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Review

Alum adjuvant: some of the tricks of the oldest adjuvant

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Alum has been the most widely used adjuvant for over 80 years. Although there have been searches for alternative adjuvants, aluminium-containing adjuvants will continue to be used for many years due to their good track record of safety, low cost and adjuvanticity with a variety of antigens. For infections that can be prevented by induction of serum antibodies, aluminium-containing adjuvants formulated under optimal conditions are the adjuvants of choice. There are also some limitations of aluminium-containing adjuvants, which include local reactions, augmentation of IgE antibody responses, ineffectiveness for some antigens and inability to augment cell-mediated immune responses, especially cytotoxic T-cell responses. In this review, we describe the current knowledge regarding the mechanisms (both cellular and molecular) by which alum employs its adjuvant effect, although the final mechanism is not yet well-defined. Furthermore, we discuss how alum's adjuvanticity could be improved.

Introduction to adjuvants

Vaccinations have been used for well over 100 years. The first reported vaccination was performed by Edward Jenner in 1796 (Jenner, 1909). He inoculated a boy with cowpox, after which this boy was protected against smallpox infection. Protection by vaccination can be achieved by giving inactivated virus particle, live-attenuated virus, or subunit vaccines. The latter does not induce a strong immune response but this can be provided by the administration of an adjuvant. The word 'adjuvant' originates from the Latin verb *adiuvare*, meaning to help or to aid.

In 1926, Alexander Glenny and colleagues reported that toxoid precipitated with aluminium potassium sulfate, referred to as alum, induced a stronger antibody production when injected into guinea pigs than soluble toxoid alone (Glenny *et al.*, 1926). This was the first report of the use of aluminium salts as an adjuvant. Since then, it has remained as the only licensed vaccine adjuvant in the USA. In Europe, however, MF59, a squalene-based oil-in-water emulsion, has been licensed as an adjuvant for influenza vaccines since 1997 (Vesikari *et al.*, 2010). Another squalene-based oil-in-water emulsion, namely adjuvant system 03 (AS03), has been used in the H5N1 influenza vaccine (Heijmans *et al.*, 2011; Leroux-Roels *et al.*, 2007). Additionally, a combination of aluminium hydroxide and monophospholipid (MPL) A, an LPS analogue, (AS04) was approved for use in vaccines against hepatitis B virus (HBV) and human papilloma virus (HPV) (Tritto *et al.*, 2009).

Currently, aluminium-containing adjuvants can be found in several childhood vaccines, like DTP (Diphtheria–Tetanus–Pertussis combination), Pediarix (DTP–HBV–Polio combination), Pentacel (DTP–*Haemophilus influenzae* B (HIB)–Polio combination), Hepatitis A, HBV, HPV, HIB, and pneumococcal vaccines.

It is important to note that aluminium salts not only serve as an adjuvant in vaccine preparations. Since they absorb proteins well, aluminium salts also stabilize vaccines by preventing the proteins in the vaccine from precipitating or sticking to the walls of the container during storage.

Cellular mechanisms induced by aluminium-containing adjuvants

The purpose of vaccination is to induce long-lasting protection against infections while avoiding unwanted side effects. Vaccine adjuvants have empirically been identified for their ability to enhance the adaptive immune response to a co-administered antigen (Schijns & Lavelle, 2011). The innate response induced by the adjuvant is important for the type and strength of the subsequent adaptive response. Aluminium-containing adjuvants induce strong innate

Abbreviations: DAMPs, damage-associated molecular patterns; DCs, dendritic cells; DTP, Diphtheria–Tetanus–Pertussis; HBV, hepatitis B virus; HIB, *Haemophilus influenzae* B; HPV, human papilloma virus; NLR, NOD-like receptor; PAMPs, pathogen-associated molecular patterns; PGE_2, prostaglandin E_2; TCR, T-cell receptor; TLR, Toll-like receptor; UA, uric acid.

immune responses (after both intramuscular and intraperitoneal injection in mouse models) that consist of an influx of neutrophils, eosinophils, NK cells, $CD11b^+$ monocytes and dendritic cells (DCs) to the site of injection (Calabro *et al.*, 2011; Kool *et al.*, 2008b; McKee *et al.*, 2009; Mosca *et al.*, 2008). Besides an influx of cells, mast cells and macrophages quickly disappear after alum injection (Kool *et al.*, 2008b; McKee *et al.*, 2009). The disappearance of macrophages is probably due to their activation and their subsequent adherence to the peritoneal wall, making it impossible to recover them in the peritoneal cavity (unpublished data; Barth *et al.*, 1995). Tissue-resident macrophages are amongst the first cells to sense a disturbance in tissue homeostasis. Through their rapid production of cytokines and chemokines, they alert the immune system and recruit other cells of the innate immune system, like neutrophils. Indeed, neutrophils are also attracted rapidly after alum injection (Kool *et al.*, 2008b; McKee *et al.*, 2009). Mast cells can directly sense alum and are amongst other cells responsible for the production of IL-1β, IL-5, CCL2 and RANTES (McKee *et al.*, 2009; Kool *et al.*, 2008b; Mosca *et al.*, 2008). IL-5 and RANTES attract eosinophils to the site of injection; however, mast cells, macrophages and eosinophils are dispensable for the adaptive immune response induced by alum adjuvant (McKee *et al.*, 2009). Besides the direct killing of pathogens, neutrophils are also important in shaping the adaptive immune response (Mantovani *et al.*, 2011). Neutrophils are able recruit inflammatory $CD11b^+$ monocytes to the site of damage/inflammation by secretion of LL-37 and azurocidin from their granules (Soehnlein *et al.*, 2008). The $CD11b^+$ monocytes that are recruited to the site of alum injection differentiate into inflammatory DCs (Kool *et al.*, 2008b; Mosca *et al.*, 2008). DCs are at the interface of the innate and adaptive immune response and are seen as nature's adjuvant (Lambrecht *et al.*, 2009; Bendelac & Medzhitov, 2002). They have the potential to recognize foreign antigens, process them into small peptides for presentation, via major histocompatibility complex (MHC) molecules, to T-cell receptors (TCRs) and provide the essential costimulatory molecules for activation of naive $CD4^+$ and $CD8^+$ T-cells (Banchereau & Steinman, 1998). Subsequently, $CD4^+$ T-cells provide help for B-cell class-switching and induction of long-lived plasma cells (Sornasse *et al.*, 1992). DCs have an immature phenotype in peripheral tissues, specialized for antigen uptake, but upon recognition of foreign material they migrate to the lymph node T-cell paracortex where they arrive as mature cells, expressing all costimulatory molecules and having lost the capacity to take up antigens (Idzko *et al.*, 2007; Shi *et al.*, 2003). The monocyte-derived inflammatory DCs are crucial for the adjuvant activity of alum (Kool *et al.*, 2008b; Mannhalter *et al.*, 1985). Conditional depletion of DCs and inflammatory monocytes in CD11c-DTR mice by diphtheria toxin injection has been shown to abolish T-cell activation and significantly reduce the production of antigen-specific IgG1 antibodies (Kool *et al.*, 2008b). DCs can be activated in different ways by pathogens; directly, through recognition of pathogen-associated molecular patterns (PAMPs) like endotoxin, peptidoglycan or unmethylated CpG motifs on Toll-like receptors (TLRs) (Takeda & Akira, 2005); or indirectly, through recognition of damage-associated molecular patterns (DAMPs) like uric acid (UA), ATP, host DNA or HMGB-1, which are released upon tissue damage (Krysko *et al.*, 2011; Marichal *et al.*, 2011; Shi *et al.*, 2003). This dual activation mechanism is important in order to understand the mechanism of action of most adjuvants.

Granulocytes are not only seen in the innate (early) response but also in late-phase response. In the spleen of mice, 7 days after injection of alum an innate $Gr\text{-}1^+$ $IL\text{-}4^+$ population has been shown to appear that primed B cells for MHC II signalling (McKee *et al.*, 2008). These splenic granulocytic cells have been shown to be IL-4-expressing eosinophils, which shape the adaptive Th2 response and induce the production of antigen-specific IgM antibodies (McKee *et al.*, 2008; Wang & Weller, 2008). Depletion of neutrophils during alum sensitization enhances T-cell and B-cell responses, indicating that their presence is of importance (Yang *et al.*, 2010). It is probable that their absence influences the antigen amount that is present for DCs and macrophages to engulf (Yang *et al.*, 2010). This leads to enhanced DC–T-cell contact and stronger stimulation/responses.

Primed macrophages will, upon stimulation with alum adjuvant, produce IL-1β, IL-6 and prostaglandin E_2 (PGE_2) (Kuroda *et al.*, 2011; McKee *et al.*, 2009). PGE_2 can influence the outcome of the T-cell response that is induced, which is a complex issue. In the case of alum injection, a Th2 response is provoked (Brewer *et al.*, 1996). Besides its effects on T-cell differentiation, PGE_2 can also directly influence B-cells and promote IgE production (Kuroda *et al.*, 2011).

The role of depot formation by alum adjuvant

Besides triggering innate immune cell responses, it has been proposed that alum adjuvant ensures a long-lasting immune response through formation of a depot. In this way, the antigen attached to the surface of alum can be slowly released, which favours a strong antibody response. Aluminium precipitates from the peritoneal cavity could transfer the adjuvant effect for as long as 1 month after injection when transplanted from one mouse to another. Around these depots of alum, an accumulation of $CD11c^+$ DCs and antigen specific T-cells has been observed, suggesting that depot formation is involved in the maintenance of a memory pool (unpublished data, Lambrecht *et al.*, 2009). However, the release of the antigen from alum precipitate is influenced by the interstitial fluid (Shi *et al.*, 2001). Upon intramuscular or subcutaneous administration, the antigen–adjuvant complex is exposed to a different environment in the interstitial fluid, after which the degree of adsorption changes following administration

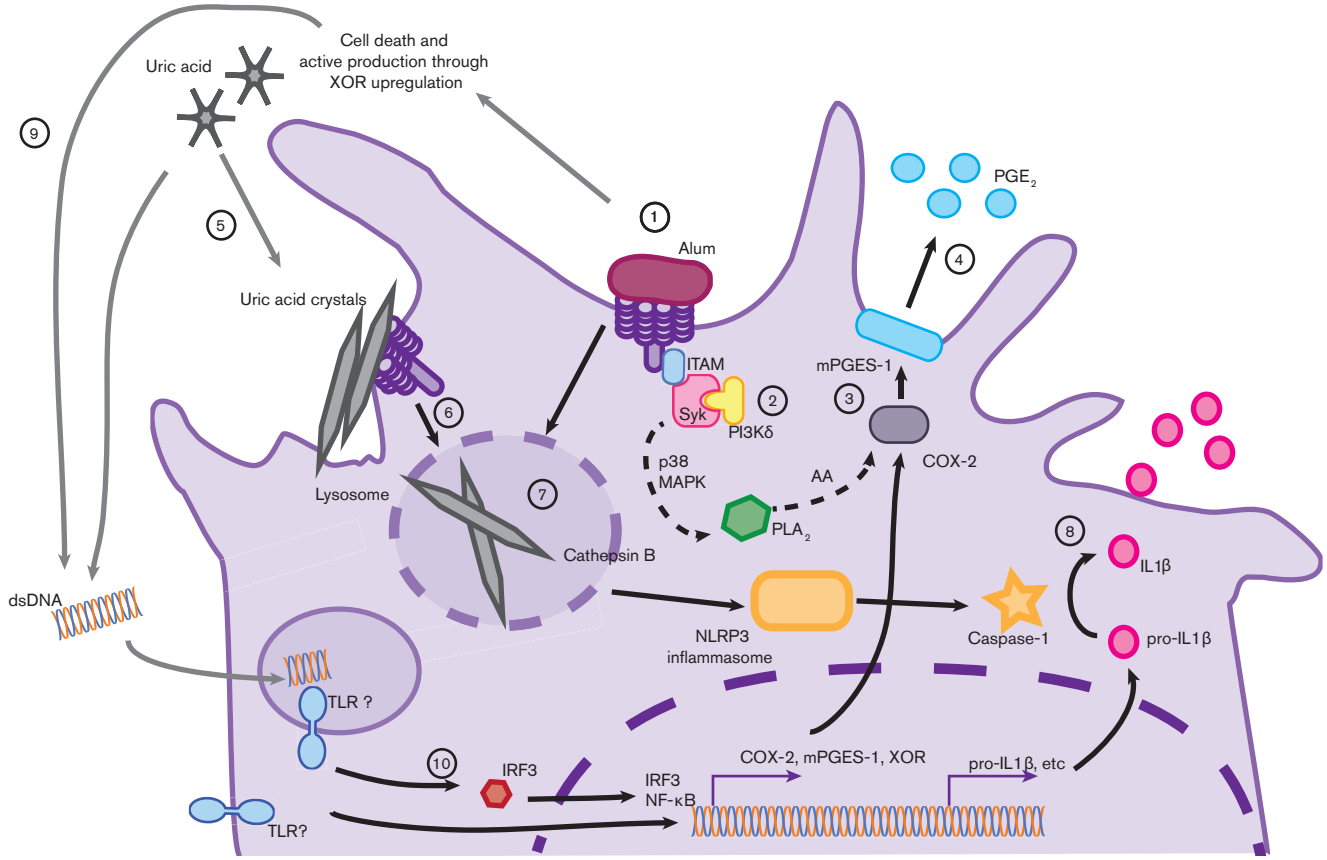

Fig. 1. Molecular pathways induced by alum in DCs and macrophages. (1) Alum binds to the cell membrane and thereby induces lipid raft formation. (2) In the lipid raft, ITAM-containing receptors cluster and activate the Syk-PI3Kδ pathway. (3) This pathway, in turn, activates cytosolic phospholipase A2 (cPLA$_2$), probably via p38 MAP kinase, resulting in the release of arachidonic acid (AA) from membrane lipids. COX-2 and membrane-associated PGE synthase-1 (mPGES-1) convert AA to prostaglandin E2 (PGE$_2$). (4) PGE$_2$ is released from the cell and instructs the Th2 response. (5) Furthermore, alum induces the release of uric acid via the induction of XOR. (6) Upon phagocytosis of alum or uric acid crystals, lysosomal damage is induced. (7) This activates the NLRP3 inflammasome by the release of enzymes like cathepsin B into the cytoplasm. (8) Activated caspase-1 mediates the proteolytic cleavage of proIL1β into biologically active IL1β. (9) Besides the release of uric acid, alum also induces the release of dsDNA, probably through cell death. (10) dsDNA activates the immune system and, more specifically, monocytes through IRF3, which is critical for the migration of inflammatory monocytes.

of the vaccine. Here, the antigen defuses very fast and is rapidly cleared from the site of administration within hours (Gupta *et al.*, 1996). This suggests that alum adjuvant has an effect on the cells and tissues at the injection site and that there is no need for depot formation. Several others have also shown that the depot of alum is not necessary for adjuvanticity (Hutchison *et al.*, 2012; Munks *et al.*, 2010). These data suggest that there is a difference in the functionality of the alum depot, probably depending on the site of injection and the local environment.

Molecular mechanism of aluminium-containing adjuvants

The cellular signalling pathways triggered by alum that activate DCs and macrophages and direct toward Th2 immune responses and effective humoral immunity against antigens have only recently been addressed (Fig 1). The field has started to evaluate a role for TLR activation by assessing the ability of several knockout mice to respond to alum. It has been shown that mice genetically lacking both MyD88 and TRIF, adaptor molecules of the TLR signalling pathway, still mount robust antigen specific antibody responses to alum adjuvant (Gavin *et al.*, 2006; Nemazee *et al.*, 2006; Palm & Medzhitov, 2009). On the contrary, there is a role for TLR signalling in the innate monocyte recruitment after aluminium-containing-adjuvant injection (Kool *et al.*, 2008b).

While TLRs sense extracellular non-self-motifs of infectious organisms and endogenous signals (DAMPs), NOD-like receptors (NLRs), which trigger inflammasome assembly, sense stimuli of microbial origin as well as DAMPs

(Lamkanfi & Kanneganti, 2010; Mariathasan & Monack, 2007; Pétrilli *et al.*, 2007a). They can recognize self-derived molecules, such DNA and RNA, which act as stress signals and abnormal self-signals, often as a result of cellular stress or damage, or danger signals, such as UA (Matzinger, 2002). NLRP3 (NALP3), a member of the NLR family, along with ASC and caspase 1, forms a molecular platform called the inflammasome (Pétrilli *et al.*, 2007b). NLRP3 can be stimulated by different molecules as described above, such as ATP, UA, gout-associated proteins, asbestos, silica and, as was recently shown, alum adjuvant. Upon activation, NLRPs are able to form a complex that eventually leads to the production of active IL-1-family members, such as IL1β and IL18. IL1β and IL18 are potent stimulators of the adaptive response and their production requires two signals (Lambrecht *et al.*, 2009). The first signal, derived from TLR agonists such as LPS and/or inflammatory cytokines, activates the NF-κB pathway and initiates transcription of the cytokine genes and accumulation of the precursor proteins like proIL1β and proIL18. Cleavage and secretion of the cytokines is mediated by caspase 1, which is activated within the inflammasome. Finally, these cytokines are released into the extracellular environment (Burns *et al.*, 2003). IL1β, in turn, secreted by DCs and macrophages, triggers another cascade of molecular events that result in inflammation. IL1β is a potent inflammatory cytokine, which is implicated in acute and chronic inflammatory disorders. IL1β, also called 'lymphocyte activating factor', based on its ability to stimulate T-cell proliferation, has classically been thought to promote Th2-cell proliferation but has also been shown to enhance Th17 differentiation in combination with other cytokines. IL1β enhances the differentiation of $CD4^+$ T-cells to Th2- and Th17-cells and boosts antibody production (Huber *et al.*, 1998). In coherence with the data obtained from the $MyD88^{KO}$ mice (MyD88 is also an adaptor molecule in the IL-1R signalling pathway), the $IL\text{-}1R^{KO}$ mice also only have a defect in the innate response after alum injection whereas their adaptive responses are normal (Ueda *et al.*, 2009).

There is controversy about the requirement of the NLRP3 inflammasome in the alum-induced response. *In vitro*, the results are coherent, as aluminium-containing adjuvants stimulate the production of IL-1β and IL18 in TLR-stimulated DCs and macrophages (Franchi & Núñez, 2008; Kool *et al.*, 2008a; Kuroda *et al.*, 2011; Li *et al.*, 2007, 2008; Sharp *et al.*, 2009) and it has been shown that the *in vitro* IL1β and IL18 production was dependent on the NLRP3 inflammasome (Franchi & Núñez, 2008; Kool *et al.*, 2008a; Li *et al.*, 2008). Alum can activate the NLRP3 inflammasome by phagosomal destabilization, lysosomal acidification, cathepsin B activity, via generation of reactive oxygen species, and/or via K^+ influx (Hornung *et al.*, 2008; Pétrilli *et al.*, 2007; Sharp *et al.*, 2009).

In contrast to the *in vitro* data, there is considerable controversy about the necessity of the inflammasome-IL1β pathway for the humoral immune response induced by alum. *In vivo*, there appears to be a discrepancy between the innate and adaptive immune response for the necessity of the NLRP3 inflammasome. Results show either an abrogation of antibody response in the absence of NLRP3 (Eisenbarth *et al.*, 2008; Li *et al.*, 2008), no need for NLRP3 (Franchi & Núñez, 2008; McKee *et al.*, 2009) or a selective need for NLRP3 (Kool *et al.*, 2008a). Alum was also shown to induce DC and T-cell activation partially through NLRP3 (Eisenbarth *et al.*, 2008; Kool *et al.*, 2008a); however, others have found no involvement of NLRP3 in DC and lymphocyte activation by alum adjuvant (Franchi & Núñez, 2008; Li *et al.*, 2008; McKee *et al.*, 2009). The difference in results obtained by the different groups is unexplained at the moment, however different types of alum, mouse strain background and different immunization protocols could contribute to the different phenotypes seen.

To add to the perplexity, the need for the NLRP3 inflammasome has been challenged as the adaptor molecules MyD88 and TRIF of the TLR/IL1R pathway are not required for alum-induced immunoglobulin production (Gavin *et al.*, 2006; Schnare *et al.*, 2001).

Recently, besides triggering the inflammasome, it was shown that alum induces signalling through ITAM-Syk-PI3Kδ which eventually leads to the production of PGE_2 (Flach *et al.*, 2011; Kuroda *et al.*, 2011). *In vitro*, alum stimulates macrophages and DCs by inducing lipid raft formation by binding to cholesterol. These lipid rafts contain ITAM-bearing receptors and this leads to activation of the Syk-PI3Kδ pathway. After interacting with alum, DCs strongly engage $CD4^+$ T-cells which is mediated via ICAM-1 and LFA-1. This will lead to subsequent B-cell activation (Flach *et al.*, 2011). Besides the Syk-PI3Kδ pathway, it has been shown that IRF3 in monocytes is also involved in the signalling response induced by aluminium-containing adjuvants (Marichal *et al.*, 2011). Whether Syk-PI3Kδ and IRF3 activation are in the same signalling pathway needs to be investigated; however, to date, there have been no reports of a link between these molecules.

Aluminium-containing adjuvants induce endogenous danger signals

As described above, vaccine formulations containing aluminium hydroxide stimulate the immune system by inducing immunological endogenous danger signals. To date, UA and host DNA have been shown to be released *in vivo* after alum injection (Kool *et al.*, 2008b; Marichal *et al.*, 2011). Uric acid is a danger signal produced during the catabolism of purines and is the end product in ureotelic mammals. Uric acid is released from injured cells, which rapidly degrade RNA and DNA, after which the liberated purines will be converted in UA. Besides degradation of RNA and DNA, UA is constitutively present in cells and increases when cells are injured. We were able to show that UA is already released 2 h after intraperitoneal administration of alum (Kool *et al.*, 2008b). When alum adjuvant is administered intramuscularly to humans, necrotic cells, a

potential source of UA, can be found at the site of injection (Goto & Akama, 1982, 1984; Goto *et al.*, 1997). Decreasing UA levels by uricase treatment decreases T-cell priming and humoral responses, indicating its functional relevance (Kool *et al.*, 2008b, 2011; Munks *et al.*, 2010). *In vitro*, both UA and alum can activate the NLRP3 inflammasome and induce the release of IL1β (Eisenbarth *et al.*, 2008; Franchi & Núñez, 2008; Kool *et al.*, 2008a, 2011; Li *et al.*, 2008; Martinon *et al.*, 2006). However, the induction of IL1β *in vitro* by alum did not depend on the presence of UA crystals as the addition of uricase had no effect on IL1β production, whereas *in vivo* it did (Kool *et al.*, 2008a, 2011; Li *et al.*, 2008). Recently, it was shown that UA crystals, when used as an adjuvant can induce Th2 responses in addition to an effective CTL response (Kool *et al.*, 2011; Shi *et al.*, 2003). In the UA crystal-induced Th2 response, neither TLR signalling nor the NLRP3-IL1 axis was required. However, the Th2 induction depended on Syk-PI3Kδ signalling in DCs (Kool *et al.*, 2011). It has to be noted that physiological UA induced by alum has not been shown to crystalize into UA crystals.

Another DAMP induced by alum is the release of host DNA due to induced cell death (Marichal *et al.*, 2011). In line with the evidence indicating a role for UA, Marichal *et al.* (2011) showed its necessity by using the rationale of Koch's postulates. First, an induction of dsDNA could be found at the site of alum injection; second, depleting DNA with DNase treatment decreased the adjuvant properties of alum; and third, dsDNA could be used to replace alum as an adjuvant (Marichal *et al.*, 2011). DNA can be sensed by TLR9, which will lead to IL1β production, via NF-κB activation, or to IFNβ production, via IRF3 activation. The humoral IgG1 response induced by alum and/or dsDNA is independent of IRF3, whereas IgE class switching and Th2 differentiation are not (Marichal *et al.*, 2011). When IRF3 signalling is blocked, the production of IL-12p80 (IL-12p40 homodimer) is reduced upon alum injection. IL-12p80 cannot induce IFNγ production in T-cells and would, thereby, favour the differentiation into Th2 cells. Besides its effect on T-cells, IL-12p80 can influence the responsiveness of monocyte-derived DCs for CCR7 chemokines (Robinson *et al.*, 2008). Since monocytes and monocyte-derived DCs play a crucial role in the induction of the adaptive response induced by alum, it is not surprising that influencing their ability to migrate alters the immune response. However, how and if the release/induction of UA and host DNA are linked needs further investigation.

Improving the use of alum adjuvant

Strategies to improve vaccine efficacy have been undertaken over recent years, whereby the use of TLR agonists have gained a lot of attention (Duthie *et al.*, 2011; Steinhagen *et al.*, 2011). There have been strategies in which aluminium-containing adjuvants are combined with a TLR agonist, like MPL or CpG motifs (Didierlaurent *et al.*, 2009; Yang *et al.*, 2009). Alum in combination with MPL, also known as AS04, has been used with success in vaccines against HBV (FENDrix), HPV (Cervarix), and Epstein–Barr virus (Kundi, 2007; Monie *et al.*, 2008; Sokal *et al.*, 2007). It is of note that adverse local events (typically mild–moderate) were generally more frequent in subjects receiving AS04 vaccines versus alum, but no serious systemic adverse events were reported. The addition of CpG motifs (TLR9 agonist) to alum adjuvant has only been tested in mice to examine its effectiveness in polio vaccines, which induced an enhanced humoral and cellular response (Yang *et al.*, 2009).

Recently, a combination of alum adjuvant with an opioid antagonist, naloxone, was demonstrated to induce stronger humoral cellular responses in mice than alum adjuvant alone (Jazani *et al.*, 2011a, b). Opioid peptides are well-known inhibitors of the immune response and vaccination against microbial agents (Carpenter & Carr, 1995; Molitor *et al.*, 1992). Besides, it was shown that naloxone was able to shift the immune response towards a Th1 profile, which is more favourable for vaccine efficacy (Sacerdote *et al.*, 2000a, b).

Taking advantage of alum's capacity to induce a strong humoral response when combined with agents to stimulate the adequate cellular response would eventually lead to an ultimate vaccine adjuvant.

In this review, we have discussed some tricks of the oldest adjuvant used in human vaccines but the mechanisms affecting the adjuvanticity of alum remain largely elusive. Nonetheless, the type and strength of the innate immune response will essentially be responsible for the ensuing adaptive response and, as a result, the strength of the adjuvant. In this process, several immune cells play an important role. Of these cells, DCs are the main bridge between innate and adaptive immunity. Activation of DCs via TLRs or NLRs, like NLRP3, has been shown not to be necessary for the adjuvanticity of alum. However, signalling molecules like Syk/PI3Kδ and IRF3 are essential mediators in the activation of DCs by alum adjuvant, both *in vitro* and *in vivo*. The receptor by which the DCs are activated still remains unclear and needs further investigation. Targeting the activation of DCs is fundamental in the working mechanism of an adjuvant, so exploiting this pathway could be used further to improve adjuvanticity.

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