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REVIEW

Local innate immune responses in the vaccine adjuvant-injected muscle

Frank Liang and Karin Loré

Inducing a high magnitude of antibodies, possibly in combination with T-cell responses that offer epitope breadth over prolonged periods of time is likely a prerequisite for effective vaccines against severe diseases such as HIV-1 infection, malaria and tuberculosis. A much better understanding of the innate immune mechanisms that are critical for inducing desired responses to vaccination would help in the design of novel vaccines. The majority of human vaccines are administered into the muscle. In this brief review, we focus on the initial innate immune events that occur locally at the site of intramuscular vaccine delivery, and how they are influenced by clinically approved vaccine adjuvants. In particular, the effects on cell mobilization, cell activation and vaccine antigen uptake are reviewed. Understanding how distinct adjuvants enhance and tailor vaccine responses would facilitate the selection of the best-suited adjuvant to improve vaccine efficacy to a given pathogen.
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INTRODUCTION

Most existing vaccines work by efficient neutralization of pathogens with low antigenic variability. Considerable challenges remain in the development of vaccines that can elicit effective protection against highly mutation-prone pathogens with large epitope variability such as HIV-1, or those with a complex life cycle such as the causative agent of malaria (*Plasmodium falciparum*). These pathogens would likely require vaccines that induce durable protection consisting of broadly neutralizing antibodies and/or efficient T-cell responses. Deciphering the mechanisms of how vaccines stimulate and shape responses is instrumental for the design of future vaccines and to find strategies to enhance the potency of candidate vaccines. Several existing vaccines consist of live attenuated or killed whole pathogens that induce robust immune responses due to their inherent immunostimulatory microbial structure and content, referred to as pathogen-associated molecular patterns (PAMPs). However, live attenuated vaccines are not suitable platforms for lethal pathogens such as HIV-1. In addition, the attenuation and inactivation processes of live pathogens are relatively lengthy, which could fail to promptly supply demands of large vaccine quantities in pandemic situations. Recombinant technology offers rapid large-scale production of highly purified protein subunit vaccines with exceptional safety profiles, but these vaccines are poorly immunogenic, as they lack and/or are depleted of PAMPs. As stimulating innate immune responses is a prerequisite for generating adaptive vaccine responses, co-formulation with adjuvants is needed for many subunit vaccines. How adjuvants work is not fully understood; however, much evidence suggests that they activate innate immune responses.

Different routes of vaccine delivery have been explored in humans, including skin and mucosal tissue, but the majority of clinical vaccines are still injected into skeletal muscle tissue. Although skin vaccination has proven to induce similar antibody titers using doses lower than those used for the intramuscular (i.m.) route (that is, a dose-sparing effect), skin vaccinations more frequently result in unwanted local reactions.[1] Vaccine delivery to the muscle is generally associated with no or minor local reactogenicity,[2] which is necessary to meet the stringent safety and tolerability requirements for clinical use. Vaccine potency and efficiency are usually estimated by antigen-specific antibody titers and T-cell responses generated weeks after vaccination. However, the series of innate immune responses occurring between the time of vaccine delivery and induction of the measurable adaptive immunity remain largely unknown. This review therefore evolves around the limited current knowledge about the early local innate immune responses *in vivo* in the muscle after injection with adjuvants used in approved human vaccines. Specific mechanisms proposed for these adjuvants are introduced and their influence on the hallmarks of innate immune responses will be discussed.

Skeletal muscle contains few immune cells.[3] Tissue resident and/or infiltrating immune cells, including potent antigen presenting cells (APCs) such as dendritic cells (DCs) encounter vaccine antigens for the first time at the site of administration. Thus, the magnitude of local innate immune responses starting at the vaccine delivery site initially controls subsequent adaptive immune responses. Induction of an efficient vaccine response requires some degree of local inflammation to trigger and support the sequence of immunological events leading to the adaptive immunity. Vaccine adjuvants administered to the muscle have a central role in inducing transient inflammation at the delivery site that promotes immune cell recruitment and activation. This inflammation likely leads to better vaccine antigen uptake by critical infiltrating cell types and migration

Immunology and Allergy Unit, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden
Correspondence: Dr F Liang, Immunology and Allergy Unit, Department of Medicine Solna, Karolinska Institutet, Stockholm 17176, Sweden.
E-mail: frank.liang@ki.se
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of vaccine-loaded cells to the draining lymph nodes (dLNs) to establish the adaptive immunity (Figure 1).

DISTINCT ADJUVANTS USED FOR APPROVED VACCINES AGAINST INFECTIOUS DISEASES

A plethora of adjuvants are assessed in preclinical studies or clinical trials, but only a few are approved for human vaccines against infectious diseases (Table 1). Adjuvants are broadly defined as carrier/delivery systems (for example, aluminum salts and emulsions), immunostimulatory molecules (for example, toll-like receptor (TLR) ligands and saponin-derived molecules) and combinations thereof. They may therefore stimulate different features of innate immunity. To this end, adjuvants provide means to enhance the durability and strength of the adaptive immunity, as well as opportunities to steer immune responses toward antibody- and/or T-cell-based immunity for optimal protection against a specific disease.[4,5]

Alum adjuvant

Aluminum hydroxide and aluminum phosphate, referred to as alum is by far the most commonly used adjuvant for clinical vaccines (for example, diphtheria, tetanus, pertussis and hepatitis A/B). Alum is an inexpensive delivery system with an exceptional safety profile. The mechanism of action of alum was originally thought to be associated with a depot effect, which would retain the vaccine antigens at the delivery site for prolonged antigen exposure and immune activation. This hypothesis was further supported by findings in guinea pigs that mounted antigen-specific antibody titers when immunized with minced tissue from the injection site of other animals that received diphtheria toxoid and alum.[6] However, the depot effect has also been proven to be dispensable for antigen-specific T-cell and B-cell responses, as the injection site (ear of the mice) can be removed after immunization without any effect on the adaptive vaccine responses.[7] Instead, alum's ability to stimulate various aspects of innate immunity has emerged as the main function for the adjuvant effect. Although intraperitoneal (i.p.) delivery of alum has been shown to activate the pro-inflammatory cytokine interleukin (IL)-1β via the multi-protein complex NLRP3 inflammasome (Table 1),[8,9] other reports have questioned this role in alum adjuvanticity.[10,11] In addition, alum delivered i.p. was shown to activate DCs, promote inflammatory monocytes, enhance antigen uptake and induce uric acid levels that potentiated the adaptive immunity.[12] Uric acid crystals have also been reported to induce NLRP3 inflammasome activity *in vitro*.[13] However, when alum was given i.m. to NLRP3-deficient or wild-type mice, respectively, similar antibody titers were observed.[11] Inflammatory responses in the muscle mediated by alum have also been associated with cell death[14] and host-derived immunostimulatory molecules, that is, danger-associated molecular patterns (DAMPs).[15,16] Of note, the innate immune responses to alum-adjuvanted antigens delivered i.p. and i.m. may not be the same.

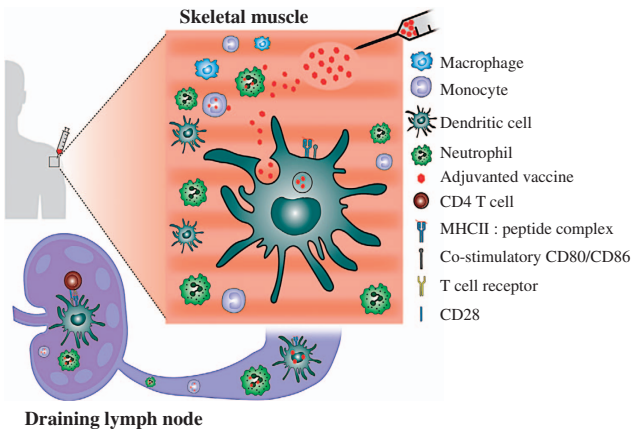

Figure 1 Innate immune responses of adjuvanted intramuscular vaccine, represented by immune cell infiltration to the delivery site, vaccine antigen uptake, cellular activation and homing to draining lymph nodes for antigen presentation and stimulation of CD4 T cells.

Emulsion adjuvants

Distinctly different from alum is the oil-in-water (O/W) squalene emulsion adjuvant MF59, approved for pandemic influenza vaccines. MF59 was reported to increase antibody titers using lower vaccine doses and to broaden antibody specificities.[18] Another squalene-based emulsion, AS03, which also contains α-tocopherol (vitamin E), enhanced the efficacy of pandemic influenza vaccines with regards to dose sparing[19] and cross-reactive antibody titers.[20] Of note, α-tocopherol alone was shown to be an efficient inducer of antibody titers.[21] As with alum, the mechanisms of action of emulsion adjuvants have only recently started to be described. The effect of MF59 delivered i.p. was shown to require myeloid differentiation primary response gene 88 (MyD88) an adapter protein for most TLR signaling pathways, but the NLRP3 inflammasome was dispensable.[22] Although referred to as a delivery system, MF59 induces an 'immunocompetent environment' at the muscle injection sites, which is a local inflammation that facilitates innate immune activity such as cell infiltration, activation and antigen uptake.[23–25] The requirement of an adjuvant-induced inflammatory milieu in the muscle was elegantly demonstrated in mice, where sequential delivery of the adjuvant followed by protein antigens to the same site was necessary to obtain antigen-specific immunity.[26–29]

Toll-like receptor ligand- and liposome-based adjuvants

In contrast to alum and emulsion adjuvants, the mechanisms of immunostimulatory adjuvants are better understood as many of them are designed to target specific TLRs. The TLRs are pattern recognition receptors that mediate cellular activation upon binding to pathogenic PAMPs or their synthetic analogs.[30] The TLR4 ligand monophosphoryl lipid A (MPL), a detoxified derivate of bacterial lipopolysaccharide (LPS), is currently the only TLR-targeting adjuvant in approved vaccines. Innate immune cell subsets including DCs, monocytes and neutrophils express TLR4,[31,32] which suggest that they are responsive to TLR4 stimulation. MPL formulated with alum is licensed as AS04 and used in the hepatitis B virus and human papilloma virus (HPV)-16/18 vaccines.[33] The recently approved AS01-adjuvanted malaria vaccine (RTS,S), resulted in a successful but modest efficacy in infants in seven malaria-endemic countries.[34] Moreover, AS01 was shown to efficiently enhance the effect of a herpes zoster subunit vaccine in a recent phase III study,[35] which may relate to the AS01-driven induction of antigen-specific $CD4^+$ T cells.[36] AS01 consists of liposomes, MPL and quillaja saponin fraction 21 (QS21), an immunostimulatory saponin-derivate from the tree bark of *Quillaja saponaria* Molina. Delivery of QS21 alone was shown to induce inflammasome-mediated IL-1β in the muscles of mice.[37] The protein antigens in the clinically approved AS01- and AS04-adjuvanted vaccines are composed of virus-like particles (VLPs), which are self-assembled recombinant protein monomers with surface protein organization and conformation reminiscent of native virus particles, but lacking the viral genome. As VLPs mimic viral structure, they may provide some adjuvant effect. Virosomes, which qualify as VLPs in

Table 1 Examples of adjuvanted clinical vaccines against infectious diseases

Adjuvant	*Type*	*Examples of potential innate modes of action*[a]	*Clinical vaccines*	*Manufacturer*
Alum	Aluminum salt	NALP3 inflammasome activity, antigen uptake, release of host DNA, monocyte activation.	Diphtheria, tetanus, pertussis, HAV, HBV and so on.	Various
MF59	O/W emulsion	MyD88 activation, release of ATP, neutrophil recruitment, moDC differentiation.	Fluad (seasonal flu) Foceteria (pandemic flu) Aflunov (pre-pandemic flu)	CSL
AS01	MPL, QS21, liposome	TLR4 stimulation, NALP3 inflammasome activity, homing of antigen$^+$ monocytes to dLNs.	Mosquirix (RTS,S malaria)	GSK
AS03	O/W emulsion and α-tocopherol	Monocyte activation, homing of antigen$^+$ granulocytes and DCs to dLNs.	Pandemrix (pandemic flu) Arepanrix (pandemic flu)	GSK
AS04	MPL and alum	TLR4 stimulation, NFκB activity, antigen uptake, DC activation.	Cervarix (HPV-16/18) Fendrix (HBV)	GSK
Virosomes	Liposome/VLP	HA-mediated antigen uptake (Virosomes), TLR activation mediated by viral-like structures.	Inflexal (seasonal flu) Epaxal (HAV) Invivac (seasonal flu)	CrucelI Solvay

Abbreviations: CSL, commonwealth serum laboratories; DC, dendritic cell; dLNs, draining lymph nodes; GSK, GlaxoSmithKline; HA, hemagglutinin of influenza virus A; HAV, hepatitis A virus; HBV, hepatitis B virus; HPV, human papilloma virus; MoDC, monocyte-derived DC; MPL, monophosphoryl lipid A; MyD88, myeloid differentiation primary response gene 88; NALP3, NACHT (neuronal apoptosis inhibitor protein, major histocompatibility complex class 2 transcription activator, heterokaryon incompatibility, and telomerase-associated protein 1), LRR (leucine rich repeat) and PYD (pyrin domain) containing protein 3; NFκB, nuclear factor kappa-light-chain enhancer of activated B cells; O/W, oil-in-water emulsion; QS21, quillaja saponin fraction 21; TLR, toll-like receptor; VLP, virus-like particles.
[a]The examples of potential modes of action are based on the publications reviewed herein.

terms of mimicking virus particles, consist of influenza virus neuraminidase and hemagglutinin integrated into phospholipid bilayer liposome. A virosomal influenza vaccine has been proven efficient when delivered into skin or to the muscle of healthy adults,[1] and the virosome platform is approved for hepatitis A and influenza vaccines without additional immunostimulatory substances. Virosomes have also shown the capacity to activate DCs *in vitro*.[38]

LOCAL INNATE IMMUNE RESPONSES IN THE MUSCLE AFTER ADJUVANT ADMINISTRATION

Inflammatory mediators in the muscle tissue after injection of adjuvants

The local inflammatory responses induced by vaccine adjuvants in the injected muscle represent the first of a series of innate immune events, leading to adaptive immune responses (Figure 1). In contrast to TLR-targeting adjuvants, the specific receptors for alum or emulsions *in vivo* are not defined, which impedes identification of the cell subsets responsible for initiating local inflammation in the vaccine-injected muscle. As mentioned earlier, muscle tissue contains relatively few resident immune cells. Thus, it is plausible that inflammatory responses following i.m. vaccination are mediated primarily by infiltrating immune cells. However, muscle fibers were found to be susceptible to MF59-mediated activation *in vivo* by upregulating the pattern recognition receptor, pentraxin 3 and the transcription factor JUNB.[23] A subsequent study demonstrated the release of ATP nucleotides, with properties of DAMPs, in MF59-injected muscles.[25] These reports suggest that muscle tissue has the ability to produce inflammation-related molecules *in vivo*, at least in response to MF59. Alum-injected muscle was shown to release DAMPs in the form of host cell DNA.[15,16] Interestingly, increase in messenger RNA for chemokines were detected in LPS-stimulated muscle fibers,[39] which indicate that muscle tissue has the necessary machinery to produce chemokines and may therefore support cell infiltration. Thus, both muscle cells and muscle-resident immune cells seem capable of initiating inflammation to promote infiltration of circulating immune cells. Indeed, chemokines and cytokines were readily detected in mouse muscles injected with distinct adjuvants as early as 3 h post injection (p.i.).[23,24,26–28] This was in line with the early gene expression of cytokines, chemokines and other effector molecules in the muscle receiving MF59[23] or AS03.[26] These emulsion adjuvants also upregulated several pro-inflammatory genes after delivery compared with alum.

The initiation of local innate immune responses in the muscle appears to occur instantly after the injection of a vaccine and could be triggered by several components in the adjuvant. AS03 lacking α-tocopherol induced high levels of several inflammatory mediators in the muscle early after injection (6 h) compared with complete AS03, which instead showed higher levels at 24 h.[26] Interestingly, one of the surfactants of MF59 was also able to induce pro-inflammatory genes on its own, albeit less efficient compared with complete MF59.[40] In AS04-injected mouse muscles, cytokines and chemokines were detected within 6 h and the MPL component alone induced the highest cytokine and chemokine levels.[28] This suggests that individual adjuvant components have intrinsic pro-inflammatory properties and kinetics, which have an impact on obtaining the optimal concentrations and/or ratio of specific components that make a potent and safe adjuvant. In line with the studies mentioned above, AS01 was also able to induce robust production of cytokines and chemokines in the muscle when compared with muscle receiving non-adjuvanted protein antigens.[27] AS01 used in this study did not contain liposomes, which may have influenced the innate immune response profile. However, liposomes alone have shown to be poor immunostimulants *in vivo*[41] and possibly only serve as a vehicle for AS01. Nonetheless, AS01 proved to be the superior vaccine adjuvant over AS02 (MPL, QS21 and O/W emulsion) when compared side by side in a phase II RTS,S malaria vaccine trial.[42] It remains to be determined whether AS01 and AS02, which both contain MPL and QS21, induce different local inflammatory mediators. Further, most cytokines and chemokines in the adjuvant-injected muscle were rapidly reduced 24 h after delivery.[24,26–28] Expression of pro-inflammatory molecules were not only restricted to the muscle, as dLNs of adjuvant-injected muscle showed higher cytokine and chemokine expression compared with LNs-draining muscle receiving phosphate-buffered saline (PBS) or alum,[26] or protein antigen alone.[27] These *in vivo* findings taken together indicate that there is a rapid and transient local inflammation, which facilitates subsequent innate

immune activities such as cell infiltration, antigen uptake and cellular activation.

Immune cell mobilization in the adjuvant-injected muscle

Recruitment of immune cells to vaccine delivery sites is important for interactions between vaccine antigens and immune cells. The clinical adjuvants mentioned above were found to induce chemokines such as CCL2 (MCP-1) and CXCL1 (murine functional homolog of human neutrophil attractant IL-8), which enhance immune cell mobilization to vaccine-injected muscle. Mice deficient of CCR2 (receptor for CCL2-mediated recruitment of, for example, monocytes from circulation to tissues) showed significantly impaired cell infiltration in muscle after MF59 administration.[43] Neutrophils and monocytes are the first cells to infiltrate antigen-exposed tissues. They were found recruited to the mouse muscle as early as 3–6 h after injection of either MF59[24] or AS01.[26] Cell infiltration was not due to the injection itself as muscles injected with PBS or non-adjuvanted protein antigens had similar cell numbers as non-injected muscles.[24] Frequencies of neutrophils and monocytes peaked at 16 and 48 h, respectively, in the MF59-injected muscles and a similar kinetics for $CD11b^+$ cells (comprising neutrophils, monocytes and DCs) in alum-injected muscle was mentioned by the authors.[24] AS01 appeared to induce faster kinetics as both neutrophil and monocyte numbers peaked already at 6 h compared with MF59- or alum-injected muscles. Increased numbers of neutrophils have been found for up to 48 h in alum-injected muscles.[44] Neutrophils or monocytes returned to steady-state levels in adjuvant-injected muscle after 5–7 days,[24,27] which correlated with the baseline levels of most cytokines and chemokines at the later time points.[26–28] This again emphasizes the rather transient local inflammation in the muscle after immunization. As robust adjuvant-driven chemokine production in muscle was detected at gene and protein level, one may expect a correlation between chemokines and cell infiltration. In this regard, multivariate analysis showed that neutrophil kinetics in the AS01-injected muscle correlated with 8 out of 12 genes encoding cytokines or chemokines.[27] In addition to neutrophils and monocytes, other immune cells including DCs, eosinophils, natural killer cells and T cells were found recruited to the adjuvant-injected muscle,[24,26,27] which likely leads to cross talk between cells to orchestrate innate immune responses.

As DCs are recognized as potent and versatile APCs,[5] their infiltration to the injection site is likely vital for mounting strong adaptive immune responses. In contrast to neutrophils and monocytes, DCs peaked at 72 h in MF59-injected muscles.[24] At day 7, a slight increase in DCs was detected in AS01-injected muscle compared with control muscles[27] and DCs were still present in low numbers in the MF59-injected muscle at day 11. MF59 has been shown to support differentiation of DCs from monocytes (moDCs) *in vitro*.[45] Whether this increase of DC numbers at the later time points represent moDCs or *bona fide* DCs, which are retained at the injection site or recruited by residual low-grade inflammation, remains elusive. However, a low level of sustained DC infiltration may have consequences if vaccination regimens consist of frequent administration to the same site, as this would locally target more DCs.

Adjuvant-driven homing of vaccine $antigen^+$ cells to the draining lymph nodes

Antigen presentation leading to adaptive vaccine responses presumably occurs in the LNs that drain the vaccination site. The numbers of vaccine $antigen^+$ cells in dLNs of the injection site may give an estimate of the levels of APCs and antigens (intact or presented on major compatibility complex (MHC) molecules as peptides) that are available for antigen-specific interactions with cognate T-cell receptors. $Antigen^+$ cells in dLNs likely represent a mixture of antigen-bearing cells emigrated from muscle and LN-resident cells that locally captured disseminated antigens. In this regard, activated moDCs exposed to MF59, alum or LPS, have been shown to efficiently migrate toward LN-homing chemokines *in vitro*.[45] $Antigen^+$ neutrophils in the dLNs of AS01- or MF59-injected muscles rapidly peaked at 3 h p.i., and declined thereafter at a similar rate already at 48 h.[24,27] Of note, AS01 was most efficient in inducing $antigen^+$ monocytes in dLNs compared with alum, MF59, AS03 and AS04. As mentioned earlier, DC numbers in muscles receiving AS01 were unchanged over time, which may suggest rapid antigen uptake and migration of DCs from the injection site to the dLNs. In fact, AS01 induced almost twice as many $antigen^+$ DCs in dLNs during the earliest time points compared with MF59. Although labeled with the same fluorophore, the protein antigens were different in these studies and intrinsic properties of these proteins (for example, size and net charge) may influence accumulation of $antigen^+$ cells in dLNs. Nonetheless, $antigen^+$ DCs levels in dLNs were on par at 24 h after delivery of AS01 or MF59. Comparatively, AS04 induced the highest levels of $antigen^+$ DCs in dLNs 24 h p.i., whereas AS03 induced the lowest when using the same protein antigen.[26,28] Importantly, compared with alum as the adjuvant, MF59, AS03 and AS04 were all more efficient in mobilizing multiple $antigen^+$-bearing cell subsets to the dLNs, and AS01 was found to induce more efficient homing $antigen^+$ cells to dLNs than non-adjuvanted proteins. The more robust influx of vaccine $antigen^+$ cells to the LNs induced by MF59, AS01, AS03 and AS04 is likely one factor that contributes to the superiority of these adjuvants compared with alum.

Interestingly, exclusion of α-tocopherol in AS03 resulted in higher monocyte and DC levels in the dLNs, whereas complete AS03 induced higher dLN homing of neutrophils and eosinophils.[26] As mentioned, a surfactant component of MF59 was able to induce low-level expression of inflammatory genes at the injection site and this inferior stimulation of inflammation was also reflected in the lower numbers of $antigen^+$ cells in dLNs.[40] The kinetics of mobilization of specific cell subset to the injected muscle and subsequent homing to dLNs induced by adjuvants is therefore multifaceted, and the profile and degree of innate immune responses vary with time and adjuvant formulation.

Enhancement of cellular activation and antigen uptake by adjuvants *in vivo*

Cellular activation of APCs concurrent with upregulation of MHCII and co-stimulatory molecules (for example, CD80, CD86 and CD40) and cytokine production are key innate immune events for efficient antigen presentation and priming of antigen-specific naive T cells. As discussed earlier, multiple studies demonstrated a robust production of chemokines and several pro-inflammatory cytokines in the muscle after adjuvant injection.[23,24,26–28] Identifying the producer cell subsets *in vivo* is challenging as chemokines and cytokines are quickly released. Instead, expression of cell membrane-associated MHCII and co-stimulatory molecules on isolated cells from the adjuvant-injected muscle or dLNs may offer a more stable assessment of cellular activation.

Although DC activation by alum *in vitro* was proposed to involve interaction with cell membrane lipids,[46] the target receptors leading to cellular activation by non-TLR-targeting adjuvants have not been determined *in vivo*.[22] DAMPs have emerged as molecules capable of cellular activation via several receptors, including TLRs.[47,48] Alum has been shown to induce local cell death in the muscle *in vivo*[14] and *in vitro* by cathepsin-mediated necrosis.[49] This finding was in line with the presence of self-DNA in alum-injected muscles.[15,16] IL-33 and uric

acids as a consequence of alum exposure are additional DAMPs detected after i.p. injection,[12,50] but whether these molecules are induced by the i.m. delivery route needs further elucidation. Cellular activation by alum-induced DAMPs likely occurs primarily at the delivery site as DCs in LNs draining alum-injected skin[49] or muscle[28] displayed modest upregulation of co-stimulatory molecules compared with PBS control injections.

DAMPs may also be involved in cellular activation mediated by emulsion adjuvants. Release of intracellular ATP was observed *in vivo* in MF59-injected muscle.[25] In this regard, APCs that acquired MF59 have been described as apoptotic and showed high expression of CD80 and CD86.[43] Models of O/W emulsions adjuvants have also been reported to induce cell death *in vitro*.[51] These findings suggest that DAMPs likely contribute to the mechanisms of emulsion adjuvants. The emulsions AS03 and MF59 are also strong activators of monocytes. AS03 induced *in vitro* release of several cytokines, preferentially by monocytes rather than DCs,[26] and MF59 upregulated MHCII expression on monocytes plus supported moDC differentiation.[40,45] Interestingly, *in vivo* antigen uptake by monocytes was significantly reduced when AS03 lacked α-tocopherol, but antigen uptake by DCs remained unchanged.

Cellular activation as a result of targeting TLR4 is best indicated by the MPL component in AS01 and AS04. TLR4-mediated stimulation leads to activation of nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) and other transcription factors.[52] High NFκB activity was detected using *in vivo* imaging of muscles and dLNs in mice receiving MPL alone or AS04.[28] Interestingly, NFκB activity was also observed in LPS-stimulated mouse skeletal cells *in vitro*,[39] which suggests that NFκB activation can be induced in muscle fibers at the injection site. Alum has been shown to increase uptake and reduce degradation of antigens *in vitro*[53] and as AS04 contains alum in addition to MPL, efficient antigen uptake *in vivo* would be expected. Indeed, AS04 induced better antigen uptake by monocytes and DCs at 24 h p.i. compared with the emulsions AS03 and MF59. Comparison between MPL-containing adjuvants showed that AS04 resulted about twice as many antigen$^+$ DCs in dLNs than AS01. However, the superior antigen uptake induced by AS04 was not entirely due to its alum content, as MPL alone induced much more antigen$^+$ cells in dLNs compared with alum alone.[28] Importantly, expression of CD86 and CD40 were highest on the DCs that also had taken up most antigens *in vivo*, which verified the efficient cellular activation and antigen uptake induced by AS04.[28] Further, the protein antigens in the currently clinically approved AS04- and AS01-adjuvanted vaccines (HPV and malaria) consist of VLPs. Although proteins are poorly immunogenic, HPV-based VLPs alone[54] or virosomes[38] have been shown to activate DCs *in vitro*. However, the innate stimulatory effects of VLP alone were not different from PBS controls *in vivo*, despite its structural resemblance to virion particles.[28] Thus, the adjuvants and not the VPLs are the dominant stimulating component of innate immunity in AS01- or AS04-adjuvanted vaccines.

Antigen presentation capacity influenced by adjuvants

Adjuvant-mediated enhancement of cellular activation, antigen uptake and accumulation of antigen$^+$ cells in the muscle and subsequently in the dLNs are essential immune events preparing for antigen presentation and generation of antigen-specific immunity. Enhancement of antigen presentation capacity as a result of innate activation by an adjuvant was demonstrated *in vitro*, where DCs stimulated with alum-adjuvanted protein antigens more efficiently upregulated MHCII, acquired antigens and presented processed antigens better when compared with protein alone stimulation.[53] This was in line with the increased proliferation and cytokine production of CD4 T cells co-cultured with moDCs exposed to MF59.[45] Further, DCs isolated from dLNs of AS04-injected muscles induced higher CD4 T-cell responses *ex vivo*, compared with DCs from dLNs of muscle receiving alum or MPL alone.[28] Of note, purified CD4 T cells alone did not respond to MPL *in vitro*, indicating T-cell activation via antigen presentation.[28] However, the magnitude of antigen presentation occurring *in vivo* cannot be fully represented by observations from experiments with specific isolated cell subsets, as antigen$^+$ cells in the dLNs *in vivo* are heterogeneous. The presence of multiple activated and antigen$^+$ cell subsets in dLNs *in vivo* after delivery of adjuvanted vaccines suggests that there are several contributors for the generation and maintenance of adaptive immune responses.

As neutrophils represent one of the antigen$^+$ cell types in the dLNs, evaluation of their role in stimulation of the adaptive immunity is warranted. Human splenic neutrophils supported antibody responses via cytokines,[55] and mouse neutrophils were able to upregulate MHCII and co-stimulatory molecules, and stimulate antigen-specific CD4 T-cell responses *in vitro*.[56] However, the contribution of neutrophils *in vivo* is controversial as neutrophil-depleted mice immunized in the footpad with alum-adjuvanted antigens resulted higher adaptive responses.[57] In contrast, no difference in antibody titers was observed in neutrophil-depleted mice receiving i.m. delivery of antigens adjuvanted by MF59[24] or alum.[44] As antigen delivery via footpad lead to subcutaneous and intradermal antigen exposure, the differences in immunization routes may contribute to these discrepancies. Antigen$^+$ monocytes were also frequent in the dLNs after vaccination. Comparison of *ex vivo* antigen presentation capacity of monocytes and DCs isolated from dLNs 24 h p.i. with AS01-adjuvanted antigen showed that DCs were much more efficient than monocytes in stimulating both CD4 and CD8 T-cell proliferation.[27] This again confirms that DCs are the most potent APC, despite AS01 delivery resulted high levels of antigen$^+$ monocytes in dLNs. The indispensible role of DCs was also demonstrated when mice depleted of CD11c$^+$ DCs before i.m. injection of AS01-adjuvanted protein antigens showed abolished antigen-specific CD4 and CD8 T-cell responses.[27] Augmentation of antigen presentation by adjuvant-mediated cellular activation was further supported by the diminished T-cell responses in mice receiving alum-adjuvanted antigens together with DNase,[16] which is likely due to degradation of host DNA DAMPs at the injection site and/or dLNs. In this regard, degradation of ATP led to significantly lower T-cell and B-cell responses in mice receiving MF59-adjuvanted antigens together with ATPase.[25] As mentioned, one of the MF59 components, AS03 without α-tocopherol and MPL showed varying degree of pro-inflammatory capacity, which suggests that individual adjuvant components might co-operate through various pathways for its total adjuvanticity. However, the magnitude of antibody and T-cell responses were substantially reduced unless the completely formulated adjuvant was used.[26,28,40] The ability of adjuvants to enhance immune responses is essential for non-live vaccines and although the mechanistic pathways to achieve their respective adjuvanticity are different, the distinct adjuvants for approved vaccines have clearly demonstrated their efficiency to stimulate innate immune activity.

CONCLUDING REMARKS

The quantity, quality and durability of the generated immune responses determine the vaccine efficacy. Considerable efforts have been focused on understanding the mechanisms of action of adjuvants. The initial immune events at the site of vaccine administration described in this review underpins the need for more

understanding of the basic mechanisms of vaccine responses, which can help to develop better vaccines. Several questions need to be further addressed, including the *in vivo* receptors for non-TLR-targeting adjuvants, the functional capacity of antigen$^+$ APCs in the dLNs that have acquired antigens at the vaccine-injected muscles versus locally in the dLNs, and the degree of cross talk and bystander activation of infiltrating cells, and immunocompetent tissue cells at the delivery site. Although transgenic mouse models more readily provide answers to theses questions, inbred rodent strains cannot fully recapitulate the stringently regulated series of innate immune responses occurring after vaccination in humans. Outbred nonhuman primates are more representative models of human vaccine responses, as there is high degree of similarities in immune cell subset distributions, receptor expression (including TLRs), immune cell functions, in addition to modeling doses and injection sites of clinical vaccines more accurately.[3,31,58,59] To conclude, vaccination is one of the most powerful ways to prevent infectious diseases. With a more in-depth knowledge of the immune mechanisms involved in generating vaccine responses, we would be better positioned to rationally design new vaccines.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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