

Immunomodulatory properties of the vaccine adjuvant alum

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Alum, the most common adjuvant in non-living vaccines, has a record of successful use in human vaccination where it promotes antibody-mediated protective immunity. However, alum is a poor inducer of cellular immune responses. The mechanism underlying the selective enhancement of humoral immunity is still not well understood. Here, to provide an insight into its mode of action, recent findings regarding innate immune responses induced by alum and their impact on adaptive immunity are described, with a particular emphasis on early recognition of alum, including NLRP3 and PI3 kinase activation, adjuvant-induced cell death and the release of endogenous danger signals. Expanding our knowledge of alum-induced immunomodulation will greatly enhance our capacity to rationally develop novel adjuvants with specific properties.

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Introduction

While many licensed vaccines consist of whole or inactivated pathogens, there has been a recent shift towards using subunit vaccines containing purified antigens which although safer are generally less immunogenic and therefore require adjuvants to trigger protective immunity. Adjuvants can enhance and/or direct immune responses against antigens [1] and have previously been referred to by the late Charles Janeway as ‘the dirty little secret of immunologists’ [2]. However, despite their pivotal role in priming the immune system, there are only a few adjuvants currently in clinical use. Aluminium adjuvants (referred as alum in this review) elicit strong humoral immune responses which are mediated primarily by secreted antigen-specific antibodies, particularly IgG1 [3,4]. Therefore, alum is incorporated into a range of vaccines against diseases where neutralising antibodies

to bacterial and viral antigens are required for protection, including diphtheria, tetanus and hepatitis B [5]. In contrast, alum is a relatively poor inducer of cell-mediated immune responses and is therefore unsuitable for use in vaccines against HIV, malaria or tuberculosis, where strong cellular immunity is essential [6]. Currently, there is great interest in the mechanism of alum-induced immunity as it is not fully understood how this adjuvant provides protection and why it is relatively ineffective in promoting cellular responses. Particular interest has been shown in alum-induced innate immunity as this system is crucial for modulating the onset and effector functions of the adaptive immune response. Here, we provide an update on how alum, the gold standard of adjuvants, can induce and direct innate immunity. This view will improve our current understanding of the mechanisms underlying the ability of adjuvants, especially particulates, to modulate immune responses.

Depot theory

In 1926 Alexander Glenny and colleagues showed that injection of diphtheria toxoid precipitated with potassium aluminium sulphate into guinea pigs induced stronger antibody responses than toxoid alone [7]. It was also reported that alum forms nodules at the site of injection [7] and when ground up and injected into a naïve animal, this material enhanced immune responses to the adsorbed antigen [8]. Based on these observations, it was proposed that alum functioned as an antigen “depot”, retaining the antigen at the injection site and facilitating its slow release. The kinetics relating to the development and composition of the alum “depot” were only described in detail recently. Alum nodules are formed within four hours after injection due to an interaction between the clotting agent fibrinogen and alum [9]. However, the current consensus is that nodule formation is dispensable for alum adjuvanticity. Fibrinogen-deficient mice, which do not form an alum depot after vaccination, were found to have similar antigen-specific IgG1 titres compared to wild-type mice [9]. Furthermore, it has been demonstrated that surgical removal of the alum “depot” as early as two hours post injection did not affect the magnitude of the antigen-specific IgG1 response up to 35 days post vaccination [10*]. While these studies prove that the antigen “depot” is dispensable for induction of short term adaptive immunity, it is unclear whether its absence might impact on memory immune responses. Furthermore, it is possible that alum particles can form an antigen “depot” or drive local immune responses in the draining lymph nodes (dLNs) following transport from the injection site. It has been reported that nanoparticles (20–200 nm) can traffic into the dLNs,

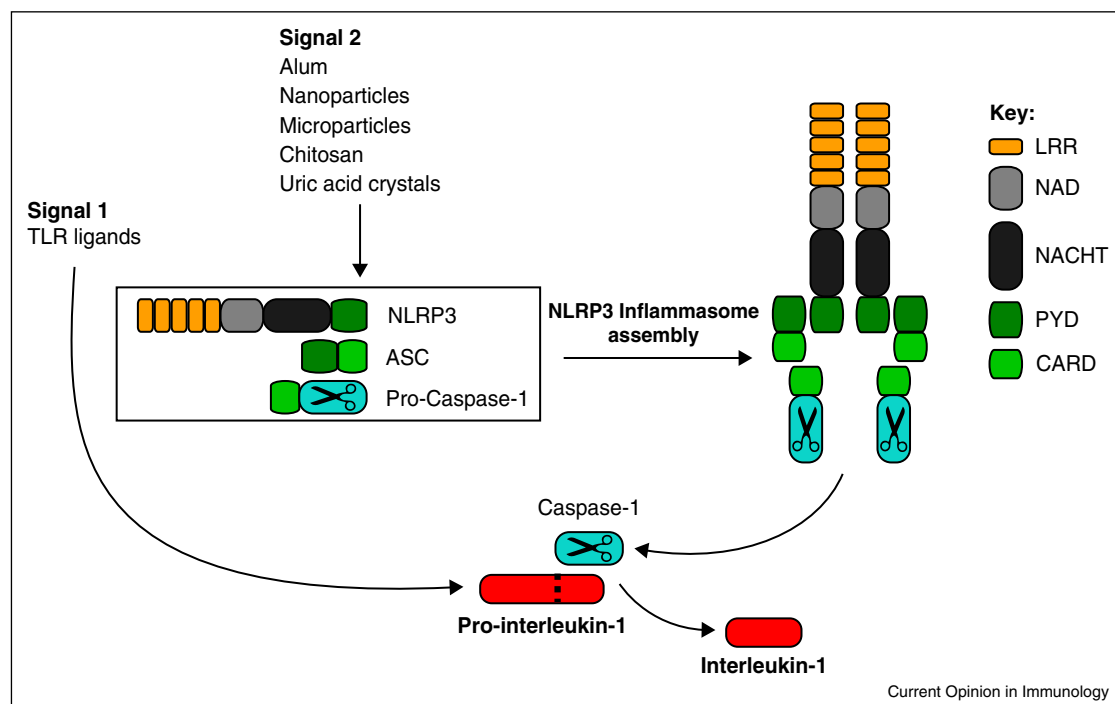
while larger particles (500–2000 nm) require phagocytosis and transport by dendritic cells (DCs) [11]. Alhydrogel (aluminium hydroxide), the most common adjuvant in human vaccination, contains fibre-like microparticles (2–12 μm in size), although the primary components are nanoparticles [12]. It is therefore possible that these alum particles may be transported to the dLNs. Indeed, it has been demonstrated that alum-adsorbed antigen can be carried by inflammatory monocytes to the dLNs [13]. However, it remains to be established whether alum itself can also be found there and how important this is for its adjuvanticity and polarisation of immune responses.

NLRP3 recognition

One of the key discoveries relating to alum and other particulate adjuvants is their ability to activate the NLRP3 inflammasome [14–16]. Upon activation, NLRP3 along with ASC and caspase-1 form an inflammasome which leads to caspase-1 activation and subsequent processing of IL-1 β and IL-18 into their biologically active mature forms (Figure 1). It was reported in 2008 that alum-induced innate and adaptive immune responses were abrogated in NLRP3-deficient mice. Specifically, NLRP3-deficient mice had impaired cell recruitment to

the site of alum injection and local secretion of IL-1 β was reduced, which was proposed to explain the reduced numbers of infiltrating neutrophils, eosinophils, monocytes and DCs [17]. Moreover, there was a decreased number of inflammatory monocytes carrying antigen into the dLNs in NLRP3-deficient mice and their activation was also impaired, as shown by reduced expression of MHC class II and the co-stimulatory molecule CD86 [17]. Furthermore, it has been proposed that NLRP3 is required for alum-induced adaptive immunity as two studies reported that NLRP3-deficient mice exhibit reduced alum-driven antigen-specific IgG1 [14,18]. While these reports appeared to have solved the mystery of alum adjuvanticity, shortly thereafter many studies contradicted these findings. For example, altered innate immune responses observed in NLRP3-deficient mice were not reproducible in caspase-1 deficient mice [19]. Moreover, several studies have challenged the role of NLRP3 in alum-induced humoral immunity [15,17,19,20]. It was shown that NLRP3^{-/-} and wild-type mice immunised with alum in combination with OVA have similar antigen-specific IgG1 titres [15]. Furthermore, NLRP3^{-/-}, caspase-1^{-/-} and wild-type mice displayed comparable numbers of antigen-specific T cells in draining lymph

Figure 1



Activation of the NLRP3 inflammasome. IL-1 β and IL-18 requires a two-step activation process to become fully active. A first signal is required to promote expression of pro-IL-1 β and pro-IL-18 in the cytoplasm, which can be provided by Toll-like receptor (TLR) ligands. Then, Nod-like receptor (NLR) family, pyrin domain-containing 3 (NLRP3) can form a multiprotein complex (inflammasome) which is able to process proforms of IL-1 β and IL-18. In brief, various stimuli, including aluminium adjuvants, other particles, chitosan and uric acid crystals, promote a conformational change in NLRP3 which allows NLRP3 to oligomerise and thus facilitate recruitment of ASC and caspase-1. Caspase-1 becomes activated and can then process pro-IL-1 β and pro-IL-18 into their active forms. LRR: leucine-rich repeat; NAD: NACHT-associated domain, NACHT: NLR family, apoptosis inhibitory protein (NAIP), class II, major histocompatibility complex, transactivator (CIITA), incompatibility locus protein from *Podospira anserinea* (HET-E) and telomerase-associated protein (TP1); PYD: pyrin domain; CARD: caspase activation and recruitment domains.

nodes and spleens, following injection with alum and OVA [19]. To date, the reason for these discrepancies remains unclear, although several factors have been postulated to be responsible, including different immunisation protocols, routes of delivery and the dose of adjuvant or assays used to determine immune responses [21].

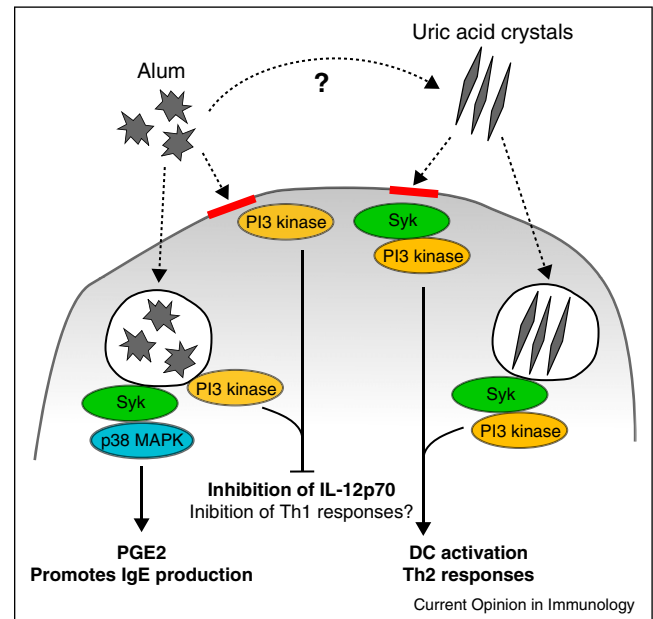
There is lack of data regarding the role of the NLRP3 inflammasome in alum-induced memory responses. While alum promotes similar antibody responses in wild-type and NLRP3^{-/-} mice shortly after immunisation, the possibility that NLRP3 might play a role in promoting memory responses cannot be discounted. It will be important to determine whether another clinically approved adjuvant, Adjuvant System 04 (AS04), which is composed of alum and the TLR4 ligand, monophosphoryl A, requires inflammasomes for its adjuvanticity as it is possible that additional components incorporated into alum might change its mode of action. Interestingly, in the case of MF59, an oil-in-water emulsion which was licensed for an influenza vaccine in Europe in 1997, inflammasome activation is not required for its adjuvanticity [20,22]. MF59 promotes antigen-specific antibodies whose production is NLRP3-independent and caspase-1-independent, but is ASC-dependent. However, it has been suggested that ASC also has inflammasome-independent roles and is required for germinal centre B-cell formation [22].

Alum promotes PI3-Syk kinase signalling

The means by which alum is initially recognised by the innate immune system has not been fully elucidated. It was recently suggested that alum is not recognised by protein receptors as DCs treated with pronase, an enzyme which cleaves surface proteins, can still interact with alum. In contrast, it was proposed that alum is sensed by plasma membrane lipids [23^{••}]. Furthermore, it has been shown that the interaction of alum with membrane lipids on the DC surface induces receptor-independent cell activation that is dependent on the Syk-PI3 kinase pathway [23^{••}] (Figure 2).

Activation of PI3 kinase signalling has been implicated in the poor induction of cellular immunity by alum. IL-12p70 is a key cytokine produced by antigen-presenting cells which drives the polarisation of naïve T cells towards a Th1 phenotype. Importantly, it has been demonstrated that PI3 kinase activation by aluminium adjuvants inhibits the secretion of IL-12p70 by DCs. Specifically, alum selectively inhibited DC expression of the IL-12p35 subunit of bioactive IL-12p70 [24^{••}]. Moreover, it has been observed that alum promotes prostaglandin E2 (PGE₂) production *in vitro* in a Syk and p38-MAPK-dependent, but NLRP3-independent manner [25]. Prostaglandins are lipid mediators involved in the induction of inflammatory responses and PGE₂ is involved in shaping immunity by suppressing Th1 responses. Mice deficient

Figure 2



Alum and uric acid crystals induce Syk-PI3 kinase signalling. Interaction of alum and uric acid crystals with membrane lipids on the cell surface may lead to activation of the Syk and PI3 kinase pathway. Alternatively, it is possible that this signalling pathway is activated following phagocytosis of particles. Syk and/or PI3 kinase activation by alum has been implicated in promoting PGE₂ secretion in macrophages and inhibition of IL-12p70 in dendritic cells (DCs). Uric acid crystals also activate DCs in a Syk-PI3 kinase-dependent manner which promotes Th2 responses. However, the crosstalk between these different effects of alum and uric acid crystals is still unknown. Overall, activation of the Syk and PI3 kinase pathways leads to induction of Th2 responses, while Th1 responses are inhibited.

in PGE synthase exhibit reduced levels of alum-induced antigen-specific IgE production *in vivo*, although antigen-specific IgG1 responses remain intact, thus suggesting a limited role for PGE₂ in alum adjuvanticity [25]. Overall, recent studies point to a significant role for Syk-PI3 kinase signalling in mediating the immunostimulatory effects of alum but additional research is required to elucidate the nature of alum-induced cell signalling and its implications for adaptive immunity.

Release of endogenous danger signals after alum injection

While it has been proposed that alum is not recognised by a specific receptor but instead promotes lipid raft formation and receptor-independent cell activation [23^{••}], the adjuvant may also modulate immunity via cytotoxic effects. The “danger theory” was first proposed by Polly Matzinger [26] and postulates that the immune system responds principally to “dangerous” situations including stress, injury and cell death. These events are associated with endogenous danger signals, otherwise known as

damage-associated molecular patterns (DAMPs), which are released by necrotic cells following loss of plasma membrane integrity and can subsequently trigger alarm and inflammation [27].

Alum promotes local necrosis in vaccinated muscle tissue [28] and in the peritoneum of mice following injection [29^{••}]. The nature of alum-induced cell death is not fully characterised but it has been reported that alum-induced macrophage death is cathepsin B/S-dependent *in vitro* [30[•]]. Furthermore, a role for endogenous danger signals released during alum-induced cell death in driving immune responses has been demonstrated. In particular, uric acid and host DNA have been shown to be released following alum injection [13,29^{••}].

Uric acid is released into the peritoneal cavity as early as two hours after injection and is believed to play an important role in alum-induced immune responses. For instance, pre-treatment of mice with uricase decreases alum-induced antigen-specific T cell and humoral responses [13]. Moreover, uric acid crystals, similarly to alum, can induce Syk kinase signalling in DCs following interactions between the crystals and the membrane lipids [31]. Furthermore, *in vivo* experiments with a Syk inhibitor and also with mice deficient in PI3 kinase δ demonstrated that this pathway is important for early recruitment of inflammatory DCs to the site of uric acid crystal injection and the subsequent onset of Th2 responses [32[•]] (Figure 2). However, it remains unknown whether alum-induced Th2 responses are similarly affected by PI3-Syk kinase signalling.

Another DAMP, host DNA, is released as early as three hours after alum injection [29^{••}] and it has been reported that the alum depot contains DNA [9] in structures which resemble extracellular traps, comprising DNA nets where bacteria can be entrapped and killed [33]. DNase pre-treatment of mice immunised with alum and antigen decreased antigen-specific IgG1 and IgE responses and immunisation with dsDNA mimics alum-induced adaptive immune responses [29^{••}]. Interestingly, another report showed that alum-induced DNA release is critical for DC-T cell interactions which can be abrogated by DNase leading to a decrease in antigen-specific CD4 T cell expansion and a partial reduction in antigen-specific IgG1 production [34^{••}]. However, mice deficient in key mediators of DNA signalling, STING or IRF3, did not exhibit reduced antigen-specific IgG1 titres. Only antigen-specific IgE was reduced in the absence of IRF3 or STING while antigen-specific CD4 T cell expansion was partially dependent on STING [29^{••},34^{••}]. Therefore, it remains unknown how DNA released during immunisation mediates alum adjuvanticity and the identity of the key DNA sensing receptor remains elusive. Cytoplasmic DNA sensing is a very dynamic field of research with a number of novel receptors recently described, so pursuing

the role of these pathways in alum adjuvanticity may prove to be an important avenue of investigation [35].

Conclusions

There has been substantial progress in understanding the mode of action of aluminium adjuvants in recent years. New signalling pathways, cytokines and endogenous danger signals have been implicated, however, it will be crucial to understand how these diverse, but simultaneously triggered signals are integrated to drive immune responses. Importantly, none of these different events fully explain how the adjuvant promotes selective protective antibody responses. Therefore, there remains a need to further pursue research in this field in order to address the remaining immunomodulatory mechanisms associated with alum adjuvanticity. However, it will be crucial to standardise the dose, route of immunisation and type of aluminium adjuvant used in order to obtain consistent results among different laboratories. While many aluminium adjuvants are available for animal studies, including Imject alum, aluminium hydroxide and aluminium phosphate, they possess different chemical and physical properties and so it will be important to adsorb antigens onto the standardised commercially available preparations, Alhydrogel or Adju-Phos, which are used for human vaccination [36]. Overall, improved understanding of alum-induced innate and adaptive immune responses will provide valuable information on how novel particulate adjuvants should be designed in order to overcome the deficiencies associated with alum.

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