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REVIEW

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Selection of adjuvants for vaccines targeting specific pathogens

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ABSTRACT

Introduction: Adjuvants form an integral component in most of the inactivated and subunit vaccine formulations. Careful and proper selection of adjuvants helps in promoting appropriate immune responses against target pathogens at both innate and adaptive levels such that protective immunity can be elicited.

Areas covered: Herein, we describe the recent progress in our understanding of the mode of action of adjuvants that are licensed for use in human vaccines or in clinical or pre-clinical stages at both innate and adaptive levels. Different pathogens have distinct characteristics, which require the host to mount an appropriate immune response against them. Adjuvants can be selected to elicit a tailor-made immune response to specific pathogens based on their unique properties. Identification of biomarkers of adjuvanticity for several candidate vaccines using omics-based technologies can unravel the mechanism of action of modern and experimental adjuvants.

Expert opinion: Adjuvant technology has been revolutionized over the last two decades. In-depth understanding of the role of adjuvants in activating the innate immune system, combined with systems vaccinology approaches, have led to the development of next-generation, novel adjuvants that can be used in vaccines against challenging pathogens and in specific target populations.

1. Introduction

Development of vaccines against infectious diseases is one of the most remarkable accomplishments in the history of mankind [1]. Smallpox has been eradicated from the world, and other diseases like diphtheria, poliomyelitis, pertussis, measles, and neonatal tetanus are significantly controlled by vaccination [1,2]. While live attenuated vaccines are immunogenic, there is a chance of live virus-induced disease progression in populations with underdeveloped or compromised immune systems [2]. In contrast, inactivated virus vaccines are safe, but unsuitable when natural infection by the pathogen itself fails to induce any long-term immunity. Recombinant subunit vaccines are considered as one of the most attractive modern vaccine types due to their high safety profiles. However, subunit vaccines are not inherently immunogenic [2,3]. To overcome this limitation, adjuvants are incorporated in subunit vaccines to enhance immunogenicity of the vaccine antigen. Adjuvants facilitate the development of vaccines targeting pathogens against which live attenuated or inactivated vaccines are ineffective or undesirable. Identification and selection of new adjuvants is thus critical, but also challenging, for successful subunit vaccine development.

2. Mode of action of adjuvants

The fact that only a few adjuvants were approved for use in human vaccines till a few years ago can be at least partially attributed to the dearth of knowledge of the mechanism of action of adjuvants. However, presently six adjuvants (Alum, AS04, MF59, AS03, AS01 and CpG ODN) are approved for use in human vaccines. This was possible because structural characterization of several adjuvants and identification of various pattern recognition receptors (PRRs) and co-stimulatory ligand receptors have enabled us to better understand the mode of action of adjuvants at a molecular level. Understanding the mode of action of adjuvants is critical in designing vaccines that elicit pathogen-specific effector and long-term memory responses and in assessing the adjuvant safety at developmental and regulatory stages. The possible mechanisms of action by which adjuvants exert their adjuvanticity are discussed in the subsequent sections and represented schematically in Figure 1.

2.1. Delivery systems

Adjuvants as a delivery system in subunit vaccines [such as liposomes, immune stimulating complexes (ISCOMs) and nanoparticles] are considered effective in stimulating protective immunity [4]. Such adjuvants prevent rapid degradation of proteins and peptides *in vivo*, thereby enhancing the dose effectiveness of the vaccine antigen.

Liposomes are used in vaccine formulations against influenza, chlamydia, malaria, and tuberculosis (TB). Co-administration of antigen with cationic liposomes leads to the induction of stronger antigen-specific immune responses than neutral or anionic liposomes [5]. Liposomes are effective vaccine delivery systems and act as carriers in adjuvants such as AS01, a liposome-based formulation consisting of monophosphoryl lipid A (MPLA) and QS-21 [6]. Improved saponin-based

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tensoactive adjuvants (ISCOM, ISCOMATRIX, and Matrix-M™) are particulate antigen delivery systems with powerful immunostimulating activity [7]. In ISCOMs, saponin, cholesterol and phospholipid form cage-like structures (40–50 nm in diameter). The adjuvant ISCOMATRIX has a structurally similar structure but without the incorporated antigen (the antigen can be formulated with ISCOMATRIX to prepare an ISCOMATRIX vaccine) [8]. Both antigen delivery and immunostimulatory properties are present in ISCOMATRIX [9]. Within the first few hours of injection, the antigen–ISCOMATRIX complex traffics into draining lymph nodes (dLNs) where antigen delivery takes place for uptake by the resident dendritic cells (DCs) and other antigen presenting cells (APCs) [9]. ISCOMs are currently used in the

development of influenza vaccines and ISCOMATRIX in hepatitis C virus (HCV), influenza and candidate cancer vaccines in humans. The Matrix-M™ adjuvant is being evaluated in vaccines against influenza, herpes simplex virus (HSV) type 2 and malaria [10]. Nanoparticles are polymeric colloidal carriers of antigens, which enable site-directed delivery and prolonged release of antigen and facilitate alternative modes of vaccine administration (such as inhalation, optical or topical delivery). Examples of polymeric nanoparticles are poly(lactic-co-glycolic acid) (PLGA), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(hydroxybutyrate) (PHB) and chitosan [11]

Aluminum salts are also used as delivery systems. Crystals of alum bind to and alter the lipids of the DC plasma membrane to trigger cell activation that facilitates delivery of antigen, without alum itself being internalized by the DCs [12]. Aluminum salts are used as adjuvants in human vaccines against diphtheria, tetanus, pertussis, rabies, anthrax, and hepatitis A and B [13]. *In vitro* studies revealed that the oil-in-water emulsion adjuvant, MF59 increases both phagocytosis and pinocytosis indirectly to promote better antigen uptake by APCs. Instead of directly targeting DCs for antigen uptake, MF59 acts upstream by promoting influx of DC precursor cells and augmenting their differentiation into DCs [13]. The safety of MF59 was demonstrated in various clinical studies with antigens from hepatitis B virus (HBV), HCV, cytomegalovirus (CMV), HSV and human immunodeficiency virus (HIV) [14]. Similar to MF59, AS03 does not directly activate DCs *in vitro*. Intramuscular injection of AS03 in mice promotes influx of monocytes, DCs, and granulocytes into the dLNs. AS03 is
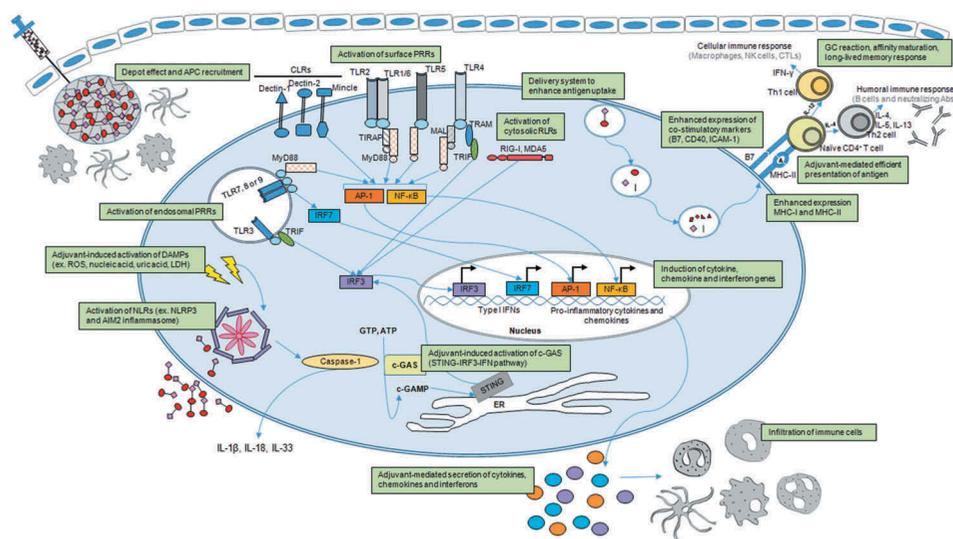

Figure 1. Schematic representation to highlight the possible mechanism of action by which adjuvants exert their adjuvanticity. Adjuvants can serve as a depot that mediates recruitment of APCs or act as a delivery system to facilitate uptake of antigen by the APCs. Adjuvants may activate innate immune responses by signaling through cell surface CLRs (such as Dectin-1, Dectin-2, Mincle), cytosolic NLRs, cell surface TLRs, endosomal TLRs or cytosolic RIG-I and MDA5. Signaling via PRRs may lead to the activation of several transcription factors, which results in the production of pro-inflammatory cytokines, chemokines and type I IFNs. Secretion of chemokines due to adjuvants may also result in the recruitment and infiltration of more immune cells. Adjuvants can activate c-GAS that participates in the STING-mediated IRF3-type I IFN pathway. Adjuvants can enhance the expression of MHC and co-stimulatory molecules to mediate efficient presentation of antigen to naïve CD4+ T cells. Depending upon the class of adjuvant, cellular (Th1) and/or humoral (Th2) immune responses may be induced. Adjuvants also play important roles in GC reaction, affinity maturation and long-lived memory responses as a part of humoral immunity.

APC: antigen presenting cell, CLR: C-type Lectin receptors, NLR: nod-like receptors, TLR: toll-like receptor; RIG-I: retinoic acid-inducible gene I, RLR: RIG-I-like receptor; IFN: interferon, c-GAMP: cyclic guanosine monophosphate-adenosine monophosphate, c-GAS: c-GAMP synthase, STING: stimulator of IFN genes, GC: germinal centre, PRR: pattern recognition receptor, DAMP: damage-associated molecular pattern, ROS: reactive oxygen species, LDH: lactate dehydrogenase, Abs: antibodies, NLRP3: NLR family pyrin domain containing 3, AIM2: absent in melanoma2, MyD88: myeloid differentiation primary response 88, TRIF: TIR-domain-containing adapter-inducing IFN-β, IRF: interferon regulatory factor, TIRAP: toll/interleukin-1 receptor domain-containing adapter protein, AP-1: activator protein 1, NF-κB: nuclear factor-κB, MAL: MyD88 adaptor-like, TRAM: TRIF-related adaptor molecule, MDA5: melanoma differentiation-associated protein 5, ER: endoplasmic reticulum, ICAM-1: intercellular adhesion molecule 1, NK: natural killer, CTL: cytotoxic T lymphocyte, MHC: major histocompatibility complex.

also responsible for enhanced antigen uptake, by monocytes in particular, and antigen presentation in the dLNs; this process is mediated by the presence of α-tocopherol in AS03 [15,16]. The safety of squalene-based adjuvants (such as MF59 and AS03) has been demonstrated by toxicological studies in animal models as well as in Phase I–III studies in humans [16–18]

2.2. Depot effect

Depot effect refers to slow and prolonged antigen release at the site of injection providing continuous stimulation of the immune system. This facilitates enhanced antigen uptake by the APCs, which correlates to induction of high antibody titers. Adjuvanticity of alum was originally thought to be due to depot effect; however, according to recent evidence, a depot effect is not the only mechanism by which it exerts its adjuvant activity [19]. In a mouse model, alum was found to rapidly induce an inflammatory environment (via induction of inflammatory chemokines) that in turn triggers neutrophil recruitment and swarming at the injection site. Furthermore, alum induces neutrophil death, resulting in the release of extracellular DNA strands (neutrophil extracellular traps or NETs) that play a significant role in the adjuvant action of alum [20]. Oil-in-water emulsions such as Emulsigen®, water-in-oil emulsions such as cationic adjuvant formulation (CAF)01 (a cationic liposome consisting of a combination of dimethyldioctadecylammonium/α,α'-trehalose 6,6'-dibehenate or DDA/TDB), as well as biodegradable micro- and nano-particles exhibit adjuvant activity via a depot effect in mice [6,21]. Cationic liposomes exhibit long depot effects at the site of injection and strong electrostatic interactions with APCs. In contrast, adjuvants such as MF59 or ISCOMs do not require depot formation to exert their adjuvant activities; rather, antigen and adjuvant are cleared rapidly from the site of administration. A biodistribution study of AS03 in mice conducted by radiolabelling each component of AS03 revealed that all constituents of AS03 infiltrated into the dLNs within 30 min of injection [20] and that 57–73% of each constituent of AS03 was cleared from the injection site 3 days post injection, suggesting dissociation of AS03 [22]. Similarly, intramuscular injection of an AS01-adjuvanted vaccine in mice indicated rapid clearance of antigen and QS-21 from the injection site and into the dLNs. Differential biodistribution dynamics and pharmacokinetics of antigen and QS-21 suggested that the AS01 and the vaccine antigen were not physically associated with each other [23]. Similarly, in matrix adjuvant formulations (consisting of nanoparticles comprised of saponin, cholesterol, and phospholipid), a physical association between matrix and antigen is not required for potent immune stimulation [24]. In contrast, electrostatic interaction of antigen with adjuvant is required for optimal adjuvant activity of CAF01. Similarly, optimal adjuvanticity of virosomes is achieved when the antigen of interest is associated with the virosome either through encapsulation or attachment to the bilayer via hydrophobic interaction [25].

2.3. Activation of PRRs and cellular signal transduction pathways

2.3.1. Toll-like receptors (TLRs)

The success of the yellow fever vaccine YF-17D, a live attenuated virus vaccine, can be attributed to its ability to activate multiple TLRs including TLR2, 7, 8 and 9, on or in DCs in mice [26]. YF-17D also activates human monocyte-derived DCs and plasmacytoid DCs (pDCs) [26]. TLR2-TLR1 complexes are activated by the lipopeptide analog Pam$_3$CSK$_4$, while TLR2-TLR6 complexes are activated by the macrophage activating lipopeptide-2 (MALP-2) from mycoplasma [27]. Poly(I:C) is a ligand for endosomal TLR3, cytosolic retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5). Poly(I:C) and its two derivatives, polyI:C$_{12}$U (Ampligen) and poly(IC:LC) (Hiltonol) are used in clinical trials against both tumors and infectious diseases such as HIV [28]. TLR4 is targeted by monophosphoryl lipid (MPL)A. AS04 (containing MPL) is approved for use in HBV (Fendrix) and human papilloma virus (HPV) vaccines (Cervarix) [29,30]. AS01 (containing MPL) is also used in a malaria vaccine (RTS,S) and the varicella zoster virus vaccine (Hz/Su) that have been found to be efficacious in phase III trials [23]. TLR5 recognizes bacterial flagellin. TLR5 signaling in CD103$^+$CD11b$^+$DCs plays an important role in intestinal IgA production and Th17 differentiation, and leads to MyD88-dependent, strong nuclear factor (NF)-κB activation and Th-2 type immune responses in mice [23,28]. In contrast, flagellin acts as a Th1-polarizing factor for CD4$^+$ T cells from human neonates and adults [23]. TLR7 and TLR8 recognizing single-stranded RNA (ssRNA) molecules are targeted by small-molecule immune potentiator (SMIP)-based adjuvants such as imiquimod and resiquimod used in HPV virus-like particle (VLP) vaccines [31]. TLR7 signaling induces B cell-mediated production of immunoglobulins (Ig), interleukin (IL)-6 and TNF-α, and natural killer (NK) cell-mediated production of IFN-γ, while TLR8 signaling induces T cell proliferation, production of IFN-γ, IL-2, and IL-10, and memory T cell activation [28]. TLR9 recognizes unmethylated CpG motif-containing microbial DNA or immunostimulatory sequences (ISS). TLR9 agonists are used in HBV vaccines to promote higher levels of protective antibodies. Consequently, fewer immunizations and lower antigen doses are needed. In mice, TLR9 signaling leads to Th1 type pro-inflammatory responses (IL-1, IL-6, IL-12, IL-18, TNF-α and IFN-γ), up-regulation of major histocompatibility complex (MHC) and co-stimulatory molecules, and increased CD8$^+$ T cell responses, while TLR9-mediated B cell activation is responsible for induction of humoral immunity and antibody class switching [28].

2.3.2. Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)

Intracellular NLRs such as NOD1 and NOD2 receptors recognize diaminopimelatic acid (DAP)-containing muropeptide from gram-negative bacteria, while NOD2 detects the muramyl dipeptide (MDP) component present in all bacterial peptidoglycans. The adjuvanticity of the mucosal adjuvant Cholera Toxin (CT) is mediated through the NOD2 receptor [32].

Adjuvants that are inducers of damage-associated molecular patterns (DAMPs) trigger innate immune responses *in vivo* by damaging the host cells, thereby resulting in the release of DAMP factors (ex. RNA, DNA) for subsequent activation of the innate immune receptors [2]. The cytosolic receptor NLRP3 is recognized by adjuvants such as Quil-A and chitosan, as well as ATP, MDP, uric acid crystals and silica. These compounds generate DAMP signals, such as reactive oxygen species (ROS) or induce potassium efflux to activate NLRP3. Alum's adjuvanticity in mice is also attributed to the activation of NLRP3/ NACHT (via swelling and rupture of the phagolysosome, release of cathepsin B into cytosol, subsequent activation of caspase 1 and release of IL-1β), Leucine-rich repeat (LRR), and PYRIN-PAAD-DAPIN domain (PYD) domains-containing protein 3 (NALP3) inflammasome, release of uric acid or activation of the stimulator of interferon (IFN) genes (STING)-IFN regulatory factor (IRF)3 pathway due to the release of DNA [16,19]. However, validation of these hypotheses for humans is warranted (ex. a direct role of IL-1β in adjuvanticity of alum in humans is debatable) [16]. For instance, it is generally concluded that NLRP3 inflammasome and caspase 1 are sometimes, but not always, required for induction of Th2 cell-associated antibody responses in response to aluminum salts *in vivo* [33] Activation of caspase-1 is NLRP3-dependent *in vitro*. However, no such role of NLRP3 is observed for QS-21 *in vivo*. QS-21 when formulated in liposomes activates human DCS by promoting cholesterol-dependent endocytosis with subsequent lysosomal destabilization and Syk activation similar to alum [16]. In mice, Endocine, a lipid adjuvant, causes cellular damage to generate DAMPs such as lactate dehydrogenase (LDH), DNA and RNA [34]. Chitosan induces release of mitochondrial DNA into the cytoplasm to activate the NLRP3 inflammasome in mice [35]. MF59 (but not aluminum hydroxide or calcium phosphate adjuvants) induces release of extracellular ATP from the muscle in mice that acts as a danger signal [16]. Other DAMP adjuvants such as hydroxypropyl-β-cyclodextrin (bCD) induce local cellular stress and death resulting in the release of the host cellular DNA that serves as a DAMP to induce Th2-type immune responses [2].

2.3.3. Other PRRs

Cytosolic dsDNAs are sensed by several other PRRs such as absent in melanoma2 (AIM2), as well as by the protein cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS), which simultaneously leads to the activation of STING-dependent TBK1-IRF3-IFN-1 pathways and RelA-TNF-α pathways [36]. STING can bind cyclic dinucleotides (CDN), cyclic di-GMP (CDG) and cyclic di-AMP (CDA). In mice, CDG appears to be a safer mucosal adjuvant than cholera toxin [37] and to promote protective immunity against H5N1 influenza, *Staphylococcus, Streptococcus* and *Klebsiella* infections. In mice, chitosan triggers release of intracellular DNA that results in the engagement of the cGAS-STING pathway in DCs to induce type 1 IFN production and ISGs, thereby promoting robust Th1 immunity. This leads to the upregulation of costimulatory immune markers and the subsequent activation of DCs, as well as induction of IgG2c and cell-mediated immunity (CMI) [35].

2.3.4. Carbohydrate-based adjuvants

Carbohydrate-based adjuvants include glucans, fructans, mannans, chitin/chitosan and other carbohydrate compounds derived from *Mycobacterium spp.* (including lipoarabinomannan, muramyldipeptide/MDP, trehalose-6–6-dimycolate/TDM), as well as LPS and saponin compounds (including QS-21, a saponin in an oil-in-water emulsion) [38]. In human monocyte-derived DCs (moDC), QS-21 (also a component of AS01) is endocytosed via the action of membrane cholesterol, with subsequent lysosomal destabilization and Syk activation to promote a pro-inflammatory transcription program. In addition, cathepsin B (a lysosomal cysteine protease) is required for activation of moDCs *in vitro* and also required for adjuvant activity of QS-21 *in vivo* [39]. In another study, LN-resident CD11b$^+$CD169$^+$ macrophages played a key role in the adjuvant activity of QS-21. Upon intramuscular injection in mice, QS-21 leads to rapid induction of local innate immune responses in the dLNs and co-localises with LN-resident macrophages that are crucial for innate and effector responses to antigens formulated with QS-21 (via Caspase 1/11 and inflammasome activation) [40].

The primary mechanism of action of carbohydrate-based adjuvants involves interaction with PRRs such as TLRs, NOD2 and C-type lectin receptors (CLRs) Dectin-1, Dectin-2 and Mincle on monocytes and APCs. This interaction activates NF-κB to induce inflammatory chemokine and cytokine responses [41]. Carbohydrate adjuvants also activate complement pathways to generate complement components acting as opsonins and chemokines. Other important mechanisms of action of carbohydrate-based adjuvants include chemotaxis of lymphocytes, inflammasome activation (e.g. zymosan and mannans), and pore-formation, facilitating antigen entry into APCs (via interaction with cholesterol in the plasma membrane, e.g. QS-21) [38].

2.3.5. Signal transduction pathways

Adjuvants induce a series of signal transduction pathways to exert their action at both innate and adaptive levels. Intramuscular injection of MPL or ASO4 in mice is responsible for NF-κB activation in the muscles and local dLNs [42]. Synthetic derivatives of MPL induce activation of TLR4 and selectively activate the p38 MAPK pathway, which is strongly associated with optimal induction of IFN-γ-induced protein 10 (IP-10), TNF-α and IL-10 in mice [43]. Injection of AS01-adjuvanted vaccine promotes release of IFN-γ by LN-resident NK cells and CD8$^+$ T cells. Pathway enrichment analysis of differentially expressed genes in injection site-dLNs revealed that the IFN-signaling pathway was most enriched at 4 and 6 h, while the IL-10-driven anti-inflammatory pathway was also triggered by AS01 at 6 h post-injection. Increased levels of IFN-γ were detected early in the serum and dLNs of humans and macaques vaccinated with AS01-adjuvanted vaccine, respectively [44]. Cellular and molecular synergy between MPL and QS-21 used in AS01 in combination was responsible for this early IFN-γ response, which in turn, enhances the immunogenicity of AS01-adjuvanted vaccines [44]. IL-21 as an adjuvant activates Janus kinase (JAK)-signal transducer and activator of transcription (STAT), phosphoinositide 3-kinase (PI3K) and

MAPK pathways, thereby promoting B-cell and T-cell differentiation via sustained activation of STAT3 and Th17 differentiation through IRF4 [45]. Subtle chemical alterations to MPLA were made to develop a designer SMIP-based TLR4-agonist known as SLA, which induces TRIF signaling to produce Th1-biased cytokines and chemokines like IFN and IP-10, and less IL-1β. SLA in oil-in-water emulsion (SLA-SE) was produced capitalizing on the knowledge that a combination of IFN and caspase-dependent inflammasome signaling leads to powerful adjuvant action [46].

Activation of the NF-κB pathway, as well as the p38 and JNK MAPK pathways, programs DCs to produce IL-12p70 and to induce Th1 responses. The extracellular signal-regulated kinase (Erk)-c-Fos MAPK pathways favor Th2-type responses, while Erk-retinaldehyde dehydrogenase (RALDH) enzymes or β-catenin program DCs to induce T regulatory (Treg) responses. Similarly, complete Freund's adjuvant (CFA) induces transcription of MHC-II and B cell activation markers via the Lyn-Syk-PI3K, the calcineurin-nuclear factor of activated T-cells (NFAT) and the Ras-MEK-ERK signaling pathways [47].

Saponin adjuvanticity relies on MyD88-mediated and IL-18 receptor-signaling pathways [48]. Chitosan engages cGAS-STING pathways to induce IgG2c and Th1 responses in mice [35]. Intact MyD88 signaling in each of the three types of APCs (DCs, macrophages and B cells) is essential for robust activity of TLR ligand-based vaccine adjuvants (PorB, a TLR2 ligand and CpG, a TLR9 ligand) such as induction of in vivo cytokine responses, germinal center (GC) formation and antibody production [49].

2.4. Induction of cytokines, chemokines and IFNs to facilitate recruitment of immune cells

Based on microarray analysis, Mosca et al. demonstrated that three potent human vaccine adjuvants, MF59, CpG ODN, and alum, modulate a common set of 168 genes ['adjuvant core response genes'] encoding cytokines, chemokines, innate immune receptors, IFN-induced proteins and adhesion molecules in mouse muscles [50]. The establishment of a local immunocompetent environment due to such non-pathogenic inflammatory responses is associated with vaccine adjuvanticity. When compared to CpG ODN and alum, MF59 was found to be the stronger inducer of adjuvant core response genes, which was reflected in enhanced and more rapid influx of MHC-II$^+$ and CD11b$^+$ cells into the injection site and more efficient transport of antigen to the dLNs [13]. Both alum and MF59 induced chemokines involved in cellular influx such as CCL2, CCL3, CCL4, and CXCL-8 [51].

The presence of α-tocopherol in AS03 promotes induction of leukocyte-recruiting chemokines (CCL2, CCL3 and CCL5), neutrophil-mobilising cytokine (granulocyte colony-stimulating factor 3 (CSF3)) and pro-inflammatory cytokine/chemokines (IL-6 and CXCL-1), in mice which is in agreement with increased recruitment of granulocytes and antigen-loaded monocytes into the dLNs [52]. Aluminum adjuvants facilitate recruitment and differentiation of inflammatory monocytes (F4/80^{int}CD11b$^+$LyG$^-$Ly6C$^+$) into inflammatory DCs, thereby enhancing both humoral and cellular immunity [53]. Many of the above results with alum and MF59 have been confirmed in non-human primates [16]. For example, vaccination with HIV vaccine adjuvanted with alum or MF59 triggered recruitment of monocytes, DCs, and neutrophils in the muscle [16]. Subcutaneous administration of ISCOMATRIX in sheep induces a rapid and transient production of cytokines (IL-6, IL-8, IFN-γ) and influx of innate cells such as NK cells, NKT cells, neutrophils, migratory DCs (CD205$^+$CD8$^-$) and CD8α$^+$ DCs to the dLNs [54]. A combination adjuvant consisting of poly(I:C), a host defense peptide and PCEP when delivered intranasally transiently induces production of chemokines and cytokines in murine respiratory tissues, which promotes infiltration and activation of DCs, macrophages, and neutrophils to generate improved mucosal and systemic immune responses [55].

2.5. Induction of humoral immunity

(a) Improvement of the quality of antibody responses: Innate immune responses play a profound role in regulating the magnitude, quality and persistence of antibody responses. The magnitude of the antibody response is critical in conferring protection against diphtheria, hepatitis A, lyme disease, tetanus, yellow fever, polio, rabies, and pneumococcal infections [56], while for RSV and meningococcal infections, the magnitude and quality of the antibody and cell-mediated response are important. Adjuvant systems such as AS01 are used in malaria (RTS,S), herpes zoster (HZ/su), TB and HIV vaccines, while AS03 is used in several influenza vaccines such as trivalent inactivated H1N1 influenza, H5N1 pre-pandemic influenza, and candidate H7N1 and H7N9 pandemic influenza vaccines. AS04 is used in licensed HPV-16/18 and HBV vaccines. Such adjuvant systems are known to augment antigen-specific T cell and antibody responses [57]. In a murine study, the ER stress-related pathway was found to potentially contribute to the adjuvanticity of AS03 in vivo [58]. Furthermore, the expression of the ER stress sensor kinase IRE1α by myeloid cells was involved in adjuvant activity of AS03. The ER stress-related pathway was required for AS03-mediated induction of IL-6, robust Tfh responses, and antigen-specific antibody affinity maturation. IL-6 plays a crucial role in differentiation of Tfh cells as well as in the expression of IL-21 by Tfh cells and in production of antibodies [58].

Alum-adjuvanted vaccines act on IL-4 producing Gr-1$^+$ cells to facilitate optimal priming, clonal expansion and antibody production by antigen-specific B cells in mice [59]. In B cells, TLR ligands as adjuvants induce upregulation of surface markers involved in antigen uptake (MHC-I and MHC-II) and surface markers involved in cross-talk with the T cells (CD40, CD80, and CD86), which ultimately leads to increased antigen-specific antibody production [28]. However, when AS04 is used as an adjuvant in the same HBV and HPV vaccine, instead of aluminum salts, higher levels of antibodies are induced in humans, indicating the added benefit of MPL (a TLR4 agonist) in humans [16]. Emulsigen®, an oil-in-water adjuvant, similar to MF59 and AS03, boosts innate responses and increases the number of CD4$^+$ T cells required for robust antibody responses [60]. MF59 supports induction of T follicular helper (Tfh) cells and GC responses to vaccination by an unknown mechanism

[61]. In humans, MF59 and AS03 promote early inflammation similar to results from animal studies [16]. Type 1 IFNs responses are induced in children as early as day 1 post-immunization with MF59-adjuvanted influenza vaccine, but in children immunized with non-adjuvanted vaccine, type I IFN responses are weaker and delayed. Such early innate immune responses are reflected in induction of antigen-specific Tfh cells 7 days post-vaccination, and enhanced antibody responses in humans immunized with MF59-adjuvanted influenza vaccine [16].

(b) Induction of GC reactions to promote memory B cell development: Immunological memory is a distinctive hallmark of the adaptive immune system that contributes to protective immunity against infectious diseases. The GC reaction is central to memory development. Induction of certain key molecules such as CD40, inducible T-cell costimulator (ICOS), IL-21, programmed death-ligand 1 (PD-1), CD95, IRF4, and B-cell lymphoma 6 protein (Bcl-6) play a critical role in regulation of GC differentiation, affinity maturation and long-lived memory responses [56]. TLRs expressed on GC B cells, follicular DCs (FDCs) and T cells have a profound effect on induction of antibody responses. Nanoparticles resembling virions in size and containing TLR ligands, MPL and R837, in combination with H5N1 hemagglutinin mediate increased persistence of GCs, which significantly influence the differentiation of memory B cells critical for long-lived antibody responses in mice [62]. A subset of CD4$^+$ T cells, ICOS$^+$CXCR3$^+$CXCR5$^+$ T cells, was associated with protective antibody responses conferred by a trivalent split-virus influenza vaccine and efficiently induced memory B cells to differentiate into plasma cells [63]. Novel adjuvants may enhance B-cell activation in GCs and bone-marrow plasma cell survival. For example, the heat-labile enterotoxin (LT) of Escherichia coli, LTK63, when administered parenterally to neonatal mice, facilitates maturation of follicular DCs and generation of GCs [64].

2.6. Induction of cellular immunity: effector Th1/Th2/Th17 and memory T cell responses

Signaling via TLR3, TLR4, TLR7, TLR8, and TLR9 promotes Th1-biased immunity, while signaling via TLR2/TLR1, TLR2/TLR6 and TLR5 promotes Th2-biased immunity. CD11c$^+$CD11b$^-$CD8α$^+$ DCs localized in the marginal zones of LNs induce Th1 responses, as well as exhibit cross-presentation of antigens in vivo and ex vivo in mice [65]. In humans, BDCA1$^+$ (CD1c$^+$) and BDCA3$^+$ (CD141$^+$) DCs (equivalent to murine CD8α$^-$ and CD8α$^+$, respectively) are involved in cross-presentation of extracellular antigens [65]. As a result of TLR3-mediated enhanced MHC-I expression and type I IFN production, poly(I:C) promotes antigen cross-presentation to CD8$^+$ T cells and antigen-specific CTLs. In contrast, alum promotes Th2 responses (strong antigen-specific IgG1 and IgE) and does not induce CD8$^+$ T cell immunity, and even inhibits Th1 immune responses in mice [28]. However, when alum is present in combination with MPLA, Th1 responses can be generated as is found for ASO4 [42]. It is unclear whether such an aluminum salt-induced Th2 bias exists in humans [16]. Rather, poor T cell responses are induced by aluminum salts in humans, possibly due to poor stimulation of the innate immune system [16]. Squalene-based oil emulsion is a potent inducer of both Th1- and Th2-mediated

immunity and is well tolerated [18]. Adjuvants such as QS-21, MF59 or CFA preferentially induce Th1-biased or a mixed Th1/Th17 and Th1/Th2 immune response. Experimental CAFs combined with immunostimulators such as TDB in TB vaccines stimulate both cellular and humoral immune responses, as well as promote efficient polyfunctional memory T cells, and Th1- and Th17-biased immune responses in mice [48]. A STING-activating adjuvant, CDN, when formulated in a subunit vaccine and delivered intranasally, promoted a robust Th17 immune response that correlated with long-lasting enhanced protection against Mycobacterium tuberculosis in mice. Adjuvanted vaccines promoting Th17 responses may protect against intracellular pathogens by recruiting protective T cells earlier during infection [66].

In neonates, CD4$^+$ T cells are polarized towards Th2 responses and reduced Th1 responses. However, novel adjuvants such as IC31 and CAF01 can induce adult-like Th1 responses in newborn mice [67]. CDG as a mucosal adjuvant induces Th1 and Th17 immune responses in mice [68]. CAF01 predominantly induces CD4$^+$ T cell responses, while CAF05 (consisting of DDA, TDB and poly(I:C)) induces both CD4$^+$ and CD8$^+$ T cell responses [48]. Replacing AS02 in an RTS,S/AS02 candidate malaria vaccine with AS01 improved antibody and CD4$^+$ T cell responses, as well as protective efficacy as demonstrated by a randomized, double-blind, Phase 2a trial [69]. Activated murine sub-capsular macrophages produce IL-18, which promotes generation of CD4$^+$ T cells, while activated APCs also produce IL-6 or IL-12, promoting generation of Tfh cells, which in turn favors production of high-avidity antibodies by B cells in both mice and humans [61]. Intramuscular injection of mice with AS01 induces early IFN-γ production by LN-resident NK cells, which is mediated by synergistic action of IL-12 and IL-18 in promoting IFN-γ responses. Early IFN-γ production by NK cells is a prerequisite for optimal activation of DCs and induction of antigen-specific CD4$^+$ T responses to AS01-adjuvanted antigens [16,44]. Such IFN-γ responses were also observed in LNs of macaques when they were injected with AS01 [16]. Elevated levels of IFN-γ in the serum at day 1 post-immunization and an increase in the number of cytokine-producing antigen-specific CD4$^+$ T cells was also observed in humans immunized with RTS,S malaria vaccine [16]. The mechanisms of action of different adjuvants are summarized in Table 1. The adjuvants that are licensed for use in human vaccines are listed in Table 2, while the adjuvants in clinical trials are listed in Table 3.

3. Selection of adjuvants based on their mechanism of action against distinct types of pathogens

3.1. Mucosal pathogens

Mucosal surfaces are an attractive target for pathogens whose port of entry are gastrointestinal (e.g. polio virus, Escherichia, Salmonella, Shigella, Vibrio and Helicobacter), respiratory (influenza virus, M. tuberculosis or Mtb, adenovirus, coronavirus, rhinovirus and RSV) or urogenital tract (HSV, HPV, HIV-1, Chlamydia and Neisseria) [75]. Mucosal adjuvants can be categorized as toxin-based (LT and CT), immunostimulatory (MPL, CpG, and QS21) and delivery system (Emulsigen® and ISCOMs). Two commonly used oral toxin-based adjuvants are

Table 1. A comprehensive list of vaccine adjuvants and their modes of action.

Adjuvant class	Examples	Mechanism of action	References
Liposome-based adjuvants	Virosome Archaeosome CAF01	Antigen delivery system; mucoadhesive; depot effect; immunostimulatory; strong antigen-specific antibody and Th1/Th2 cell responses	[4–6,75,97]
Tensoactive	Saponin-based (ISCOMs, ISCOMATRIX, Matrix-M™ and QS-21)	Antigen delivery system; immunostimulatory; induction of cytokines and cellular influx; induction of Th1- or mixed Th1/Th17-, Th1/Th2-type as well as strong antibody responses	[4,7,10,48]
Particulates	Polymeric nanoparticles: e.g. PLGA, PLG, PLA, PGA and PHB Inorganic nanoparticles: e.g. Gold nanorods, gold nanoparticles, carbon nanoparticles, silica-based nanoparticles like mesoporous silica nanoparticles (MSN) Polyphosphazene	Antigen delivery system; depot effect; mucoadhesive; strong antigen-specific Th1/Th2, CD8+ T cell and antibody responses; potent inducer of 'adjuvant core response genes' as well as induction of cytokines, chemokines and enhanced cellular influx (PCEP)	[4,11,19,70]
Mineral salts	Aluminum salt (phosphate or hydroxide, Alum) Calcium salt Iron salt Zirconium salt	Antigen delivery systems; source of DAMP; potent inducer of 'adjuvant core response genes' (alum); cytokine, chemokine, antibody, and Th2 responses	[12,28,50,51,59,75]
Emulsions	Complete Freund's adjuvant (CFA), Montanide ISA51, Incomplete Freund's adjuvant (IFA) MF59 (Squalene+Tween80+ Span 85), Emulsigen® GLA-SE	Antigen delivery system (MF59); depot effect (Emulsigen®); enhanced antigen uptake by APCs; potent inducer of 'adjuvant core response genes' as well as cytokine, chemokine, and antibody responses; Th1- or mixed Th1/Th17- and Th1/Th2-type immune responses (MF59 and CFA); induction of CD4+ T cells to promote antibody responses (Emulsigen®); strong Th1 responses (GLA-SE)	[13,28,50,51,60,70,75]
Nucleic acid/Nucleotides	dsRNA: e.g. poly(I:C), poly:C12U (Ampligen), poly(IC:LC) (Hiltonol), M8, defective interfering (DI) RNA CpG ODNs: e.g. IC31 (unmethylated CpG DNA) Cyclic dinucleotide: e.g. CDG	PRR activation; potent inducer of 'adjuvant core response genes' (CpG); type I IFN induction; pro-inflammatory cytokine/chemokine/antibody/CD4+/CD8+ T cell responses; mucosal adjuvant inducing Th1 and Th17 immune responses (CDG)	[50,75]
Toxins	Cholera toxin (CT) and CTA1-DD (CT derivative) Heat-labile toxin (LT) and mutant LT toxin (LTK63) Clostridium difficile toxin Shiga toxin Staphylococcal enterotoxins LPS (endotoxin or lipoglycan) MPLA	Mucosal adjuvants; immunostimulatory; PRR activation; induction of cytokine responses and cellular influx (LPS and MPLA); induction of strong mucosal IgA, Th1, Th2, Th17 and CTL responses	[32,64,75]
Carbohydrate/Polysaccharide	Glucans: α-glucans (e.g. Dextran), β-glucans (e.g. Zymosan), lentinan, algal glucan, β-glucan particles Fructans Mannans Chitin/Chitosan Zymosan	Site-directed delivery of antigens; source of DAMP (chitosan); mucosal (chitosan); PRR activation; upregulation of co-stimulatory molecules; activation of complement pathways; chemotaxis; activation of inflammasome; penetration enhancer; induction of pro-inflammatory cytokines and secretory antibody responses; Th2 and mucosal IgA responses	[35,38,81]
Peptides/Lipopeptides/Peptidoglycans	Muramyl dipeptide (MDP) Lipopeptides: e.g. MALP-2 Lipopeptide analogs: e.g. Pam3CSK4 Host defense peptides: e.g. IDR1001, IDR1002, LL-37, and Defensin	PRR activation; mucosal IgA; pro-inflammatory cytokine; induction of Th1 (GLA-SE), Th2 (MALP-2 and Pam3CSK4), mucosal Th17 (Pam3CSK4) and antibody responses	[27,28,71,75]
Proteins	Flagellin Profilin	PRR activation; strong mucosal IgA/Th2/Th17 responses	[72]
Lipids	α-GalCer (Glycosphingolipids) RC529 (lipid A mimetic) Monophosphoryl lipid A Endocine	PRR activation; source of DAMP (Endocine); Th1/CTL and mucosal IgA responses	[34,75]
Cytokines and chemokines	GM-CSF Type I IFNs IL-1, IL-2, IL-6, IL-12, IL-15, IL-18 and IL-21 CCL2	Induction of Th1/Th2/CD8+ T and mucosal IgA responses; B and T cell differentiation (IL-21); activation of DCs as well as increased migration and antigen presentation to CD4+ T cells; cross-priming of CD8+ T cells; activation of B cells and NK cells; generation of Th1 biased CD4+ T cells (IFNs); mucosal IgA and CTL responses (CCL2)	[45,77]

(Continued)

Table 1. (Continued).

Adjuvant class	Examples	Mechanism of action	References
Small molecule immune potentiators (SMIPs)	SMIPs for TLR7/8: e.g. Imiquimod or R837, Resiquimod or R848, Gardiquimod SMIPs for TLR4 (SLA, substituted pyrimido[5,4-b]indoles) Second-generation SMIP-based adjuvants for TLR7/8 formulated with Al(OH)₃	Localized innate immune activation; short in vivo residence time and PRR activation (second-generation SMIP-based adjuvants for TLR7/8); induction of Th1-biased cytokines and chemokines (SMIPs for TLR4)	[31,46,75]
Adjuvant Systems (AS)	AS01 (MPL, QS-21 and liposome) AS03 (Squalene, polysorbate 80 and α-tocopherol) AS04 (MPL adsorbed onto aluminum hydroxide or aluminum phosphate)	Transient induction of cytokines at the site of injection or in dLNs; recruitment of granulocytes and monocytes; increased influx of antigen-loaded monocytes in dLNs.	[70, 15,16]

PRR: pattern recognition receptor, DAMP: damage-associated molecular pattern, APC: antigen presenting cell, IFN: interferon, CAF: cationic adjuvant formulation, Th: Thelper, ISCOM: immune stimulating complexes, PLGA: poly (lactic-co-glycolic acid), PLA: poly(lactic acid), PGA: poly(glycolic acid), PHB: poly(hydroxybutyrate), PCEP: poly[di(sodium carboxylatoethylphenoxy)-phosphazene, PCPP: poly[di(sodium carboxylatophenoxy)]-phosphazene, GLA-SE: glycopyranosyl lipid adjuvant (GLA) in combination with squalene (SE), ds: double-stranded, ODN: oligodeoxynucleotide, CDG: cyclic di-GMP, LPS: lipopolysaccharide, MPLA: monophosphoryl lipid A, MALP-2: macrophage activating lipopeptide-2, IDR: innate defense regulator, GM-CSF: granulocyte-macrophage colony-stimulating factor.

Table 2. Adjuvants used in licensed vaccines.

Adjuvants	Licensed vaccines	References
Alum	Various	[121]
MF59	Seasonal influenza vaccine Pandemic influenza vaccine Avian influenza vaccine	[16]
CpG ODN	HBV vaccine	[16]
AS01	Malaria vaccine (RTS,S or Mosquirix) Herpes zoster vaccine (HZ/su or Shingrix)	[15]
AS03	Pre-pandemic H5N1 vaccine Pandemic H1N1 influenza vaccine	[15]
AS04	HPV vaccine (Cervarix) HBV vaccine (Fendrix)	[15]

Table 3. Adjuvants currently in use in Phase I, II, and III vaccine trials.

Adjuvants	Experimental vaccines	References
AS01	Haemophilus influenza vaccine Moraxella catarrhalis vaccine Tuberculosis vaccine HIV vaccine	[15]
CpG ODN	Cancer vaccine for patients with melanoma (Phase I)	[73]
Flagellin	Plague vaccine (Phase I) Influenza vaccine (Phase I/II)	[74]
PolyI:C₁₂U (Ampligen)	H5N1 influenza vaccine (Phase III) Cancer vaccine (Phase I/II)	[83,84]
CAF01	Chlamydia trachomatis vaccine (Phase I) Tuberculosis vaccine (Phase I)	NIH NIH
IC31	Tuberculosis vaccine (Phase I/II)	NIH
ISCOMATRIX	Tumor cell vaccine (Phase I) Melanoma (Phase II)	NIH NIH

a modified versions of CT lacking the A subunit (CTB) from *Vibrio cholerae* and a double mutant of LT (dmLT) from *Escherichia coli* [76]. Both are potent, but also toxic, when used as mucosal adjuvants. Protective efficacy was attained when intranasal vaccines containing mutant LT adjuvants were used against HSV, *Bordetella pertussis* and *Streptococcus pneumoniae* [77]. Natural infection with RSV induces poor antibody responses with impaired effector functions, and perturbs localization and persistence of effector and memory T cells [78]. Induction of a potent, local mucosal immune response is required to prevent infection and a high systemic antibody response is also required to interrupt disease progression. Nanoemulsions are safe for intranasal delivery and induce Th1 and Th17 responses in mice with no significant inflammation [41]. Sastry et al. tested multiple adjuvants such as Sigma adjuvant system (SAS, an oil-in-water adjuvant), carbopol, alum, Adjuplex, poly(I:C), poly(IC:LC), MPLA, AddaVax and Montanide ISA, and found that the adjuvant-mediated increase in RSV-specific neutralizing antibody responses was context-dependent (i.e. whether pre-existing immunity was present or not) and species-specific (i.e. mice vs. calves) [79]. Formulation of the RSV fusion (F) protein with a combination adjuvant, TriAdj, elicited protective, mucosal and systemic, immune responses against RSV in mice and cotton rats [80].

The mucosal epithelial barrier limits the bioavailability of vaccine antigens for sampling by APCs. Adjuvants such as polyethyleneimine and chitosan are used as penetration enhancers and immunostimulants to augment binding to the mucosal surfaces and activate innate immunity in a mouse model [81]. Chitosan polymeric nanoparticles stimulate the

nasal-associated lymphoid tissues (NALT) to produce mucosal secretory IgA, IgG, TNF-α, IL-6, and IFN-γ. Pro-inflammatory cytokines and chemokines also act as mucosal adjuvants. Pro-inflammatory cytokines such as IL-1, IL-6, IL-12, IL-15, and IL-18 induce mucosal CD8$^+$ CTLs and antigen-specific IgA. Similarly, chemokines such as CCL2 (or MCP-1) enhance mucosal IgA and CTL responses [77]. Neutralizing antibodies may protect against some acute self-limiting mucosal pathogens, but for highly invasive pathogens causing chronic infections (such as HIV, HCV, herpesviruses, and mycobacteria), mucosal innate and adaptive immune responses including CD4$^+$, Th17, and CD8$^+$ CTLs, as well as secretory IgA and IgG1 neutralizing antibodies at the port of pathogen entry, are required for effective and optimal protection [82].

Mucosal adjuvant-containing vaccines elicit both local and systemic immune responses, effective at local as well as distant sites [76]. To control enteropathogens, orally administered vaccines must overcome challenges of antigen degradation and immune tolerance [41]. Biodegradable micro- or nanoparticles are required that are resistant to low pH and can target antigen to M cells. U-Omp19, a bacterial protease inhibitor from *Brucella abortus*, is an oral adjuvant suitable for subunit vaccine formulation, which can inhibit stomach and gut proteases and delay antigen digestion at the lysosome to enhance antigen presentation and recruitment of immune cells to gastrointestinal mucosa [76]. Intranasal immunization of mice with poly-I:C$_{12}$U (Ampligen) in an H5N1 influenza vaccine promoted increased levels of protective, mucosal IgA and systemic IgG [83].

However, only a few mucosal vaccines are licensed for humans, primarily due to a dearth of safe and effective mucosal adjuvants [81]. Although during the last few decades there has been a constant development of new and effective mucosal adjuvants, most of them are toxic. For example, LTK63 when intranasally injected can trigger transient facial nerve paralysis or Bell's palsy [81]. Thus, there is an urgent need to address safety issues of mucosal adjuvants. In a phase III trial, poly-I:C$_{12}$U (Ampligen) was demonstrated to be safe [83]. A randomized phase I/II trial was conducted to determine the safety and efficacy of Ampligen in patients with stage II-IV human epidermal growth factor receptor 2 (HER2)-positive breast cancer. The result from this trial will give important insight into the application of Ampligen in therapeutic cancer vaccines [84].

3.2. Pathogens with complex life cycles

Pathogenic fungi and protozoan parasites have complex life cycles and switch among several different forms during their life. *Histoplasma capsulatum* grows as a mold in the soil at low temperature, but upon inhalation into the lungs, it switches to yeast form and causes histoplasmosis. Interaction of infected macrophages with CD4$^+$ and CD8$^+$ T cells leads to increased production of Th1 cytokines, IL-12, IFN-γ, and TNF-α, that are critically important in generating protective immunity against *H. capsulatum* infection in mice. Leukotrienes, lipid mediators derived from arachidonic acid metabolism, are found to be potent adjuvants against such fungal infections [85].

Malaria vaccine development is impeded by the complex life cycle of *Plasmodium spp.*, the intracellular stage in its life cycle, large physical size, surface antigenic diversity and enormous genetic and genomic plasticity [86]. The parasite replicates intracellularly (and thus is partially protected from immune recognition) and also sequesters any innate immune ligand away from PRRs in the sporozoite and gametocyte stages of their life cycle. A malaria vaccine needs to establish humoral immunity to prevent merozoites from entering the erythrocytes and the liver or destroy the merozoites through opsonization and CMI. RTS,S/AS01 (Mosquirix) is a malaria candidate vaccine targeted against the infectious sporozoite stage and designed to enhance both antigen-specific humoral and cellular immunity. Th1 effector cells are essential to target asexual blood stages, while eventual control and/or clearance of the parasites requires antibody-mediated responses [87]. MPL and QS-21, the two components used in AS01 have important functions. MPL is a TLR4 agonist that induces production of IFN-γ by T cells and antibody isotype switching to IgG2a/c in mice, while QS-21 induces neutralizing antibodies and cytotoxic T cell responses [88]. AS01 requires synergistic activities of both MPL and QS-21 for optimal adjuvant activity. AS01 in combination with *Plasmodium* antigens induces rapid and transient innate immune responses in the injection site and dLNs, activates immune cells (including APCs), as well as generates 20-fold higher antibody titers when compared to natural exposure [87]. However, in a large phase III trial in 8922 children and 6537 young infants in seven sub-Saharan African countries, although RTS,S/AS01 prevented a considerable number of cases of clinical malaria in infants and young children over 3–4 years, the vaccine efficacy declined with subsequent follow-ups in the infants, and did not provide significant protection against severe malaria [89]. Nonetheless, RTS,S/AS01 plays a significant role in the control of malaria in areas of high transmission when used in conjunction with other effective preventive measures (RTS,S Clinical Trials Partnership). Poly(I:C) and its derivatives are of great importance for vaccines that need to induce a Th1/CTL immune response against various viruses and pathogens including *P. falciparum* [90]. Pam$_3$CSK$_4$ was used in a malaria vaccine containing *P. falciparum* circumsporozoite protein B cell epitopes and universal T cell epitopes, which resulted in the induction of high titers of antigen-specific IgG1, IgG3 and IgG4 in immunized volunteers [28].

3.3. Pathogens with latent disease phase

Herpesviruses are large viruses with a complex genome. Primary infection with varicella zoster virus (VZV) causes varicella (chickenpox) and may go into latent phase in human cranial and dorsal root ganglia. Aging or immune dampening results in decline of VZV-specific CMI, which may induce reactivation of the virus and cause shingles. Hence, CMI is necessary to prevent reactivation of the latent virus. The VZV vaccine HZ/su (Shingrix) composed of the VZV glycoprotein E subunit (gE) antigen and AS01$_B$ was recently approved for the prevention of herpes zoster in adults aged 50 years or older [91]. AS01 was selected as the adjuvant for the VZV

vaccine, because compared to other adjuvant systems, AS01 induced higher numbers of IFN-γ secreting CD4$^+$ T cells, and thus improved T cell as well as antibody responses, with acceptable clinical safety profiles [92].

HPV effectively evades innate immunity by inhibiting the IFN receptor signaling pathways and activation of ISGs via the E6 and E7 proteins. HPV also downregulates TLR9 and does not induce any danger signal to alert the immune system [93]. This prolongs the duration of infection and delays the onset of adaptive immunity. Thus, an effective CMI is required to clear and control HPV infection. Effective vaccine-induced immunity against HPV should consist of CMI to the early proteins, E2 and E6, and neutralizing antibodies against the virus coat protein L1. Two currently approved HPV vaccines, Cervarix (a bivalent HPV 16/18 vaccine, GSK) and Gardasil (a quadrivalent HPV 6/11/16/18 vaccine, Merck) are highly protective against HPV 6, 11, 16 and 18 [94]. Both are LI VLPs; however, Cervarix is AS04-adjuvanted, while Gardasil is AAHS (amorphous aluminum hydroxyphosphate sulfate)-adjuvanted. VLPs strongly activate the stromal DCs in the injection site that migrate to the dLNs, or may directly bind to the surface of APCs or other immune cells and migrate to the LNs, where they prime naïve B cells [95]. According to a recent study in girls aged 9–14 years, two doses of Cervarix elicited superior HPV-16/18 antibody responses compared to two or three doses of Gardasil. The differences in immunogenicity between the two vaccines may be due to the different types of adjuvants used. AS04 enhances humoral immune responses and CMI by triggering local and transient cytokine responses that promote enhanced activation and presentation ability of APCs [96]. Significantly higher antibody titers are induced in mice immunized with HPV-16 L1 VLPs adsorbed onto AAHS as compared to VLPs adsorbed onto aluminum hydroxide along with induction of an improved L1-specific IFN-γ secreting T cell response [96].

3.4. Intracellular pathogens

Mtb causing TB is an intracellular pathogen that has the ability to survive within the hostile environment of the alveolar macrophages after being phagocytosed and to multiply unchecked. CD4$^+$ T cells, CD8$^+$ T cells, CTLs, Th17 cells, NK cells, and activated macrophages are critical in controlling *Mtb* infections. Bacillus Calmette-Guérin (BCG) vaccine fails to protect adults from pulmonary TB and prevent transmission of *Mtb* in adolescents and adults [97]. Thus, there is an urgent need for improved vaccines against TB. One of the potential vaccine strategies against *Mtb* is to eliminate or control latent infection and prevent reactivation or progression to clinical TB in latently infected patients. This may be accomplished by incorporating adjuvants that are capable of inducing both CD4$^+$ and CD8$^+$ T cell responses in both immunocompetent and immunocompromised individuals.

Mechanisms of antibody-mediated protection against TB include opsonization, complement activation, and Fc receptor engagement. Current research is focused on adjuvants that act on innate lymphoid cells (ILCs), NK cells and non-classical T cells such as CD1, MR1, HLA-E and γδ T cells present in large numbers in the circulation and mucosa [98]. Although the immune correlates of protection from TB disease are not

validated yet, vaccines currently in clinical development predominantly focus on generating CD4$^+$ and CD8$^+$ Th1-type immune responses. Adjuvants such as mineral salts, saponin, Emulsigen®, micro- or nanoparticles, toxin derivatives, cationic lipids, CpG DNA, adjuvant systems and cytokines have been tested in subunit vaccine preparations, either alone or in combination with BCG in a prime-boost strategy [97]. The strongest Th1-inducing adjuvants for TB are unmethylated mycobacterial DNA and CpG ODN, which promote CTL activation and IFN-γ production [97]. TLR2/1 and TLR2/6 ligands are presented on the surface of *Mtb* (triacylated and diacylated forms of mycobacterial p19 lipoprotein) or secreted by the bacterium, while NLRs such as NOD2 are responsible for intracellular recognition of mycobacteria [99]. Novel adjuvants, including DDA, TDB, IC31, poly(I:C), gelatin, CpG ODN, MPLA, glycopyranosyl lipid adjuvant (GLA) in combination with squalene (SE) known as GLA-SE, MF59, CAF01, and AS01B are also being clinically tested. DDA promotes generating humoral, cell-mediated and IFN-γ responses against *Mtb*, while AS01 and MF59 induce strong Th1 immunity against *Mtb*. All these adjuvanted subunit vaccines induce protective immunity and enhance BCG-primed immunity in animal models [100]. In a randomized, double-blind, phase 2b trial, a candidate tuberculosis vaccine, M72/AS01$_E$, demonstrated a clinically acceptable safety profile and conferred 54% protection against active pulmonary tuberculosis in adults with latent *Mtb* infection [101]. Nanoparticle-based vaccines are critical for the induction of protective Th1-type immune responses to intracellular pathogens. The liposomal CAF01 adjuvant promoted Th1 and long-lasting memory T cell response in human TB vaccination trials. CAF01-adjuvanted TB vaccine stimulates the CLR, and Mincle, and triggers the Syk/Card9 signaling cascade to activate the Th17 signaling pathway [48].

4. New approaches to study adjuvant modes of action

One of the biggest challenges in vaccine development is the fact that the immunological mechanisms that govern vaccine safety and efficacy are still largely unknown. In recent years, systems vaccinology has emerged as an interdisciplinary approach that relies on high-throughput omics-based techniques to study vaccine-induced changes in the entire genome, set of transcripts, proteins, and metabolites in various tissues. A systems vaccinology approach has been used to elucidate immune responses to vaccines against yellow fever [102], influenza [103], malaria [104], smallpox [105] and HIV [106]. In addition, a systems vaccinology approach identified molecular and cellular immune signatures of a vaccine against *Bordetella pertussis* [107]. IP-10 was identified as an early innate immune signature that correlated with antibody responses to an Ebola vaccine (rVSV-ZEBOV) [108].

4.1. Mechanisms of adjuvanticity: identification of biomarkers

Computational analysis of the transcriptomic profile in human peripheral blood mononuclear cells (PBMCs) induced by

yellow fever vaccine YF-17D identified two molecular signatures: eukaryotic translation initiation factor 2 alpha kinase 4 (EIF2AK4) and TNFRSF17, encoding the receptor for the B-cell growth factor BLyS-BAFF [102]. EIF2AK4 correlated with the magnitude of the CD8$^+$ T cell responses, while TNFRSF17 correlated with the magnitude of neutralizing antibody responses. Other genes such as calreticulin, c-Jun, and glucocorticoid receptor were also induced by YF-17D, and this induction correlated with CD8$^+$ T cell responses [102]. In another study with young healthy adults, intramuscularly administered TIV induced higher antibody levels and plasmablasts when compared to intranasally delivered live attenuated influenza vaccine (LAIV) with induction of distinct transcriptional signatures such as enhanced expression of type 1 IFN genes in LAIV recipients, but not in TIV recipients [109]. Based on a systems vaccinology approach, TLR5 agonists as adjuvants were found to potently enhance the immunogenicity of influenza vaccine, resulting in an improved antibody response in humans [109]. The longevity of the immunoglobulin response post vaccination could be predicted from the ability of the adjuvanted vaccine to induce proliferation of antigen-specific IL-21$^+$ICOS1$^+$CXCR5$^-$CD4$^+$ T cells in the peripheral blood.

Systems vaccinology also identified two biomarkers (*Junb* and *Ptx3*) of MF59 and the skeletal muscle tissue cells (in addition to APCs) as direct target of MF59 for its adjuvant action in mice [50]. Caproni et al. investigated molecular signatures induced by different TLR-dependent (CpG ODN, Resiquimod and Pam$_3$CSK$_4$) and TLR-independent (MF59 and alum) adjuvants in influenza subunit vaccines to establish the innate immune correlates of adjuvanticity by using DNA microarrays in a mouse model [110]. Two adjuvants, MF59 and Pam$_3$CSK$_4$ increased overall antibody and HAI titers, and induced active infiltration of CD11b$^+$ cells, especially neutrophils, to the injection site. This suggests early induction of CD11b$^+$ cells due to an emulsion-based adjuvant to be predictive of subsequent robust humoral immunity.

Systems vaccinology has also been applied to identify novel mechanisms of induction of Th2 responses by an adjuvant. For instance, the Th2-promoting adjuvant activity of cysteine protease allergen is dependent on the production of ROS by DCs. As a result of induction of ROS, oxidized lipids are induced that in turn promote epithelial cell-mediated production of thymic stromal lymphopoietin (TSLP), resulting in the recruitment of IL-4$^+$ basophils to the LNs for induction of Th2-type immune responses in mice [111]. Genes associated with memory B cell formation and productive antibody responses such as *Bcl2, Bcl11a, Tank, Plcg2*, and *Cd38* are induced when mice are immunized with ovalbumin (OVA) adjuvanted with TLR7 and TLR4 agonists [112]. In a study with the candidate malaria vaccine RTS,S/AS01B in human subjects, enhanced expression of genes involved in immunoproteasome formation, *PSME2* in particular, was found to be responsible for conferring protection from parasitemia. Induction of the immunoproteasome enhances MHC antigen presentation, which in turn, indirectly enhances antibody responses and directly augments CD4$^+$ T cell development and production of IFN-γ, TNF-α, IL-2, and CD40L. The above immune signatures may contribute to the protective efficacy of the candidate malaria vaccine [104,112].

A comparative systems analysis of four vaccine adjuvants, GLA-SE, IC31, CAF01, and alum, in mice revealed distinct molecular signatures. GLA-SE induced massive changes in the transcriptomic profile in the whole blood and dLNs that correlated with increased cellular influx (such as CD11c$^+$GR1$^+$ mDCs) in the dLNs, in contrast to limited transcriptomic changes induced by other adjuvants. Co-expression analysis of differentially expressed genes in whole blood revealed that CAF01 and GLA-SE (but not IC31) induced transcriptional signatures related to innate immune responses. The analysis also revealed modules enriched for genes associated with Tfh and GC-mediated B cell responses; for example, GLA-SE induced *Nfatc1, Nfatc2*, and *IL21R*; CAF01 induced *Batf* and IC31 induced *Pou2af1*. A systemic analysis of protective immune responses to three RTS,S vaccinations with a subsequent controlled human malaria challenge of the vaccine recipients with *Plasmodium*-infected mosquitoes was carried out. Molecular signatures of B cell and plasma cells in human PBMCs were found to be positively correlated to protection, while the NK cell signatures correlated negatively with protection, indicating multiple mechanisms of protective immunity against *P. falciparum* [113].

In a study by Burny et al., different adjuvants [AS01$_B$, AS01$_E$, AS03, AS04, and alum/Al(OH)$_3$] induced common innate pathways and were responsible for improved adaptive responses when used with a model antigen (HBV surface antigen or HBsAg) in humans. AS01$_B$, AS01$_E$, and AS03 induced comparable innate profiles and so did AS04 and alum. Furthermore, the ability to activate innate immunity (IFN-signaling pathway, in particular) was linked to enhanced adaptive responses elicited by AS01- and AS03-adjuvanted vaccine. Early changes in immune markers, such as CRP, IL-6, IFN-γ, and IP-10, correlated with the magnitude of the adaptive responses [57].

4.2. Identification of factors controlling vaccine safety and efficacy

Systems vaccinology also identifies signatures of vaccine safety and efficacy. Non-specific adverse side effects observed for vaccines that fail in human clinical trials are frequently associated with over-stimulation of certain components of the innate immune system. Systems vaccinology can be applied to screen adjuvants to help design protective and safe vaccines [114]. Correlates of protection have been established for a number of licensed vaccines as reviewed by Tomaras et al. [115]. However, attempts to identify correlates of protection are still ongoing for TB, while the commonly assumed immune correlates often fail to correctly predict an individual's risk of developing malaria [116]. For HIV, complex immune correlates of protection characterized by multiple types of immune responses are found to be involved in controlling HIV-1 transmission [115]. For vaccines for which the immune correlates of protection are unknown, systems vaccinology approaches can be used to identify signatures induced rapidly after vaccination that will help to predict the later immune outcome. A systems vaccinology approach can also help in identifying vaccine non-responders as well as vaccine high- and low-responders [117].

Innate and adaptive immune responses are profoundly influenced by any significant changes in metabolic activity. Inflammation triggered by vaccine adjuvants results in a shift in energy supply leading to metabolic acidosis and impaired oxygen supply, which in turn results in phenotypic shifts. These phenotypic shifts heavily affect the metabolic state of an individual. Lipid metabolism plays an important role in inflammation. Liquid chromatography-mass spectrometry (LC-MS) is employed to identify and quantify cell- or tissue-specific metabolites [117]. Metabolite immune-correlates such as nucleotides, amino acids, lipids, fatty acids, and anti-oxidants may represent inflammatory mediators and/or biomarkers that profoundly influence several inflammatory processes such as cellular infiltration, activation of signaling pathways and oxidative stress [118]. Thus, a comprehensive understanding of the molecular signatures induced by adjuvants early after vaccination will help to predict the later adaptive immune responses in humans. Furthermore, such knowledge will also improve or help in (re-)designing next-generation adjuvants and drive the development of next-generation vaccines with the concerted effort of vaccinologists, clinicians, systems biologists, statisticians, as well as industrial and regulatory authorities.

The relationship between adjuvants, innate pathways/receptors activated, immune responses triggered, and the type of pathogens ideal for such adjuvant-mediated immune responses are summarized in Table 4.

5. Conclusion

In this review article, we have summarized the mechanism of action of different classes of adjuvants. We also discussed why this knowledge is important in context of distinct disease targets and how this knowledge can be utilized to improve the development of adjuvanted vaccines against challenging pathogens. We also briefly highlighted the important role of the new-age systems vaccinology approaches in better understanding an adjuvant's mode of action, and identification of unique cellular and molecular biomarkers of adjuvanticity. It is important to note here that mouse models offer flexibility and accessibility to study intricate facets of the mechanism of action of adjuvants and responses to immunization with adjuvanted vaccines. While the results from animal studies often overlap with the results from human studies, there are several dissimilarities as well. For instance, MF59-adjuvanted vaccine in mice induces both cell-mediated and humoral immune responses, which is not observed in humans at any age, rather they tend to develop a Th0-Th1 response.

Even in humans, immune responses need to be investigated not only in the serum but also in the dLNs and other lymphoid organs; not only at the priming site but also in the distant effector sites such that a holistic and reliable assessment of mechanism of action of adjuvant and/or vaccine can be made encompassing all possible immune parameters. Even the immunological correlates that have recently been identified by gene profiling or systems vaccinology for different adjuvants/vaccines are defined more at the population level and much less at the individual level [16]. All these considerations must be taken into account while designing effective and safe vaccines/adjuvants.

6. Expert opinion

Recent advancements have allowed researchers to conclude that clinical-grade adjuvants have distinct immunological profiles and signatures, which can be used to target different pathogens. Based on pathogen-specific immune response requirements (i.e. Th1, Th2 or Th17 responses, or mixed Th1/Th2 or Th1/Th17 responses, etc.), next-generation adjuvants can be rationally developed and incorporated into human vaccines. Currently, all approved human adjuvants mostly induce only antibody responses. However, recent adjuvant research has led to the development of novel adjuvants capable of inducing CMI (especially required for malaria, TB and HIV), as well as antibody responses. New immunostimulatory adjuvants or immunomodulatory compounds are under investigation to induce CMI and high antibody titers. Novel combination adjuvants are being tested in candidate human vaccines with promising results that have strong implications for use in vaccines against challenging infectious pathogens and different target populations. This is potentially due to activation of multiple innate immune sensing signal transduction pathways by combination adjuvants. Novel adjuvants are required that can target emerging new pathogens or re-emerging old pathogens. Such pathogens often have a more complex host–pathogen interaction, which needs better understanding and further characterization. Among these new-generation adjuvants, several are proprietary, which may make it difficult to purchase them and conduct independent parallel trials. Factors such as genetic background, pre-exposure to pathogens or vaccine antigens, age, nutritional and immunological status of vaccine recipients, all dictate the final effectiveness of adjuvanted vaccines. Nevertheless, with the aid of structural, systems and reverse vaccinology, epitope prediction and other technological advancements, adjuvant technology is now gradually progressing towards a more personalized approach.

There has been an exponential growth in the field of adjuvant research. While alum was historically used as the only licensed adjuvant for more than 70 years, six new adjuvants were approved in the last 20 years. The next five years will see substantial progress in obtaining licensure for more varied types of adjuvants. Understanding innate immunity and the role of PRR agonists as adjuvants in stimulating the innate immune system has revolutionized adjuvant technology. Systems biology has immense contribution in the development of effective and potent adjuvants, and will continue to do so in the coming years. Correlates of adjuvanticity or immune signatures, and biomarkers of adjuvant safety and protective efficacy will further streamline adjuvant research. Tailor-made adjuvants will find their use against distinct pathogens and in specific target populations. Better characterization of adjuvants by new omics-based technologies will facilitate licensing of new adjuvants. Since there is a pressing need for developing vaccines against a multitude of very virulent/emerging pathogens, we need to develop subunit vaccines and not live vaccines, and hence adjuvant selection is critical.

The mucosal surface is the preferred route of entry for most pathogens. Therefore, mucosal immunization is considered to

Table 4. Relationship between adjuvants, innate pathways/receptors activated, immune responses triggered and the type of pathogen targets for such adjuvant-mediated immune responses.

Adjuvants	Innate pathways/receptors	Immune responses triggered	Type of target pathogens
Alum	Phagolysosomal rupture, NLRP3/NACHT/NALP3, NET, STING-IRF3	Ab, Th2 (+Th1 in humans)	Toxin-producing, extracellular pathogen
MF59 and AS03	Inflammation (without any defined receptors), ASC, MyD88 and ER stress-related pathway	Ab, Th1 + Th2	Intracellular and extracellular
AS04	TLR4	Ab, Th1	Pathogens with latent disease phase, extracellular
AS01, AS02 [MPL, QS-21]	TLR4 (MPL), lysosomal destabilization & Syk activation (QS-21)	Ab, Mucosal IgA, and Th1	Pathogens with complex life cycles, pathogens with latent disease phase, intracellular, extracellular
Poly-IC or Poly(IC:LC), poly(I:C$_{12}$U)	TLR3, MDA5	Type I IFN, pro-inflammatory cytokines, Ab, Th1, CD8$^+$ T cells	Mucosal, pathogens with complex life cycles, intracellular
Flagellin or Flagellin-Ag fusion proteins	TLR5	Mucosal IgA, Th1 + Th2	Mucosal
Chitosan	Tight junctions	Mucosal IgA	Mucosal
Imiquimod, Resiquimod, Gardiquimod	TLR7, TLR8 or both	Type I IFN, pro-inflammatory cytokines, Ab, Th1, CD8$^+$ T cells (when conjugated)	Intracellular
CpG ODNs (IC31)	TLR9	Type I IFN, pro-inflammatory cytokines, Ab, Th1, CD8$^+$ T cells (when conjugated)	Intracellular
CAF01	CLR, Mincle, Syk/Card9	Ab, Th1, Th17	Intracellular, extracellular
ISCOMS, ISCOMATRIX	Undefined	Ab, Th1+ Th2, CD8$^+$ T cells	Mucosal, intracellular
CFA	NLR, inflammasome, Mincle, Lyn-Syk-PI3K, NFAT, Ras-MEK-ERK	Ab, Th1, Th17	Intracellular, extracellular
CDN (CDA & CDG)	STING	Type 1 IFN, Th17	Mucosal, intracellular

Based on Coffman R L et al. [119] and Kim S-H et al. [75].

be most effective in preventing mucosally transmitted infections. However, the major hurdle in the development of mucosal vaccines is the lack of safe and effective mucosal adjuvants due to toxicity issues. There is specific need for standardized, more comprehensive and pertinent methodologies for safety evaluation to enable development of safe mucosal adjuvants [81]. Mucosal adjuvants are also required to promote bioavailability of vaccine antigen. Another requirement is the development of mucosal adjuvants with an optimal targeting ability so as to reduce undesirable adverse side effects. Since efficacy and toxicity of most mucosal adjuvants appear to be intrinsically linked, a risk–benefit ratio needs to be ascertained for these adjuvants. Attention must also be directed to studying antigen-adjuvant interactions instead of irrational mixing of an adjuvant in a vaccine formulation [120]. Oil-in-water emulsions are very promising adjuvants and characterization/analysis of components added in emulsion preparations in more detail will facilitate improvement of such adjuvants [121].

Acknowledgments

Funding

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

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