening of the skin) and granulomatous inflammation (accumulation of phagocytes causing a local inflammatory response).[123-125]

However, the consensus seems to be that the side effects are minor in comparison to the health benefits: despite the fact that alum has been the only licensed vaccine adjuvant for most of the past 80 years, persuasive evidence of widespread side effects is notable by its absence, and the FDA emphasises that it has been safely administered to humans since 1932.[126]

2.2 Alternative inorganic adjuvants

Although alum is by far the most commonly used adjuvant, a range of other systems have been explored for use in vaccines. These are discussed below.

2.2.1 Calcium phosphate

Calcium phosphate (CaP) is a compound which occurs naturally within the human body: it is thus biocompatible, which results in reduced immunotoxicity compared to alum, although strong haemolytic responses are observed following its administration.[127] CaP has been found to stimulate a reduction in IgE antibody responses and elevated IgG1 production following administration of diphtheria, tetanus or pertussis toxoid antigen to mice. [128] A study in guinea pigs indicated that CaP was as potent or less potent than an Al hydroxide adjuvant for diphtheria-tetanus vaccines.[129] However, in a human trial vaccinating against the same diseases the CaP adjuvant was found to have higher efficacy but also to lead to more adverse reactions.[130] CaP is commonly administered as a vaccine adjuvant in the diphtheria-pertussis-tetanus vaccine in France.[32]

Detailed chemical characterisation studies of a commercially available CaP adjuvant found it to comprise not $Ca_3(PO_4)_2$ as would be intuitively expected, but instead to have the chemical formula $Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$ (with x varying between 0 and 2).[131] The CaP sample analysed was found to be poorly crystalline with a point of zero charge of 5.5, indicating that at physiological pH it is likely to adsorb positively charged antigens. Crystals of calcium phosphate have been shown to induce IL-1β secretion through inflammasome activation,[132] suggesting a similar mechanism of action to that proposed for alum adjuvants. However, the mechanism of CaP adjuvanticity has not been studied in detail so questions remain.

A range of studies have been performed investigating the adjuvant effect of CaP, for instance in vaccines for influenza[133] or herpes simplex.[134, 135] In the latter case CaP yielded higher IgG2a titres than alum while provoking minimal IgE production.[135] In a murine model CaP could also induce systemic immunity to herpes simplex through mucosal administration.[134] CaP has also been explored for tolerogenic vaccination, for instance to pollen extracts,[136] and in veterinary vaccines [137, 138].

In a very recent study, Olmedo *et al.* explored the response in mice to *Bothos asper* snake venom.[139] When antigen was adsorbed onto the surface of the adjuvant CaP was found to be superior to

aluminium hydroxide adjuvant, leading to enhanced leukocyte recruitment and greater antibody responses. An alternative approach was explored in which the antigen was co-precipiated with the CaP; these samples resulted in the highest antigen loading, phagocytosis by macrophages, leukocyte recruitment and antibody response. The approach of co-precipitating antigen and CaP together is a popular one, having been used by He *et al.* in their 2002 study[134] and also by Joyappa and co-workers for DNA vaccines.[140] The latter study used a DNA antigen for foot and mouth disease, which was incorporated into CaP nanoparticles through co-precipitation. The transfection efficiency of the resultant systems was found to be very high, and high antibody titres were also observed.

Co-precipitation of CaP with antigen has been shown to lead to long-lasting CD8+ T cell responses in mice.[141] In Zhou's work, OVA was co-precipitated with CaP. The CaP-OVA particles were comparable to soluble OVA in terms of antibody responses, but led to high levels of IFN-γ production and a potent CTL response in mice. Combinations of CaP and protein-coated microcrystals (co-precipitated protein and amino acid or sugar formulations) have also been investigated, with CaP inclusion found to enhance phagocytosis in the J774.2 murine monocyte/macrophage cell line.[142] CaP modification also decreased the IgG1: IgG2a ratio in NIH mice, indicating that its inclusion can help skew the immune response to the Th1 pathway.

Zn- and Mg-containing CaP adjuvants were reported by Wang *et al.* in 2013.[143] The adjuvants were loaded with a hydrothermal extract of a human tubercle bacillus and explored for cancer immunotherapy. The authors hypothesised that the inclusion of Zn and Mg would enhance the adjuvanticity of the CaP materials owing to the ability of Zn to enhance the activity of antigen presenting cells, and Mg's role in macrophage modulation. *In vitro* GM-CSF production by macrophage-like cells was increased by the inclusion of Zn or Mg, and certain of the systems were found to have enhanced anti-tumour activity. Flagellin-functionalised CaP nanoparticles have also been reported, and shown to effectively activate the innate immune system.[144]

The influence of CaP particle size and shape has also been explored. Crystalline needle-like particles were proposed to elicit a greater immune response than that achieved with spherical nanoparticles, with smaller particle sizes preferred.[145]

2.2.2 Zinc oxide

Zinc oxide is a non-toxic entity commonly used as a dietary supplement. It could thus be an ideal biocompatible adjuvant candidate.[146] There is a small literature in which zinc oxide (ZnO) nanoparticles have been explored as potential adjuvants. Matsumura and colleagues inoculated BDA/1J mice with the model antigen OVA and 1 – 3 mg of ZnO, and then sacrificed the animals 21 days after immunisation.[147] The addition of ZnO was found to significantly increase the production of anti-OVA IgG1 and IgE antibodies compared to mice injected with PBS alone. An increase in OVA-specific splenocyte proliferation was also observed with higher doses (3 mg) of ZnO. Enhanced splenocyte proliferation of IL-4, IL-5, and IL-17 were also observed 21 days after inoculation (although no statistical tests were presented). In contrast, ZnO did not yield an uplift in IgG2a production, nor of IFN-γ production. The authors thus concluded that ZnO provokes a Th-2 type response, similar to alum adjuvants. In the experiments reported, ZnO was found to perform approximately on a par with Imject alum used as a control. It was postulated that a small amount of dissolution may occur, resulting in Zn^{2+} being present in solution. This has previously been found to cause IL-4 production by T cells.[148]

A second study was performed by Roy *et al.* in 2014.[146] These authors used nano-sized ZnO (< 50 nm) and also immunized mice (Balb/c) with ZnO and OVA. The results were largely in agreement with those of Matsumura: OVA-specific IgG1 and IgE levels were significantly enhanced compared to OVA-alone when ZnO was co-administered with OVA (see Figure 11). Studies on splenocytes revealed that the use of ZnO resulted in increased IL-2, IL-4, IL-6 and IL-17 production, while IL-10 and TNF-α production declined. In the presence of ZnO, increased numbers of eosinophils and mast cells were detected in the lungs and spleen, and macrophages and B cells were shown to mature to a greater extent. T cell proliferation was also observed. These data further support the stimulation of a Th2 response when ZnO is used as an adjuvant.

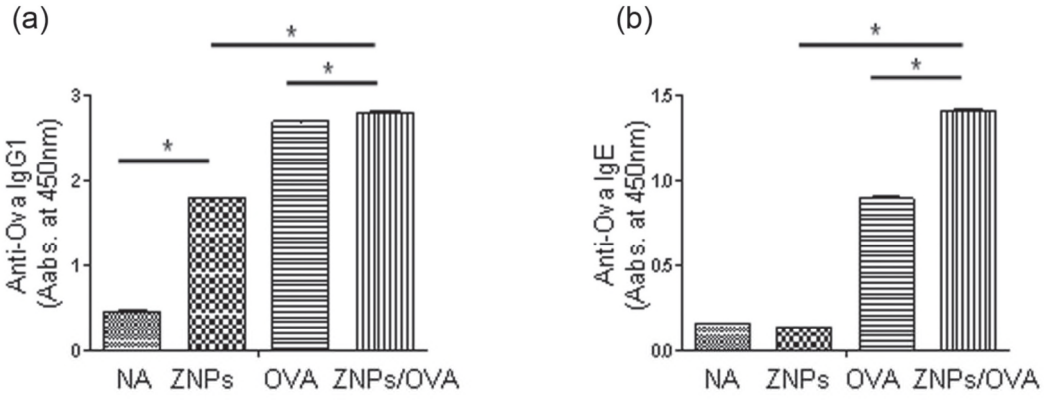


Figure 11: Serum antibody levels of anti-OVA (a) IgG1 and (b) IgE by Balb/c mice immunised with 0.25mg of ZnO nanoparticles (ZNPs) and 100 μg OVA. An increase in production of both antibodies can be seen when ZnO is used as an adjuvant with OVA. Taken with permission from [147]. Copyright Oxford University Press 2014.

2.2.3 Cobalt oxide

Cobalt oxide (Co_3O_4) nanoparticles have also attracted attention as possible adjuvants.[149] In a study with C57Bl/6 mice, Cho and colleagues vaccinated with OVA and Co_3O_4 nanoparticles (CNPs), using Imject alum as a comparator adjuvant. They found that the CNPs stimulated a more balanced Th1 and Th2 ratio than ZnO or Imject alum. While anti-OVA IgE and IgG1 production were less pronounced with CNPs than with Imject, CNPs initiated greater IgG2a production. IFN-γ levels in the peritoneal lavage fluid collected 7 days after antigen administration was also significantly higher with CNPs than with Imject alum, as depicted in Figure 12.

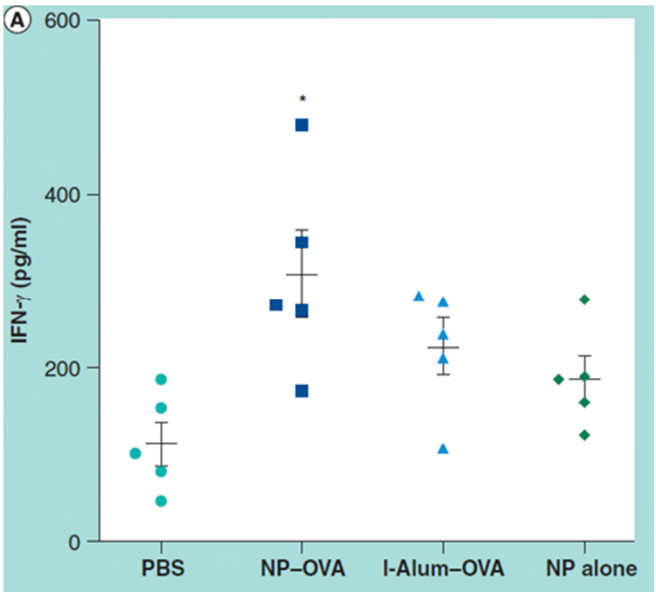


Figure 12: IFN-γ levels in in the intraperitoneal lavage fluid of mice 7 days after administration of antigen. PBS: saline alone; NP-OVA: OVA and Co_3O_4 NPs; I-Alum-OVA: OVA and Imject alum; NP alone: CNPs alone. Reproduced with permission from [149]. Copyright Future Science Group 2012.

The reduction in IgE production with CNPs indicates that there is less risk of allergic responses to the antigen when this is used as an adjuvant. Reduced lymphatic inflammation was also noted with CNPs.[149] Lung exposure to CNPs has also been shown to induce Th-1 and Th-17 hypersensitivity in Wistar rats,[150] as have NiO nanoparticles.

2.2.4 Other metal oxides

Other metal salts have generally received minimal attention as adjuvants, but a very preliminary investigation of SiO_2, $Mg_3Si_4O_{10}(OH)_2$, Al_2O_3, SnO_2, ZrO_2, Fe_2O_3 and Fe_3O_4 was performed by Naim and co-workers in the 1990s.[151] Rats were immunised with the metal oxide and OVA, or with PBS alone as a control. Attention was paid to the surface area of the adjuvants, and two separate samples of each of SiO_2 and ZrO_2 with different areas were explored. The SiO_2 and $Mg_3Si_4O_{10}(OH)_2$ materials appeared to promote inflammation and OVA-specific antibody production, while Al_2O_3 and the Fe oxides stimulated an intermediate response and the other oxides showed no effect.

Mesoporous silica (SiO_2) materials have been explored for the induction of a Th-1 anti-tumour response.[152, 153] Their immunogenicity has been reported to be dependent both on the particle and pore size of the silica. PAMPs were immobilised on apatite (Ap) within the silica pores and *in vitro* and *in vivo* experiments performed to probe the adjuvanticity of the resultant composites. The SiO_2-Ap-PAMP samples showed greater *in vitro* immunity than a composite of alum and PAMP, and could effectively inhibit tumour recurrence in mice. Particles of 30 – 200 nm were prepared, and those of 200 nm found to have minimum *in vitro* cytotoxicity[153] When GM-CSF production by macrophages derived from THP-1 cells was explored, all the SiO_2-Ap-PAMP samples caused a similar uplift in production. When pore size was explored, mesoporous silicas with 7 or 10 nm pore sizes caused a greater increase in GM-CSF production by THP-1-derived macrophages, at least at lower doses.[152] It should be noted that, while these studies claim that immunogenicity is particle-size and pore-size dependent, the only direct comparisons of the different samples are in limited *in vitro* studies, and the effects of neither parameter appear to be very pronounced in these measures. Although these papers show the silicas to be effective at hindering tumour recurrence, in each case only one silica sample was studied *in vivo*, making direct comparisons difficult.

2.2.5 Layered double hydroxides

Layered double hydroxides (LDHs) are a broad family of materials comprising positively charged mixed-metal hydroxide layers, with charge balancing anions between the layers. The general formula of an LDH is $[M_{1-x}^{2+}M_x^{3+}(OH)_2]^{x+}(A^{n-})_{x/n}\cdot yH_2O$, where M^{2+} represents a divalent metal cation, M^{3+} a trivalent metal cation and A^{n-} an anion. *x* ranges from 0.15 to 0.33 for pure LDH formation, and *y* is typically of the order 1 – 2. In essence the structure comprises a sandwich of metal hydroxide layers, with ions located between them. The structure of LDHs is given in Figure 13: it is clear that it has strong similarities with that of AlOOH (see Figure 2).

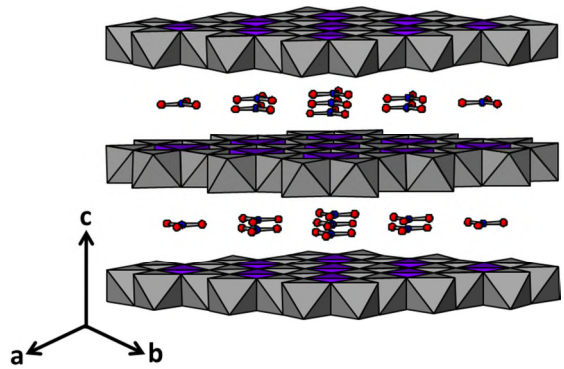


Figure 13: The generic structure of a layered double hydroxide. A material of composition $[M^{2+}_2M'^{3+}(OH)_6]NO_3{\cdot}yH_2O$ is shown; grey and purple polyhedra represent $M(OH)_6$ and $M'(OH)_6$ octahedra. N atoms are shown in blue, and O atoms in red. The metal cations and interlayer anions are considered to be fully ordered here (that this is not always the case). Interlayer water molecules are omitted for clarity.

The first study to explore LDHs as adjuvants was published in 2010 by a team from Shanghai.[154] These authors synthesised three different LDHs containing Mg and Al, with nitrate as the interlayer ion: $[Mg_3Al(OH)_8]NO_3{\cdot}yH_2O$ (Mg_3Al-NO_3), $[Mg_2Al(OH)_6]NO_3{\cdot}yH_2O$ (Mg_2Al-NO_3), and $[MgAl(OH)_4]NO_3{\cdot}yH_2O$ ($MgAl$-NO_3) with particle sizes of *ca.* 60 nm. They then investigated the influence of these materials on murine dendritic cells. The LDH particles were taken up by dendritic cells, with maximum uptake reached after around 2h at 37 °C. The co-stimulatory molecules CD86 and CD40 (but not CD80 or MHC-II) were upregulated upon exposure to the $MgAl$-NO_3 LDH. In contrast, no such upregulation was observed with the Mg_2Al-NO_3 or Mg_3Al-NO_3 materials. Consistent results were observed for the production of cytokines by DC in response to the LDH samples: the $MgAl$-NO_3 LDH causes a significant and dose-dependent increase in the production of TNF-α and IL-12p70, while no change is seen when DC are exposed to the other LDHs (see Figure 14).

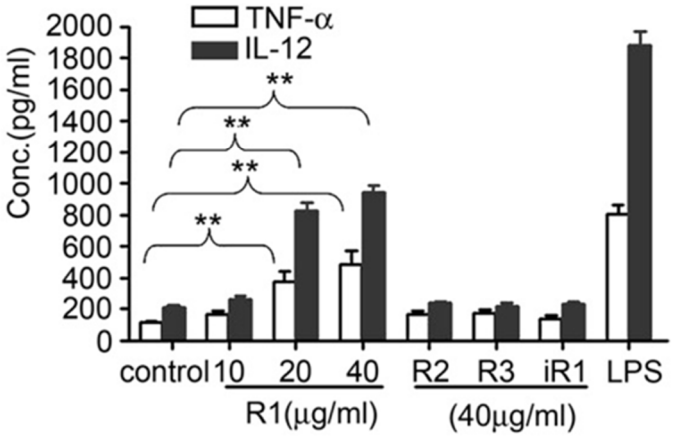


Figure 14: DC production of TNF-α and IL-12p70 after 24 hours exposure to $MgAl$-NO_3 (R1), Mg_2Al-NO_3 (R2) and Mg_3Al-NO_3 (R3). Reproduced with permission from ref [154]. Copyright Elsevier 2010.

The migration of DCs towards CCL21 (as assessed in transwell plates) was also seen to increase following treatment with $MgAl$-NO_3, but not with the other LDHs. This phenomenon was found to be

CCR7 dependent [154]. It should be noted, however, that the MgAl-NO_3 material is not phase pure: a significant amount of $Al(OH)_3$ was observed to be present in the sample as well as the LDH. It is thus unclear to what extent the immunogenicity of this sample was due to the presence of LDH, or whether the impurity is important in this regard.

Li *et al.* have built on these early studies to explore the utility of LDHs in DNA vaccines.[155] MgAl-NO_3 was found to readily form complexes with DNA, and the resultant complex had high transfection efficiency as evidenced using a green-fluorescent protein plasmid as a reporter. When C57Bl/6 mice were immunised with OVA and the DNA/LDH complex and subsequently challenged with B16-OVA melanoma cells, the complex was found to induce significant increases in serum anti-OVA IgG1 and IgG2c titres over mice immunised with DNA-alone. Survival rates were also improved by use of the LDH. IgG2c was produced in greater amounts than IgG1, suggesting that a Th-1 polarised response was being provoked. This was confirmed by the observation of an increase in IFN-γ (but not IL-4) production by spleenocytes. OVA-specific CD8+ T cell activity was also increased in the presence of the LDH adjuvant.[155] Again, however, the LDH sample used shows clear signs of phase impurity.

Another advance in the use of LDHs as adjuvants was reported by Wang and co-workers in 2014 [156]. Core-shell nanoparticles of SiO_2 (core) and Mg/Al LDH (shell) were prepared (denoted SiO_2@LDH; the ratio of Mg:Al is unclear in this work). They were found to be non-cytotoxic, and to protect DNA molecules from degradation when incubated with DNAse enzymes. When macrophages (RAW264.7 cell line) were exposed to the SiO_2@LDH particles, increased levels of IFN-γ, IL-12p70 and TNF-α were observed; IL-6 production was also enhanced at higher doses. CD86 and MHC-II were also upregulated. BALB/c mice were immunised with the SiO_2@LDH nanoparticles and Hepatitis B viral (HBV) DNA, which was found to result in an increase in anti-Hepatitis B IgG titres.

The most recent work in this area was reported by Williams and co-workers.[157] These authors exploited the fact that LDHs have huge structural and chemical diversity, and investigated the immune response to a range of LDHs both *in vitro* (using human monocyte-derived DC and macrophages) and *in vivo* (with OVA in C57Bl/6 mice). A systems vaccinology approach was implemented in an attempt to ascertain whether the immune response could be correlated with the LDH physicochemical properties. This approach is illustrated in Figure 15.

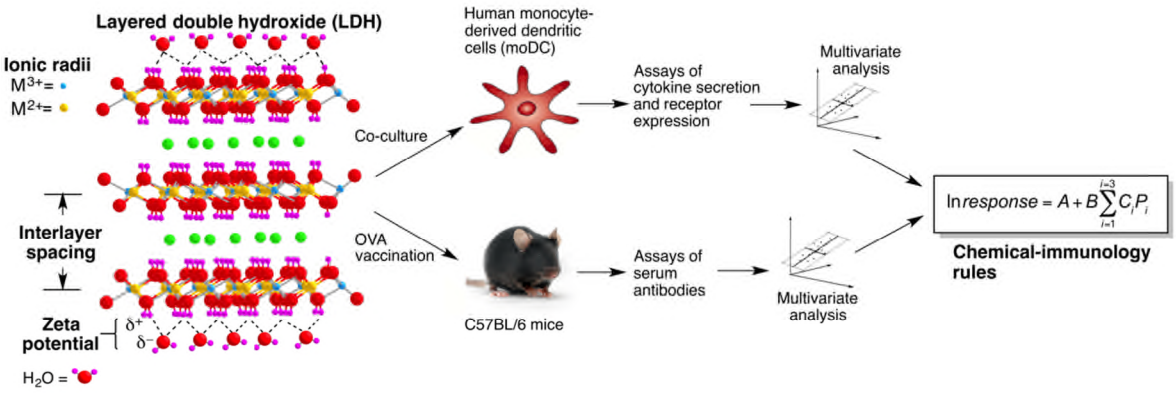


Figure 15: The systems vaccinology approach adopted by Williams *et al.* A series of LDH materials was prepared, and the response of human DC in co-culture or of mice upon OVA vaccination quantified. The physicochemical properties of the LDHs were then considered to be causative of the immune response, and structure/property relationships elucidated. Reproduced with permission from ref [157]. Copyright Rockefeller University Press 2014.

It was observed that different LDHs promote quite distinct immune responses, with some (notably $[LiAl_2(OH)_6]_2CO_3{\cdot}yH_2O$) stimulating very significant cytokine production and co-stimulatory molecule upregulation by DC while others ($[Mg_2Fe(OH)_6]Cl{\cdot}yH_2O$, Mg_2Fe-Cl) have almost no effect. Similarly *in vivo*, certain LDHs caused large uplifts in antibody titres, while Mg_2Fe-Cl was largely ineffective. The LDHs generally performed in these assays at least as well as Imject alum and Alhydrogel. *In vivo*, some LDHs were found to produce greater amounts of anti-OVA IgG1 and IgE (Th-2 type antibodies) than the standard commercial adjuvants, while others ($[Mg_2Fe(OH)_6]_2CO_3{\cdot}yH_2O$ in particular) yielded high IgG2c titres, indicative of a Th1 polarised response.

Most remarkably, it was found that the immune response (both *in vitro* for DC and macrophages and *in vivo*) was very highly correlated with three of the physicochemical properties of the LDHs: the zeta potential, the interlayer spacing, and the radius of the monovalent or divalent metal cation (see Figure 15). An equation could be devised which allowed the magnitude of a particular immunological output to be calculated from these LDH properties (see Figure 15). To test the robustness of this approach, the authors performed blinded trials, where they predicted the responses to two new, untested, LDHs using the mathematical model described above before performing immunological assays *in vitro*. The responses observed were found to be extremely close to those predicted mathematically as depicted in Figure 16, showing that it is possible to predict *a priori* the immune response to an LDH if its physicochemical properties are known.

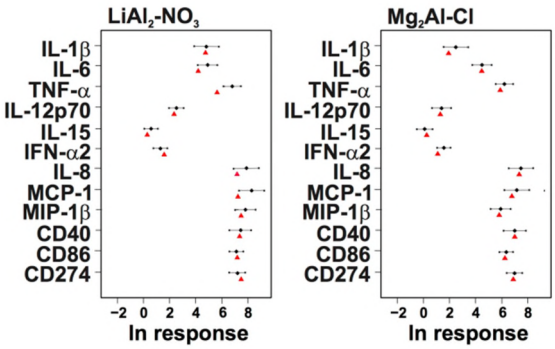


Figure 16: Data showing that the *in vitro* DC response to an LDH can be accurately predicted from its physicochemical properties. The production of a range of different cytokines and co-stimulatory molecules was quantified, with mean results shown as black squares and 95 % confidence intervals as bars. The red triangles indicated the response predicted mathematically; it is clear that these lie within the confidence intervals in the majority of cases. Reproduced with permission from [157]. Copyright Rockefeller University Press 2014.

These most recent results suggest that LDHs have enormous potential as bespoke adjuvants. The radii of metal ions are well documented, and interlayer spacing and zeta potential can be easily measured for a sample within a few minutes. Interlayer spacing can also be calculated *in silico*; while this is not yet possible to calculate zeta potential in this way, work is ongoing to remedy this issue. Ultimately, *in silico* screening of thousands of possible LDHs should be possible, permitting the material required to deliver a particular immune response to be identified, and subsequently synthesised and tested *in vitro* and *in vivo*.

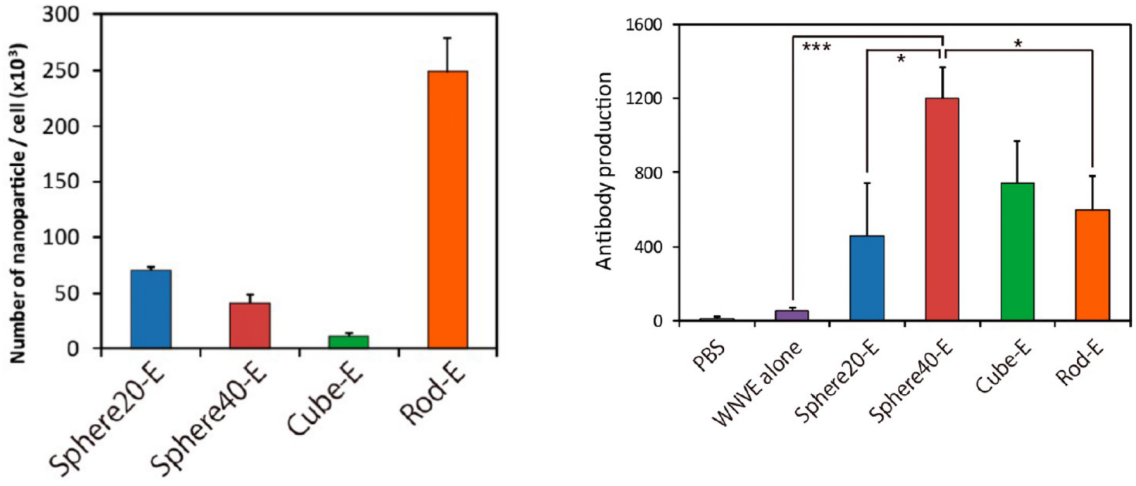
2.2.6 Gold nanoparticles

Gold nanoparticles (Au NPs) have in recent years attracted increasing attention as potential adjuvants. A number of studies have used them as antigen-carriers. For instance, Bastus and co-workers conjugated the amyloid growth inhibitory peptide and the sweet arrow peptide on 10 nm Au particles, and discovered that the NPs were able to be recognised by macrophages via the TLR-4 PRR.[158] In another study, 15 nm Au NPs loaded with a peptide were explored for use in spinal cord injury therapeutic vaccination.[159] The influence of Au NP size has been explored in the context of foot-and-mouth disease (FMD).[160] A range of NPs between 2 and 50 nm were conjugated with a synthetic peptide antigen for the FMD-virus, and the production of anti-FMD antibodies by BALB/c mice measured. Maximum antibody titres were observed when the particle size was between 8 and 17 nm. No anti-Au NP antibody response was seen in any set of experiments.

The surface engineering of Au nanorods has been posited to be potentially useful in DNA vaccines for HIV-1 [161]. However, in some cases it appears that Au NPs are insufficiently immunogenic to illicit a response: Parween *et al.* report that for the C-terminal 19 kDa fragment of merozoite surface protein it is necessary to use both Au NPs and alum to stimulate a robust antibody response [162]. Consistent with this, a recent study demonstrated that Au nanoparticles loaded with the matrix 2 protein and mixed with the CpG adjuvant can induce protective immunity against with influenza A

virus, while NPs alone are much less potent.[163] Huang *et al.* have directly conjugated CpG to Au NPs,[164] and found the conjugate to effectively increase cytokine (IL-6 and TNF-α) secretion in RAW264.7 cells. This study concluded that Au NP-CpG composites could act as efficient TLR-9 agonists. 15 nm NPs were more potent than those of size 30 nm. However, it should be noted that a separate study has indicated that Au NPs inhibit the TLR-9 pathway in the same cell line, with small NPs of 4 nm being more inhibitory than those of 11, 19, 35 or 45 nm.[165]

A very recent study explored the effect of different shapes and sizes of Au nanoparticles on the immune response both *in vitro* and *in vivo*.[166] Spherical (20 and 40 nm diameter), rod-shaped (40 x 10 nm) and cubic (40 nm) NPs were prepared. The rod-shaped AuNPs were internalised to the greatest extent by both the murine macrophage cell line RAW264.7 (Figure 17(a)) and bone-marrow derived DCs. Once internalised, the rod-shaped AuNPs stimulated the production and subsequent release of inflammasome-related cytokines (IL-1β and IL-18) from bone-marrow derived DCs (BMDCs). The 40nm-diameter spherical AuNPs and cubic AuNPs were not readily uptaken by the murine RAW264.7 macrophage cell line; however, they did cause increased secretion of the pro-inflammatory cytokines TNF-α, IL-6, IL-12 and GM-CSF. The 40 nm spherical particles were most effective in the induction of IgG antibodies to West Nile virus (WNV) when the NPs were complexed with WNV protein and injected into C3H/HeNJc1 mice. Thus, it was hypothesised that the 40nm-diameter spherical and cubic particles activate serum antibody responses via secretion of pro-inflammatory cytokines (see Figure 17(b)), whereas the rod AuNPs act via the inflammasome.[166]



(a) (b)

Figure 17: (a) Nanoparticles internalisatio by the murine macrophage cell line RAW264.7; (b) comparison of serum antibody levels in response to innoculation with PBS, West Nile virus (WNV) and the Au NP / WNV protein complexes with different morphologies; graphical data emphasises how the 40nm-diameter spherical particles stimulate the greatest antibody production. Reproduced with permission from ref [166]. Copyright American Chemical Society 2013.

2.2.7 Silver nanoparticles

Silver nanoparticles (AgNPs) have been shown to induce a pro-inflammatory response in human THP-1-derived macrophages.[167] This is characterised by elevated expression of IL-6, IL-10 and TNF-α, as shown in Figure 18. *In vivo* studies of Au NP adjuvanticity have also been undertaken [168]. Balb/c mice were immunised with either Ag NP/OVA or Ag NP/bovine serum albumin (BSA). The Ag NPs induced a Th-2 skewed immune response, similar to alum. Using FITC-labelled OVA, Xu showed that the presence of Ag NPs did not enhance OVA-uptake by macrophages; they thus hypothesised that AgNPs are likely to elicit their adjuvanticity via chemotaxis of leukocytes, which sees migration of white bloods cells to the site of injection via chemokine secretion.[168]

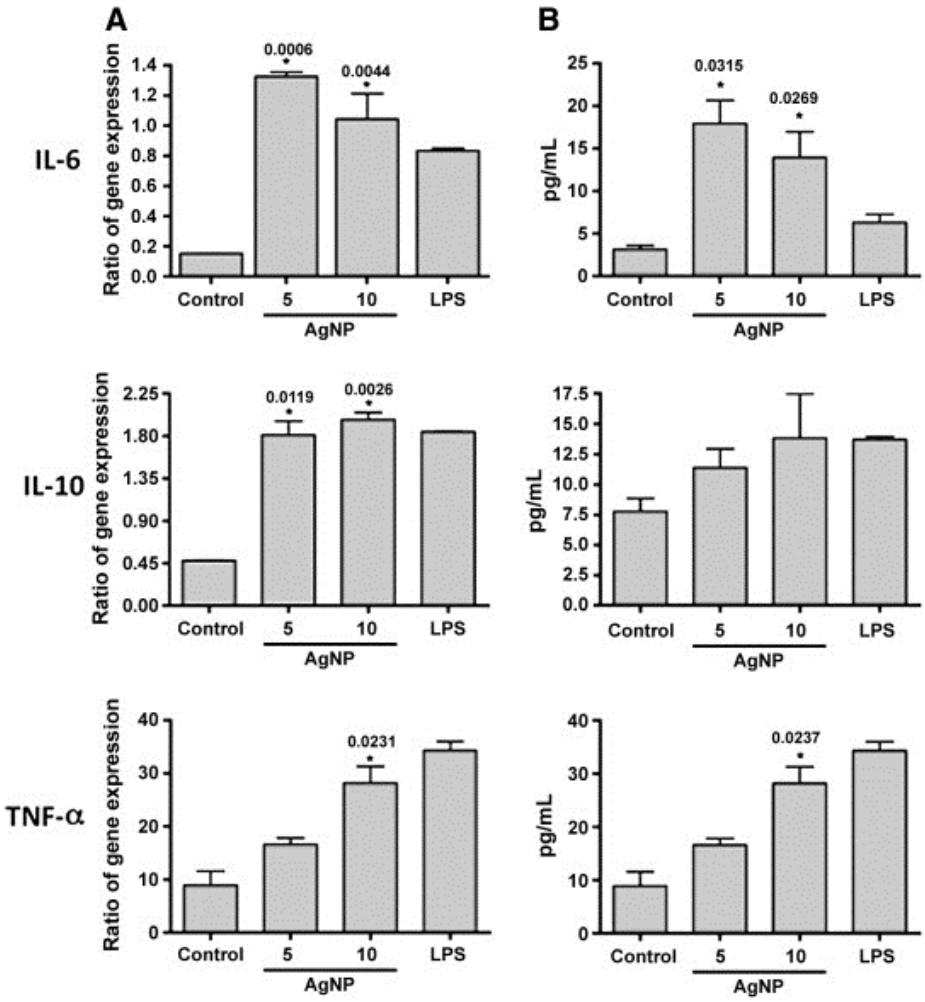


Figure 18: The inflammatory effects of Au NPs on THP-1 derived macrophages. (A) gene expression and (B) secreted cytokine levels were quantified for 5 and 10 μg/ml concentrations of 24 nm Ag NPs, with lipopolysaccharide (LPS) used as a positive control. Reproduced with permission from ref [167]. Copyright Elsevier 2012.

3. Conclusions and future outlook

In this review, the role of inorganic particulate matter as vaccine adjuvants is considered. Attention is paid to alum, a family of Al-containing adjuvants which, although often poorly defined and chemically inhomogenous, have saved countless lives since they were first used in 1932. The chemistry, utility, and proposed mechanisms of action for alum are discussed, together with recent developments in combining alum with other adjuvants to elicit improved immune responses. Despite its length of use, and significant enhancement in understanding over the past 8 years or so, the route by which alum acts as an adjuvant is still opaque. In part, this is due to variation in the nature of the alums used for research investigations, and differences in experimental protocols. Research work aiming to improve alum adjuvanticity through particle size and shape engineering is highlighted. From this, it is evident that the immune response provoked by alum can be controlled through alterations in its particle size and shape, leading to advantages such as increased antibody production and reduced local inflammation at the injection site.

Calcium phosphate, another commercially used inorganic adjuvant, is briefly discussed and current efforts to enhance its efficacy by co-precipitating it with other adjuvants or antigens are elucidated. Several other metal oxides, including ZnO, have been considered to be potential adjuvants, and a brief survey of these is given. The past four years has seen exploration of layered double hydroxides (LDHs), a family of mixed-metal hydroxides with huge structural variability, as adjuvants, and the work on these to date is explored. Very recent results showing that LDH adjuvanticity can be predicted if key LDH physicochemical properties are known offer the exciting potential to design bespoke adjuvants *in silico*, leading to reductions in required animal experimentation and reduced side effects. Finally, the adjuvant potential of metal nanoparticles is discussed.

It is clear that in recent years in particular there has been a huge explosion of research interest in the use of inorganic particulate materials as adjuvants. Given the limitations of alum, in that it can only induce strong humoral (antibody) immune responses, but not the cellular immunity required to combat intracellular pathogens or tumours, this work is vitally important. The use of particle size and shape engineering has tremendous potential to generate adjuvants which can trigger enhanced or bespoke immune responses, as does chemical composition control. It is the view of the authors that use of these approaches will expand exponentially in the years to come, hopefully leading to the exploration of these new materials in the clinic. It is truly an exciting time to be involved in adjuvant development.

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