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Mechanism of action of licensed vaccine adjuvants

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ABSTRACT

Despite the fact that alum and oil-in-water emulsions have been used for decades as human vaccine adjuvants in a large number of individuals, their mechanism of action is not completely understood. It has been reported that these particulate adjuvants act by increasing antigen availability and uptake by immune cells. However, recent work on alum and on the squalene-based emulsion MF59, has demonstrated that besides antigen delivery functions, these classes of adjuvants can also activate innate immunity pathways *in vivo*, generating an immunocompetent environment at injection site. Interestingly, it has been demonstrated that alum adjuvanticity depends on the activation of a protein complex called NLPR3/inflammasome, which is required for the correct processing of a number of pro-inflammatory cytokines, including IL1β. More work needs to be performed to investigate if the inflammasome is also required for the activity of MF59 and of other particulate vaccine adjuvants.

1. Introduction

Vaccine adjuvants are used to improve the potency of the immune response to co-administered antigens. It has been shown that the inclusion of an adjuvant in the vaccine formulation can enhance immunological memory and coverage and allows for antigen sparing and reduced number of doses. Different classes of compounds display adjuvant activity in pre-clinical models; among them, bacterial products, mineral salts, emulsions, microparticles, nucleic acids, small molecules, saponins and liposomes. However, only a few of them have been licensed for human use, while the vast majority failed due to an unacceptable safety profile. Alum adjuvanticity was demonstrated in the early 1920s. Since then, alum was incorporated in several human vaccines in the form of particulate aluminium salts, such as $Al(OH)_3$ and $AlPO_4$, and is still the only adjuvant approved in the USA. In other countries, including members of the EU, other vaccine adjuvants have been approved for human use. MF59, a squalene-based oil-in-water emulsion, was licensed for a flu vaccine formulation (Fluad) a decade ago. More recently, AS03, another oil-in-water emulsion, was approved as a component of a pre-pandemic H5N1 vaccine (Prepandrix). Finally, a combination of two adjuvants, monophosphoryl lipid A (MPL) and aluminium hydroxide, named AS04, was approved for use in HBV (Fendrix) and HPV (Cervarix) vaccines.

The molecular and cellular targets of the human licensed adjuvants described above are not always understood. MPL, a derivative of the bacterial component lipopolysaccharide (LPS), is probably the most characterized. MPL activates Toll-like receptor (TLR)-4, a receptor expressed on antigen presenting cells (APCs). Engagement of TLR4 promotes cytokine expression, antigen presentation and migration of APCs to the T cell area of draining lymph nodes, allowing for an efficient priming of naïve T cells. Other TLR agonists, such as flagellin and poly(I:C) double stranded RNA induce a similar process and are validated vaccine adjuvants in pre-clinical models [1]. Notably, CpG oligonucleotides, which target TLR9, have been also very efficiently used in humans in a number of vaccine trials [2]. In summary, the molecular mechanisms of TLR-dependent vaccine adjuvants are very well characterized. On the other hand, the mechanism of action of particulate adjuvants like alum and oil-in-water emulsions, used to vaccinate millions of individuals for decades, is not fully understood. Recently, several studies conducted on alum and MF59 have proposed novel models on how particulate adjuvants work.

2. Alum promotes antigen uptake and activates innate immune reactions at injection site

Alum is the most widely used vaccine adjuvant employed in Diphteria, Tetanus, Pertussis and Hepatitis A and B vaccines. Alum adjuvanticity is associated with enhanced antibody responses; however, its exact mechanism of action is still largely unknown. It has been proposed that alum acts through the formation of a depot that induces the gradual release of the adsorbed antigen at the injection site. This process may allow for longer exposure of the antigen to the immune system, therefore increasing the likelihood of APC–antigen interaction. The very long half-life of alum at injection site supports the depot effect model. In a Cynomolgus monkey model, alum was detected in the muscle up to 6 months after intra-

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muscular (i.m.) injection of a DT-TT vaccine and it was hypothesized that the alum-adsorbed antigen could also persist longer than if it was administered alone [3]. However, the depot-effect of alum has been challenged. It was shown that ^{14}C-labeled TT is released very rapidly from the alum complex at injection site [4]. Another study demonstrated that the excision of the DT-alum treated site soon after sub-cute vaccination did not change the immune response to the antigen [5]. It has also been proposed that antigens adsorbed to alum are presented in a particulate multivalent form, which is more immunogenic and more efficiently internalized by APCs. *In vitro* experiments support this model. It has been showed that although DCs can engulf antigens in solution through macropinocitosis, they are more efficient in internalizing alum-adsorbed antigen through phagocytosis in a process dependent on the size of the aggregate [6]. Other suggested mechanisms of alum adjuvanticity include the activation of the complement cascade and the generation of a local inflammatory environment at injection site characterized by the recruitment of blood cells [7,8]. Several *in vitro* studies have tried to identify the primary target cells of alum immunostimulating activity. Unlike TLR agonists, alum does not activate directly DCs *in vitro*, but acts on macrophages inducing their differentiation into DC-like cells with enhanced antigen presenting capacity [9]. Monocytes purified from human PBMCs after treatment with alum produced pro-inflammatory cytokines, up-regulated MHC class II, CD86 and CD83, and down-modulated CD14 expression, thus reflecting the acquisition of mature DC phenotype [9–11]. Two studies in the mouse model have highlighted the importance of the immunostimulatory activities of alum for its adjuvanticity. It has been shown that activation of IL4 secretion in Gr1$^+$ cells is required for optimal B cell priming in the spleen following alum administration [12]. Furthermore, the adjuvanticity of alum after intraperitoneal challenge was associated with the recruitment of blood cells including inflammatory monocytes at injection site [13]. Similarly to what was shown *in vitro*, alum promoted the differentiation of inflammatory monocytes into DCs. Interestingly, peritoneal monocytes recruited by alum expressed costimulatory molecules and MHC class II and acquired an enhanced capacity to take up the antigen. In addition, antigen-loaded monocyte-derived DCs efficiently migrated to the draining LNs and induced strong T cell proliferation.

It has been hypothesized that TLR-stimulation of the immune system is a prerequisite for initiation of a T cell-dependent immune response, since it allows for full maturation of DCs and delivery of costimulatory signals to T helper (Th) cells. Accordingly, a study carried out in MyD88-deficient mice showed that stimulation of B cells through TLR was necessary for the generation of T cell-mediated antibody responses [14]. The fact that alum is unable to activate directly DCs to express costimulatory molecules and release pro-inflammatory cytokines *in vitro*, suggests that it does not act via TLR-dependent signalling. Experiments performed in mice deficient for both MyD88 and TRIF showed that alum could bypass TLR signalling and still induce strong antibody responses against a co-administered T cell-dependent antigen [15]. These findings demonstrate that adaptive immune responses can be induced even in the absence of a functional TLR pathway and that alum works in a TLR-independent manner.

3. Alum adjuvanticity depends on the activation of the inflammasome complex

The data described above suggest that alum acts by at least two independent mechanisms: (i) it enhances antigen immunogenicity and delivery to APCs by assembling the antigen in a particulate multivalent form and (ii) it promotes, by a TLR-independent mechanism, a local pro-inflammatory environment, which results in blood cell recruitment and DC differentiation. However, the molecular target for alum pro-inflammatory activity was still unknown. Recent data from different laboratories demonstrated that this target is NOD-like receptor protein 3 (NLRP3).

NLRP3 is a member of the NLR family that interacts with the CARD-domain-containing adaptor protein ASC and the protease caspase 1 to form a protein complex called inflammasome. When the inflammasome is activated it processes pro-inflammatory cytokines including IL1β, IL18 and IL33, into their mature form. IL1β is not constitutively expressed but it is transcriptionally activated by several adjuvants, including TLR agonists. *In vitro*, alum activated caspase 1 through NLRP3 and synergized with TLR agonists like LPS or Pam_3CSK for IL1β production [16,17]. This process was blocked by actin polymerization inhibitors suggesting that NALP3 activation required phagocytosis [16].

It has also been demonstrated that NLRP3 activation contributes to alum adjuvanticity. Antibody responses to either a DT/TT or OVA antigen adjuvanted with alum were significantly reduced in NLRP3 deficient mice compared to wild-type animals [17]. In an independent study, Th2 cell priming and antibody responses to OVA or human serum albumin (HSA) co-administered with alum were impaired in NLRP3-, ASC- or caspase 1-deficient mice [18]. These data were challenged by another report in which injection of HSA in alum in NLRP3-deficient mice resulted in comparable antibody responses to those elicited in wild-type animals, suggesting that NLRP3 is not required for alum adjuvanticity [19]. These conflicting data might be the result of different immunization routes and different protocols for measuring immune responses.

It has been hypothesized that alum might activate NLRP3 through the induction of necrosis, which causes the release of danger signals such as uric acid and ATP. Indeed, uric acid concentration increased locally after alum administration in the peritoneum. Furthermore, uricase treatment decreased T cell priming induced by alum, suggesting that uric acid release at injection site is required for alum adjuvanticity [20]. However, necrosis, as a mechanism of alum adjuvanticity, has been questioned by *in vitro* data showing that macrophage treatment with uricase does not affect the response to alum [17,18]. More insights on how alum could activate NLRP3 came from very recent reports showing that phagocytosis is essential for NLRP3 activation and IL1β release by alum, silica crystals or fibrillar amyloid-β [21,22]. Phagocytosis of alum crystals induced phagosomal swelling, destabilization and rupture, finally resulting in the release of lysosomal proteins into the cytosol. Cathepsin B, a lysosomal protease, was shown to be involved in caspase 1 activation and IL1β release in macrophages in response to alum and silica [21]. These results suggest that phagosome destabilization is the missing link between alum and NLRP3 inflammasome activation.

4. Modulation of innate immune responses by MF59

Clinical trials have shown that MF59 is safe and enhances human humoral and cellular immune responses to various antigens derived by different pathogens such as influenza virus, HSV and HIV [23]. Postmarketing surveillance data have confirmed the good safety profile of MF59 [24]. An influenza adjuvanted vaccine (Fluad) was developed combining the MF59 adjuvant emulsion with the two main influenza antigens, HA and NA. This vaccine was used in more than 20 countries over the last decade. MF59-adjuvanted vaccine showed significantly increased antibody titers and enhanced cross-reactivity compared to non-adjuvanted vaccine formulations [25]. MF59 was also evaluated as an adjuvant for a potential pandemic vaccine. Human subjects receiving the MF59-adjuvanted pandemic vaccine had significantly higher antigen-specific antibody responses and superior cross-neutralization compared to subjects receiving antigen alone or formulated with alum [26,27].

Despite the fact that MF59 is widely used as a flu vaccine adjuvant, its mechanism of action is only partially understood.

Immunofluorescence analysis has shown that MF59 promotes antigen uptake by DCs after intramuscular injection [28]. Indeed, although at 3 h after injection MF59 formed small extracellular droplets, after 48 h it was internalized by cells expressing DEC-205 and MHC class II molecules, markers of activated DCs. Furthermore, at the same time point, MF59 co-administered antigen localized inside the DC vesicular organelles more efficiently than antigen administered alone. These data suggest that MF59, similarly to alum, enhances antigen uptake by APCs *in vivo*. However, it has been shown that antigen bio-distribution and clearance from the injection site are not modified by MF59, suggesting that MF59 does not induce any depot [29]. Intramuscular administration of MF59 induced recruitment of mononuclear cells expressing the surface markers CD11b and F4/80 [30]. In addition, 2 days after injection, MF59 was shown to localize in the subcapsular sinus of draining lymph nodes in cells expressing the CD80 and CD86 costimulatory molecules, and the I-A^d, CD11c and CD11b markers. These data suggest that MF59 induces in the muscle an infiltration and activation of mature macrophages, which engulf the antigen and transport it to the draining lymph nodes where they differentiate into DCs. Mononuclear cell recruitment was shown to be dependent on chemokine receptor 2 (CCR2). However, other factors may also be involved, since influx of $CD11b^+$ and $F4/80^+$ cells at injection site was not completely abolished in $CCR2^{-/-}$ mice compared to wild-type animals [30].

These data are in agreement with recent *in vitro* results confirming that MF59 stimulates human macrophages, monocytes and granulocytes to release monocyte and granulocyte attracting chemokines like CCL2, CCL3 and CCL4 and CXCL8 [11]. In addition, flow cytometric analysis of human PBMCs, showed that MF59 induces the differentiation of monocytes towards DCs as revealed by the up-regulation of the costimulatory molecule CD86 and the down-regulation of the monocyte marker CD14. MF59 also enhanced CD86 up-regulation and CD14 loss in pure monocyte cultures supplemented with GM-CSF and IL4, factors which induce the differentiation of monocytes into immature DCs.

Recently, an *in vivo* study using genome wide microarray analysis compared the effect of MF59, alum and CpG after i.m. injection in mice. All of these adjuvants modulated a cluster of common genes named "adjuvant core response genes" characterized by the up-regulation of cytokines, chemokines and adhesion molecules [31]. MF59 was more potent in inducing these genes compared to the other adjuvants. Interestingly, MF59 was shown to induce the up-regulation of IL1β and of other genes involved in IL1β processing, such as caspase 1. MF59 also induced the up-regulation of genes coding for Ccr2 and its ligands (Ccl2, Ccl7 and Ccl8) supporting previous data showing that cell recruitment at injection site is driven by CCR2. Moreover, the same study showed that MF59 promoted a more rapid influx of $CD11b^+$ cells in the muscle compared to other adjuvants. MHC class II^+ cells were also recruited in the muscle at 4 days, suggesting that $CD11b^+$ cells differentiate into functional inflammatory DCs expressing high levels of MHC class II. Some of the early genes up-regulated by MF59 were used as biomarkers to identify MF59 target cells. Proteins encoded by two early reponse genes, JunB and Pentraxin 3, were up-regulated in muscle fibers following MF59 treatment, suggesting that muscle cells are a target of MF59 *in vivo*. MF59 might exert part of its adjuvant activity after i.m. injection through direct engagement of the muscle. Indeed, skeletal muscle is known to participate in local immune reactions expressing pro-inflammatory cytokines and chemokines. In summary, this study has shown that both alum and MF59, although not capable of activating directly DCs *in vitro*, could trigger a local immunostimulatory environment characterized by the expression of several cytokines, which may indirectly activate DCs through a TLR-independent mechanism.

5. Conclusions and future perspectives

In recent years, the knowledge of the mechanism of action of vaccine adjuvants has largely increased. TLR-dependent pathways are well characterized at molecular level and significant progress has been made in understanding how particulate TLR-independent adjuvants work. Recent data on alum and MF59 suggest that particulate adjuvants have multiple mechanisms of action: they enhance antigen uptake, activate innate immune pathways and induce a local recruitment of blood cells. More work must be performed to dissect the differential contribution of each of these mechanisms in the generation of an optimal adaptive immune response.

New insights on the implication of NLRP3 in alum's adjuvanticity give some hint on new experiments to be undertaken to elucidate the mechanism of action of other TLR-independent adjuvants. It has been reported that other two vaccine adjuvants, QuilA saponin and chitosan, can activate IL1β secretion *in vitro* in a NLRP3-dependent mechanism. However, it is not known if also oil-in-water emulsions and other particulate adjuvants such as microparticles or liposomes can activate NLRP3.

The immunostimulating activity of particulate adjuvants has to be considered in the design of novel adjuvant formulations that, similarly to AS04, combine alum or emulsions with novel compounds targeting TLRs. The interference between the two adjuvant components of the vaccine must be carefully addressed in preclinical models to determine the therapeutic window allowing for superior efficacy without increasing local reactogenicity or systemic toxicity.

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