TIMELINE

Towards an understanding of the adjuvant action of aluminium

Philippa Marrack, Amy S. McKee and Michael W. Munks

Abstract | The efficacy of vaccines depends on the presence of an adjuvant in conjunction with the antigen. Of these adjuvants, the ones that contain aluminium, which were first discovered empirically in 1926, are currently the most widely used. However, a detailed understanding of their mechanism of action has only started to be revealed. In this Timeline article, we briefly describe the initial discovery of aluminium adjuvants and discuss historically important advances. We also summarize recent progress in the field and discuss their implications and the remaining questions on how these adjuvants work.

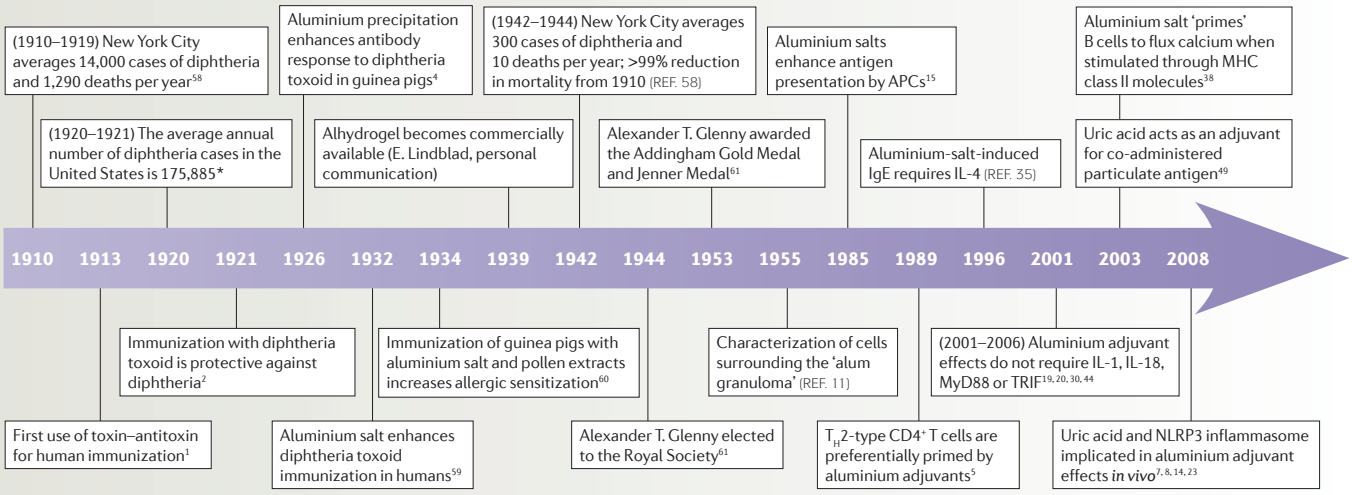
Vaccines that consist of attenuated pathogens, such as the Sabin 'live' polio vaccine, or killed pathogens, such as the Salk inactivated polio vaccine, contain endogenous adjuvants. However, vaccines that contain purified antigens, such as the diphtheria–tetanus–pertussis vaccine or the hepatitis A and hepatitis B vaccines, usually require the addition of an exogenous adjuvant to increase the immune response to the antigens following immunization.

In the early 1900s, infections by *Clostridium tetani* and *Corynebacterium diphtheriae* were serious health issues owing to the pathology that is induced by tetanus and diphtheria toxins, respectively (TIMELINE). Immunization with conjugates of toxin and antibody, which were referred to as toxin–antitoxin, yielded better protection and fewer side effects than low-dose toxin alone[1], and it was proposed that the antitoxin enhanced the immune response of the recipient by slowly releasing antigen over time. In the following years, further improvement came with the production of toxins that had been inactivated with formalin or heat (referred to as toxoids), which could be used for immunization[2,3].

In 1926, Alexander T. Glenny and colleagues reported that precipitation of antigen onto insoluble particles of aluminium potassium sulphate (BOX 1), known as 'potash alum', before immunization produced better antibody responses than soluble antigen alone, providing the first clue to the adjuvant properties of aluminium salts[4]. Following this discovery, aluminium salts were used in vaccine preparations with tetanus and diphtheria toxoids to protect against *C. tetani* and *C. diphtheriae*, respectively, and today insoluble aluminium salts are used worldwide as the principle adjuvants in clinical vaccines.

Glenny believed that aluminium salts were effective adjuvants because they allowed antigen to remain in the body for a long time and because the antigen was slowly released from the insoluble salt

Timeline | **History and important scientific advances of aluminium adjuvants**



*See Centers for disease control and prevention website. APC, antigen-presenting cell; IL, interleukin; MyD88, myeloid differentiation primary-response gene 88; NLRP3, NLR family, pyrin domain containing 3; T_H2, T helper 2; TRIF, TIR-domain-containing adaptor protein inducing IFNβ.

Box 1 | **Different insoluble aluminium salts have distinct characteristics**

Several insoluble aluminium salts are used as adjuvants in human and animal vaccines, but the term 'alum' only applies to aluminium potassium sulphate (reviewed in REF. 46) (see the table). Alum was used by Alexander Glenny and captures antigen by precipitation when the solution is neutralized. Owing to problems in manufacturing reproducibility, alum has been almost completely replaced by aluminium hydroxide and aluminium phosphate for commercial vaccines. These can be prepared in a more standardized manner and capture antigen by direct adsorption. Many types of adsorptive interactions occur between the antigen and the adjuvant, but two adsorption forces seem to predominate[46]. The first, electrostatic interactions, occurs most strongly between negatively charged proteins and aluminium hydroxide, and between positively charged proteins and aluminium phosphate. The second, anionic ligand exchange, is a covalent interaction that occurs when phosphates from an antigen substitute for hydroxyl groups on the adjuvant.

Common name	Used in humans?	Chemical formula	Chemical name
Alum	Yes	$AlK(SO_4)_2$	Aluminium potassium sulphate
Alhydrogel	Yes	$Al(OH)_3$	Aluminium hydroxide
Adju-Phos	Yes	$Al(PO_4)_3$	Aluminium phosphate
Imject Alum	No	$Al(OH)_3$ and $Mg(OH)_2$	Aluminium hydroxide and magnesium hydroxide

particles, which allowed prolonged and effective stimulation of the immune system, an effect referred to as the 'depot effect'. For approximately 60 years, this explanation was dogmatically accepted within the field, and only a few basic research papers on aluminium salts were published, often decades apart.

In the past two decades or so, interest in aluminium salts has reignited, and since 2007 there has been a flurry of activity aimed at understanding the adjuvant action of these compounds. In addition to or in contrast to the depot effect, insoluble aluminium salts activate innate immune cells in a manner that ultimately results in a T helper 2 (T_H2)-type immune response[5]. In 2008 it was suggested that the cytotoxicity of aluminium salts leads to the release of uric acid *in vivo*, which acts as a damage-associated molecular pattern (DAMP, reviewed in REF. 6) that is required for the adjuvant activity of aluminium[7]. A separate paper showed a requirement for caspase 1 activation *in vivo*, which is mediated by NLR family, pyrin domain containing 3 (NLRP3; also known as NALP3) and apoptosis-associated speck-like protein containing a CARD (ASC; also known as PYCARD), collectively known as the NLRP3 inflammasome[8] (BOX 2).

However, the field is not without controversy. Other studies have failed to confirm a requirement for uric acid *in vitro* or for activation of the NLRP3 inflammasome *in vivo*, and research in these areas is ongoing. In addition, several unresolved issues remain. For example, it is not completely clear how aluminium salts activate the inflammasome, why the Toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS) is sometimes required for inflammasome activation *in vitro*, what the potentially relevant downstream targets of caspase 1 are and why aluminium salts induce T_H2-type rather than T_H1-type responses. Here, we describe what is currently known and discuss what remains to be discovered about the effects of aluminium salts on innate and adaptive immune responses.

Challenges to the depot theory

Glenny's proposal that the adjuvant activity of aluminium salts was due to antigen persistence and prolonged release was not without basis. Initial evidence showed that precipitated toxoid persisted for several days[9], much longer than soluble toxoid. A subsequent study found that 7 weeks after immunization, alum nodules that were excised from one guinea pig and were ground up could be used to immunize a second guinea pig[10].

Experiments by White *et al.*[11] showed that immunization of rabbits with antigen plus aluminium salts induced the appearance of B-cell blasts in the draining lymph nodes within 7 days of immunization and at the site of the granuloma by day 14. However, few B-cell blasts remained in the draining lymph nodes after 3 weeks, which implies that antigen is no longer being presented in the lymph nodes and that negligible amounts of antigen escape the site of injection after ~2 weeks[11]. These results are consistent with data from other studies that showed that removal of the antigen–aluminium salt nodules 14 days after immunization had no effect on the subsequent antibody titres[12]. Therefore, these data indicate that aluminium salts do not function simply by providing a long-lived antigen depot.

More recent experiments have shown that within hours of administration of aluminium salts to mice, pro-inflammatory mediators, such as interleukin-1β (IL-1β)[13], CC-chemokine ligand 2 (CCL2; also known as MCP1), CCL11 (also known as eotaxin)[7], histamine and IL-5 (A.S.M., unpublished observations), are detectable. Also, innate inflammatory cells, such as neutrophils, eosinophils, inflammatory monocytes, myeloid dendritic cells (DCs) and plasmacytoid DCs, are recruited to the site of aluminium salt injection within 1 day[7]. Although the mediators that contribute to the recruitment of these infiltrating cell types have not yet been completely defined these data clearly show that aluminium salts have additional effects that account for their adjuvant properties.

Aluminium salts and innate immunity

Effects of aluminium salts on antigen-presenting cells. The ability of aluminium salts to promote antigen uptake and presentation by human macrophages was first demonstrated over 20 years ago[15]. More recently, it has been shown that exposure of peripheral blood mononuclear cells (PBMCs) to aluminium salts *in vitro* leads to their upregulation of MHC class II molecules, CD40 and CD86 (REF. 16). Studies using mouse DCs *in vitro* have yielded some conflicting results, with one study finding that aluminium salts did not induce the expression of co-stimulatory molecules or enhance antigen presentation[17], but another showing that CD86 was upregulated and antigen presentation by DCs was increased[18]. An *in vivo* study found that peritoneal DCs and B cells internalized antigen that was adsorbed to aluminium salts more readily than soluble antigen alone, and aluminium salt increased the expression of CD86 by DCs[7]. In addition, peritoneal injection of aluminium salt induced the recruitment of monocytes to the peritoneum. In the presence of aluminium salts, these monocytes acquired antigen, trafficked to the draining lymph node and differentiated into $CD11c^+$ MHC class II^+ DCs in a myeloid differentiation primary-response gene 88 (MyD88)-dependent manner. These recruited monocytic DC precursor cells (and not lymph-node-resident or spleen-resident DCs) were shown to be responsible for priming naive $CD4^+$ T cells in the lymph node.

Is there a specific receptor? The activity of many immunological adjuvants (for example, LPS and unmethylated CpG motifs) requires signalling through TLRs and activation of one or both of their downstream adaptor molecules, MyD88 and TIR-domain-containing adaptor protein inducing IFNβ (TRIF; also known as TICAM2). Therefore, investigators thought that aluminium salts might function by acting as ligands for one or more TLRs. However, two studies indicate that aluminium salts do not act through TLRs[19,20]. In the first study, MyD88-deficient mice produced normal amounts of IgG1 and, interestingly, excessive amounts of IgE in response to immunization with antigen and aluminium salts[19]. The second study used mice that were deficient for both MyD88 and TRIF, which therefore could not signal through TLRs[20]. In this case, antigen-specific antibody responses of all isotypes induced following vaccination with the antigen trinitrophenyl–haemocyanin and aluminium salt were comparable to those observed in control mice. Although the use of haemocyanin slightly confounds the interpretation of these results, as haemocyanin itself has adjuvant properties that might be TLR independent, together these studies strongly suggest that the antigen-specific antibody response to aluminium salts does not depend on TLRs.

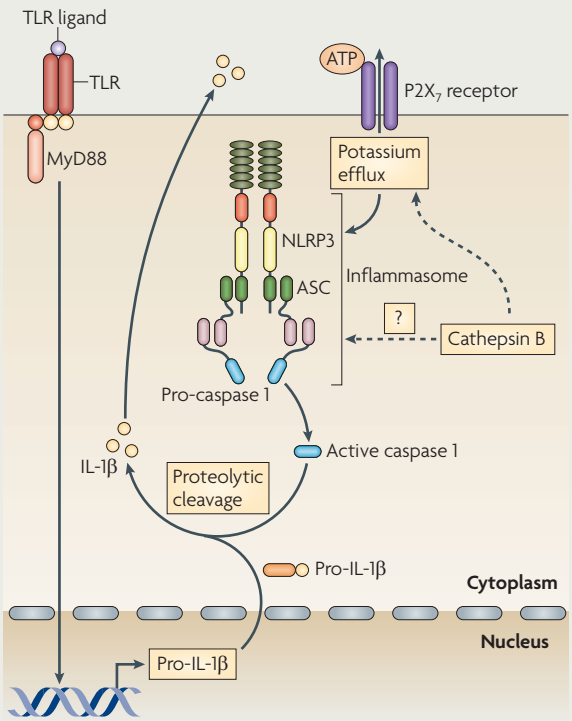
It was recently shown that monosodium urate (MSU) crystals, which are formed when the concentration of uric acid is saturating (BOX 2), bind to DCs in an unconventional manner[21]. Using atomic force microscopy to measure interaction force, MSU crystals were found to interact strongly with the plasma membrane, even when surface proteins were removed. This occurred through a direct interaction with cholesterol and potentially other lipids. So, it is possible that DCs or other phagocytic cells bind to aluminium salts through a similar mechanism, but this has yet to be determined experimentally. Latex beads were also shown to interact strongly with the membrane of phagocytes, but basic calcium phosphate crystals and allopurinol crystals did not produce detectable interaction forces[21]. Therefore, it is currently an open question whether aluminium salts interact with cells through a specific and unidentified receptor or through a less conventional manner, similarly to MSU crystals.

NLRP3 inflammasome activation: direct or indirect? Several reports showed that *in vitro* exposure of cells to aluminium salts induced the activation of caspase 1 and the release of its known downstream targets, IL-1β, IL-18 and IL-33 (REFS 18,22,23). These reports and subsequent studies[8,14,24,25] showed that NLRP3 and ASC are required for the activation of caspase 1 and its target cytokines. Macrophages primed with LPS and exposed to aluminium salts *in vitro* produced IL-1β, as well as IL-18 and IL-33 in studies that examined the production of these cytokines, in a NLRP3- and caspase 1-dependent manner[8,23,24] (FIG. 1). Intact endocytic machinery[8,22] and subsequent potassium efflux[8], which is a known co-activator of caspase 1, are thought to be required for this IL-1β production. However, the ATP receptor $P2X_7$ (a purinergic receptor), which is known to also have a role in the activation of the NLRP3 inflammasome, was not involved in IL-1β production in these studies[8,24]. Related reports have shown that other particulate materials, such as silica, asbestos and MSU crystals, also act through the NLRP3 inflammasome to produce IL-1β[14,26,27,52]. Together, these data suggest that aluminium salts and other particulates can initiate caspase 1 activation through the NLRP3 inflammasome. However, given that aluminium salts can induce an antibody response in the absence of TLR signalling adaptor molecules[19,20], it remains unclear why a TLR signal, such as that induced by LPS, is required for the production of pro-IL-1β and the activation of caspase 1 *in vitro*[8,22–24].

Box 2 | **The NLRP3 inflammasome and the immunomodulatory activity of uric acid**

NLR family, pyrin domain containing 3 (NLRP3; also known as NALP3) is a member of the nucleotide-binding domain and leucine-rich repeat containing gene (NLR) family and is an intracellular protein that can sense various pathogens, such as *Listeria monocytogenes* and *Staphylococcus aureus*, pathogen products, such as lipopolysaccharide (LPS) and muramyl dipeptide, and crystals, such as monosodium urate (MSU) and calcium pyrophosphate dihydrate[47]. When activated, NLRP3 associates with the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC; also known as PYCARD), which interacts with inactive pro-caspase 1. This facilitates the autocatalysis of pro-caspase 1 and results in the generation of active caspase 1. *In vitro*, expression of pro-interleukin-1β (pro-IL-1β) and activation of caspase 1 depend on Toll-like receptor (TLR) signalling, which is usually triggered by LPS. Caspase 1 cleaves pro-interluekin-1β (IL-1β), pro-IL-18 and pro-IL-33 to generate the active cytokines, which are then secreted through a non-canonical secretion pathway that is poorly understood. For a simplified version of what is known about the action of the NLRP3 inflammasome, see the figure.

Uric acid is a catabolic product of nucleotides and is soluble at high concentrations within cells. Cell damage or death causes the release of uric acid into the extracellular milieu, where it is much less soluble and forms MSU crystals, which are the causative agent of gout[48]. Uric acid has been shown to have adjuvant activity when co-injected with particulate antigen[49]. However, this activity only occurred above saturating levels, strongly implying that MSU crystals and not soluble uric acid function as the adjuvant. Uric acid promotes $CD4^+$ and $CD8^+$ T-cell responses[50,51], as well as antibody responses[28]. *In vitro*, stimulation of a monocytic cell line or of peritoneal macrophages with MSU crystals led to the secretion of IL-1β and IL-18, and this required all three components of the NLRP3 inflammasome: NLRP3, ASC and pro-caspase 1 (REF. 52). MyD88, myeloid differentiation primary-response gene 88; $P2X_7$, a purinergic receptor.



How do aluminium salts activate the NLRP3 inflammasome? At present there are two different models, both of which are compatible with the finding that phagocytosis is required for this process. In the direct activation model, phagocytic cells directly engage and engulf aluminium salt particles[14]. It is currently unclear whether this occurs through the interaction of aluminium salt particles with a specific receptor on the surface of phagocytic cells or through their non-specific adsorption to cell-surface molecules (see above), as has been shown with MSU crystals[21]. This engulfment leads to lysosomal damage and rupture, followed by the release of antigen and lysosomal enzymes such as cathepsin B into the cytoplasm. In some studies, the release of IL-1β was found to depend partially on cathepsin B activity and to depend strongly on NLRP3 and ASC[14,13]. Rupture of endolysosomes by silica crystals and osmotic shock produced similar results, suggesting that aluminium-salt-induced lysosomal damage may activate a default pathway that senses intracellular danger.

The indirect activation model proposes that aluminium salt cytotoxicity leads to the release of endogenous DAMPs such as uric acid (BOX 2), which results in the activation of the NLRP3 inflammasome. Following intraperitoneal injection of aluminium salts and antigen into mice, the local concentration of uric acid was shown to increase substantially[7]. Furthermore, pre-treatment of mice with uricase to degrade uric acid inhibited $CD4^+$ T-cell priming. Presumably, aluminium salts cytotoxicity causes uric acid release and the formation of MSU crystals, which could then be sensed by macrophages or other leukocytes to initiate innate and adaptive immune responses.

Notably, the study did not report what effect, if any, the uricase pre-treatment had on antibody titres, even though MSU crystals had been previously reported to enhance antibody responses to co-injected antigen[28] and antibodies are thought to provide the primary protective mechanism induced by aluminium salts. There are also other questions regarding the role of uric acid. First, *in vitro* treatment of cells with uricase did not prevent aluminium-salt-induced IL-1β release[8,14,25]. Second, the induction of inflammation and the adjuvant action of MSU crystals is reported to require IL-1–IL-1 receptor (IL-1R) engagement and the MyD88 signalling pathway[29]. However, the adjuvant activity of aluminium salts does not seem to require either MyD88 (REF. 20) (see above) or IL-1–IL-1R engagement *in vivo*[30,31] (see below). Therefore, it remains to be elucidated why uric acid is dispensable for aluminium-salt-induced inflammation *in vitro* but is required *in vivo* and whether there is any requirement for uric acid in antibody production following immunization with aluminium salts.

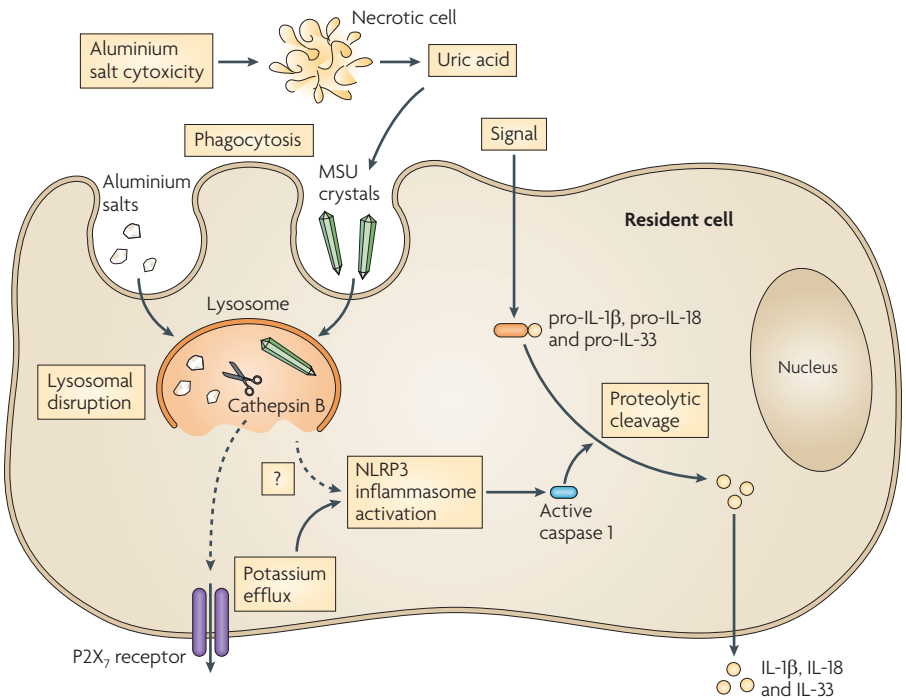


Figure 1 | **Activation of the NLRP3 inflammasome by aluminium salts.** Aluminium salt cytotoxicity leads to the release of danger-associated molecular patterns such as uric acid by the necrotic cell. At high concentrations uric acid forms monosodium urate (MSU) crystals, which are phagocytosed by resident cells. In addition, aluminium salts are directly phagocytosed by the resident cells. MSU crystals or aluminium salts disrupt lysosomes, which results in the release of cathepsin B. Cathepsin B may directly or indirectly induce potassium efflux, which activates the NLR family, pyrin domain containing 3 (NLRP3; also known as NALP3) inflammasome. A signal of unknown origin induces the production of pro-interleukin-1β (pro-IL-1β), pro-IL-18 and pro-IL-33. Caspase 1, which is activated by the NLRP3 inflammasome, cleaves pro-IL-1β, pro-IL-18 and pro-IL-33, thereby inducing the release of the active cytokines and promoting their secretion. $P2X_7$, a purinergic receptor.

Aluminium salts and adaptive immunity

IL-4 and the T_H2-type bias. It has been known for many years that aluminium salts induce robust antibody responses. The discoveries that antibody production depends on T-cell help[32] and that T_H-cell subsets with different functions exist[33] led to studies to determine the effects of aluminium salts on different T_H-cell subsets. Aluminium salts were found to preferentially induce T_H2 cells[5], which mediate the differentiation of B cells that secrete T_H2-cell-associated antibody isotypes IgG1 and IgE.

Because IL-4 production is a key feature of T_H2-cell responses *in vivo*[34], its role in immune responses that are induced by aluminium salts was evaluated[35,36]. Although immunization of IL-4-, IL-4Rα- or signal transducer and activator of transcription 6 (STAT6)-deficient mice with antigen in the presence of aluminium salts did not trigger the production of IgE, the T_H2-cell-associated antibody isotype IgG1 was present in the serum, albeit at reduced levels. However, these mice also produced high titres of the T_H1-cell-associated antibody isotype IgG2a in response to aluminium salt, which was in contrast to wild-type mice. Mirroring this change in antibody isotype production, antigen-specific T cells from IL-4-, IL-4Rα- or STAT6-deficient mice produced normal levels of the T_H2-type cytokine IL-5, but more interferon-γ (IFNγ) than control mice. Therefore, IL-4 induced by aluminium salts has a role in promoting T_H2-cell-associated antibody production and T_H2-cell responses, but has a more important role in inhibiting T_H1-cell-associated antibody production and T_H1-cell responses. Subsequent work has shown that following immunization with antigen and aluminium salts, IL-6 also inhibits

T_H1-cell-associated antibody production and T_H1-cell responses. Unlike IL-4-deficient mice, however, IL-6-deficient mice had increased T_H2-cell responses, suggesting that IL-6 suppresses both T_H1- and T_H2-cell responses to aluminium-salt-containing vaccines[37].

But what cells produce IL-4 early in response to aluminium salts? One study[38] showed that aluminium salts induce the accumulation of a population of $GR1^+$ IL-4-producing cells in the spleen 6 days following immunization. This population comprised eosinophils and a myeloid-cell type, although more recent evidence argues that it is composed mainly (~80%) of eosinophils[39]. These cells appear at the site of injection within 24 hours[40], and IL-4 produced by these cells was shown to increase B-cell proliferation and to promote an increase in the production of IgM[38,39]. In addition, depletion of $GR1^+$ cells in mice followed by administration of an aluminium-salt-containing vaccine resulted in the generation of IgG2c-producing B cells[40]; neutralization of IL-4 had the same effect.

Together, these studies indicate that aluminium salts promote the recruitment of IL-4-producing eosinophils to the spleen, which in turn mediate the production of early IgM. IL-4 may also be involved in increasing the accessibility of the B-cell IgE locus to factors that are required for class switching and in suppressing the events that are required for T_H1 cells to develop during priming[41] (BOX 3, FIG. 2).

Controversy on the NLRP3 inflammasome. A link between aluminium-salt-induced activation of the inflammasome and the induction of antibody responses was first provided by a study by Eisenbarth *et al.*[8] showing that vaccination with antigen and aluminium salt required NLRP3, ASC and caspase 1 to induce antigen-specific IgG1 production[8]. Soon after, a minor but important role for NLRP3 in aluminium-salt-induced IgG1 production was reported by Li *et al.*[23]. These studies suggested that unlike IL-4, which primarily inhibits T_H1-type responses, the NLRP3 inflammasome promotes T_H2-type responses. However, a subsequent study by Franchi *et al.*[24] reported the opposite result: NLRP3 deficiency did not alter the production of IgM, IgA and IgG antibodies of any isotype following immunization with aluminium salt and an antigen. Our own experiments in caspase 1-deficient mice have also failed to find a defect in either T-cell or antibody responses in response to aluminium salts (A.S.M., unpublished observations). Adding further confusion, Kool *et al.*[25] recently showed that NLRP3-deficient mice were partially defective at priming antigen-specific T-cell-receptor-transgenic T cells but had normal levels of IgG1. It was also shown that IgG2c antibody levels were slightly increased, whereas IgE antibodies were completely absent, suggesting that NLRP3 promotes class-switching to IgE at the expense of IgG2c.

The reason for these contrasting results is unclear, but they could be the result of differences in the timing of immunizations, the forms of aluminium salt used or the genetic background of the mice. All five studies examined secondary antibody responses after priming and boosting with antigen and adjuvant. The three papers that showed a role for NLRP3 in the antibody response to aluminium salts[8,23,25] used mice on a C57BL/6 background, whereas Franchi *et al.*[24] used mice of a mixed C57BL/6–129 background. However, in our study we used caspase 1-deficient mice also on a C57BL/6 background (A.S.M. unpublished observations) and did not find a requirement for caspase 1 in the induction in antibody responses. The Eisenbarth *et al.*, Li *et al.*, and Kool *et al.* papers also used Imject Alum (Pierce, USA), which is a non-clinical adjuvant that contains magnesium hydroxide in addition to aluminium hydroxide (BOX 1), whereas Franchi *et al.* used aluminium hydroxide as the adjuvant and we used aluminium potassium sulphate. Current work is underway in our laboratory to determine whether differences in immunization protocols, the form of the adjuvant or the background of the mice contribute to these discrepant results.

Based on the data discussed above, we conclude that activation of the NLRP3 inflammasome is sometimes, but not always, required for aluminium salts to induce T_H2-type immune responses *in vivo*.

Role for caspase 1 signalling mediators. As discussed earlier, the activation of the inflammasome by aluminium salts results in the production of IL-1β and IL-18, and therefore it was thought that these cytokines may be involved in aluminium-salt-induced adaptive immune responses. Indeed, these cytokines are known to have a role in asthma, a disease that is generally considered to be driven by T_H2 cells. In addition, airway hyperresponsiveness (AHR) can be induced by sensitization with antigen and aluminium salts[42].

However, earlier studies on the role of IL-1–IL-1R signalling in AHR in the presence or absence of aluminium salts indicated that this signalling pathway was only required for the development of airway inflammation in the absence of aluminium salts[30,31]. Indeed, in the presence of aluminium salts, IL-1–IL-1R signalling was not required for bronchoconstriction, pulmonary eosinophilia, T-cell priming or antibody responses.

IL-18, which is a pro-inflammatory member of the IL-1 family, can have either T_H1- or T_H2-cell-promoting properties depending on the system used in the study[43]. Following vaccination with antigen and aluminium salts, IL-18 was found to be partially required for IL-4 production, whereas it was not required for antigen-specific IgG1 and IgE production, or for T-cell responses[44].

So, at present, the requirement for the NLRP3 inflammasome and caspase 1 for the induction of T_H2-cell-associated antibody

Box 3 | Clues to the adjuvant action of aluminium salts from other adjuvants

Important clues about the adjuvant action of aluminium salts can be gained from substances with related properties. Other particles, such as seemingly inert sepharose beads or the structural biopolymer chitin, have been shown to induce rapid eosinophilia and basophilia[53,54]. Importantly, degradation of chitin *in vitro* and *in vivo* abrogated leukocyte infiltration[54]. Egg extract from the helminth parasite *Schistosoma mansoni* also induces eosinophilia[40]. Similar to the adjuvant action of aluminium salts (see main text), these egg extracts induce interleukin-4 (IL-4)-dependent B-cell priming and enhance the early IgM response to co-administered antigen, although they were shown to not act as a $CD4^+$ T-cell adjuvant[40].

Furthermore, parasite proteases, such as the GP63 proteins that are produced by *Leishmania* spp. are important for parasite growth and infective ability *in vivo*[55]. In addition, many protein allergens are proteases, and a recent study found that papain directly activates basophils to secrete IL-4 and thymic stromal lymphopoietin[56], which are known to promote T_H2-cell responses. In line with this observation, *Leishmania mexicana* that lacked cysteine protease activity induced less antigen-specific IgE production than the wild-type parasite in BALB/c mice[57].

Together, it seems that these particles and proteases share some properties with aluminium salts. This suggests that the adjuvant activity of aluminium salts may be partially due to their particle properties, which induce the release of lysosomal host proteases, and/or to some as-yet-unknown mechanisms that work synergistically to induce its T_H2-cell biased adjuvant effects.

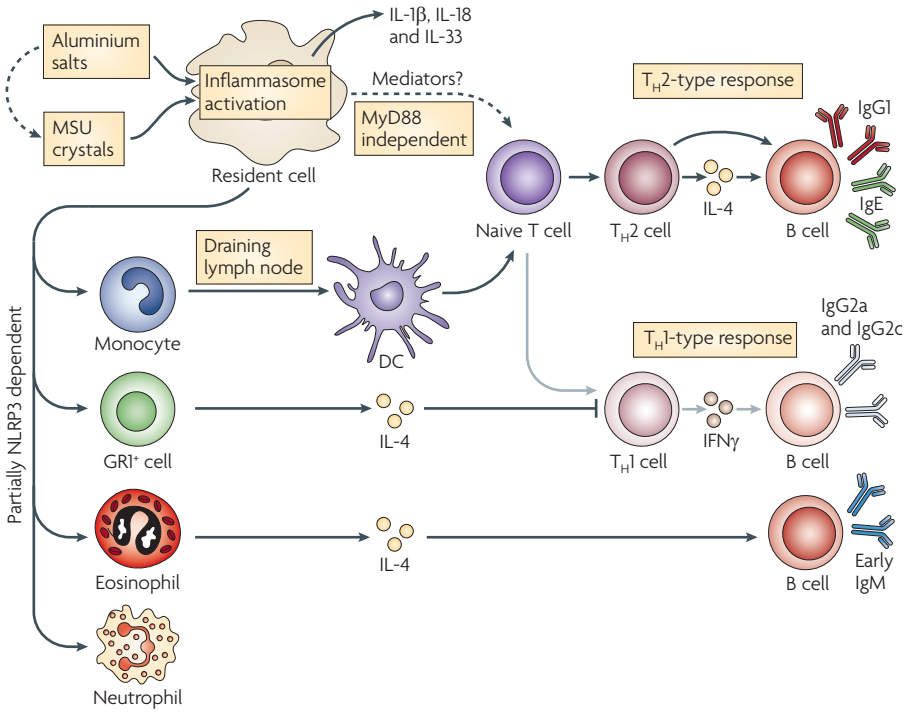


Figure 2 | **Stimulation of adaptive immune responses by aluminium salts.** Aluminium salts and/or monosodium urate (MSU) crystals that are induced by aluminium salts activate resident phagocytes partly through the NLR family, pyrin domain containing 3 (NLRP3; also known as NALP3) inflammasome. If and how NLRP3 inflammasome activation contributes to induction of adaptive T helper 2 (T_H2)-type responses, such as interleukin-4 (IL-4) production by T_H2 cells and IgG1 and IgE production by B cells, is still controversial and under investigation. Aluminium salts also induce the accumulation of monocytes, GR1$^+$ cells, eosinophils and neutrophils to the site of injection, and eosinophils also accumulate in the spleen. Monocytes mature into CD11c$^+$ dendritic cells (DCs) as they traffic to the draining lymph nodes, where they activate naive CD4$^+$ T cells. GR1$^+$ cells secrete IL-4, which suppresses T_H1-cell responses, and eosinophils enhance early IgM responses by B cells. Although neutrophils accumulate at the injection site, they have no known role in adaptive immunity. IFNγ, interferon-γ; MyD88, myeloid differentiation primary-response gene 88.

production in response to aluminium salts is still a matter of debate. However, it seems that IL-1β and IL-18, which are the best-studied caspase 1 targets, are not required for the induction of adaptive immune responses in response to aluminium salts. It is possible that IL-1β and IL-18 act redundantly in this capacity or that another member of the family (for example, IL-33) or a target of caspase 1 that has yet to be identified[45] are the crucial factors.

Outstanding questions

Recent studies have increased our knowledge of the biological events that are induced following the administration of aluminium salts. However, the mechanisms that are required for the subsequent induction of the adaptive immune response are still debated. Some additional questions remain to be answered and some important discrepancies need to be resolved: is there a specific cellular receptor that recognizes aluminium salts? *In vivo*, does aluminium-salt-induced inflammation occur indirectly owing to cytotoxicity and the release of DAMPs such as uric acid, which form MSU crystals, or directly by triggering phagocytosis and lysosomal disruption? LPS is required to upregulate *Il1b* mRNA expression and to activate caspase 1 *in vitro*, but what induces these effects *in vivo*? Is there a biological role for the induction of IL-1β secretion and for neutrophil accumulation? Is the activation of the NLRP3 inflammasome and the activation of caspase 1 crucial for the adjuvant activity of aluminium salts? If they do have a role, which caspase 1 target (or targets) is required for the induction of an adaptive immune response? Finally, after over 80 years of research, there is still no definitive proof for Glenny's original hypothesis that the depot effect of aluminium salts contributes to their adjuvant action.

Aluminium adjuvants have been successfully used in hundreds of millions of humans since 1932, greatly decreasing morbidity and mortality with minimal toxicity. Although there are instances in which aluminium adjuvants are not protective, such as vaccination against pathogens that require T_H1-cell-mediated immunity, it is hoped that the answers to these questions will aid in improving the effectiveness of aluminium salts and speed the development of alternative adjuvants.

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DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
ASC | caspase 1 | IL-1β | IL-4 | IL-18 | IL-33 | MyD88 | NLRP3 | TRIF

FURTHER INFORMATION
Philippa Marrack's homepage: http://www.kmlab.njc.org

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OPINION

Models of haematopoiesis: seeing the wood for the trees

Rhodri Ceredig, Antonius G. Rolink and Geoffrey Brown

Abstract | Many models of haematopoiesis dictate that haematopoietic stem cells make an early and irrevocable choice between lymphoid and myeloid cell fates. However, recent data show that intermediate progenitors have both lymphoid and myeloid potential, thereby refuting early theories and leading to a lack of consensus in the field. In this Opinion article we present a simple pairwise relationships model of cell fate determination that does not depend on the underlying developmental branch points that in other models dictate a preferred route (or routes) to a particular cell fate.

Haematopoiesis is an ideal system for investigating the developmental relationships between cells of an organ system: haematopoiesis was the first system for which a tissue-specific stem cell was identified, and all haematopoietic lineages can be reconstituted from a single bone-marrow-derived cell, the haematopoietic stem cell (HSC). The study of haematopoiesis began in the 1960s, when Till and McCulloch showed that bone marrow cells injected into irradiated mice gave rise to spleen colonies[1], and when Bradley and Metcalf showed that bone marrow cells dispersed in semi-solid medium formed heterogeneous colonies[2]. Later, it was realized that haematopoiesis involves interactions between developing cells and mesenchyme-derived stromal cells, which led investigators