Understanding Mechanisms of Actions for Vaccine Adjuvants Critical for Designing Effective Vaccines: *A narrative review*

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Summary

INTRODUCTION

Vaccine adjuvants enhance immunogenicity of antigens through one or a combination of mechanisms that include; improved antigen delivery to the innate immune system or by providing signals that activate the innate immune system. Activation may lead to induction of *cytokines* and *chemokines*, recruitment of immune cells to the site of vaccine inoculation or trafficking of innate immune cells to draining lymph nodes. These events culminate in activation of adaptive immunity.

OBJECTIVE

To identify the Current status of knowledge on action modes for vaccine adjuvants, modes under investigation and future directions in this important area of biomedical research. influence on molecular and physical interactions between vaccine components and innate immune cells that affect the degree of immune responses given first priority.

METHODOLOGY

Published studies in English language on vaccines and adjuvants were identified by key words from Comprehensive searches with no formal assessments for risk of biases. The type of publications included basic research using experimental animals and clinical research in human. A recent study using recombinant *hemmaglutinin* (rH5) protein of highly pathogenic avian influenza (HPAI) virus as antigen demonstrated a quicker antibody production. IL -17 and IFN-γ when adjuvant combinations of CpG and nanoemulsion were used in comparison to nanoemulsion alone [25]. Equally, potential vaccine adjuvants including pathogen associated molecular patterns (PAMPs) derived from microbes with their synthetic analogs like *cytosine* and *guanine* (CpG) *oligodeoxynucleotide* that targeted toll-like receptors (TLRs) and mast cell activating compound such as compound 48/80 (C48/80) [23, 24]. Combination of cationic peptide HH2 with CpG induced IgG1 (Th2) and IgG2a (Th1) type antibodies in experimental animals [30]

Other vaccine adjuvants developed for human use included *Monophosphoryl* lipid A (MPLA) and MF59 (oil in water emulsion). MPLA is a detoxified form of bacterial cell wall lipid A from *Salmonella* Minnesota R595 combined with alum to augment immunogenicity of subunit vaccines. When mediated through activation of TLR4 induced Th1 type immune responses [16]. Combined with alum for hepatitis B virus (FENDrix) Human *Papilloma* Virus (Cervarix) [15] and then combined with a water-soluble triterpene glucoside as adjuvants for malaria vaccine trials in human [17]. Combination of a *mucopolysaccharide chitosan* as a mucosal vaccine with Norwalk norovirus demonstrated induction of antigen-specific antibody.

Cytomegalovirus Glycoprotein B antigen when adjuvanted with MF59 induced a higher antibody titer using a lower antigen dose compared to antibody titer induced by a higher dose of the same antigen [32] Inactivated hepatitis A antigen induced a significantly higher seroconversion rate at two weeks after the first injection with 100 U of antigen compared to 50 or 25 U of the antigen [31]. Intranasal meningococcal subtype B vaccine induced highly bactericidal immunity with day 0, 7, 28 and 56 schedules than same vaccine given on days 0, 28 and 56 [34]. Induction of dsDNA released when bound by adjuvants formed a complex and trans-located into the endosome to activate TLR9 cascading to MyD88 adaptor molecule [29, 51] immunity [43].

RESULTS (DATA SYNTHESIS)

This literature review has shown that, several strategies exist that can be applied in order to maximize immune activation and improve vaccine efficacy. Such strategies include combination of adjuvants that activate different pathways of the innate immunity. Combination of adjuvants produced synergistic effect in immune responses and pathogen clearance, but individual adjuvants more often provided a response that was narrow in its effect, being either Th1 or Th2 biased.

CONCLUSION

Selection of the right adjuvant for a vaccine antigen requires knowledge on the mode of action of the adjuvant. Different adjuvants and different routes of vaccine administration could generate various types of immune responses. The route of vaccine administration might influence the type of cells in the innate immunity activated by an adjuvant. Antibodies with high avidity strongly bound to antigenic determinants on the pathogen inducing destructive processes against the pathogen. Intranasal adjuvants can be suitable for mass vaccination against respiratory infections such as influenza and CORONA viruses (SARS Cov-2 (COVID-19)). The ratio between the antigen and adjuvant in a vaccine would influence the structure of the final complex formed and its biological activities.

RECOMMENDATIONS

To effectively design an adjuvant within a vaccine formulation, first understand its mechanisms of activity in order to develop a potent, effective and safe vaccine. Induce sufficiently mature (high avidity) antibodies by a vaccine to avoid lack of protection.

Key words: Vaccine adjuvants, mechanisms of actions of vaccines, mechanisms of actions of adjuvants, vaccines design and development.

[***Afr. J.*** Health Sci. 2020 33(3) : 19 - 29]

Introduction

Vaccination is the most successful way of prevention against human infectious diseases [1, 2]. The main goal was to induce pathogen-specific immune responses using inactivated pathogen molecules that generate protective immunity against future infections by similar virulent pathogens [3].

Vaccine antigens may exist in multiple forms such as;

- live-attenuated microorganisms,
- inactivated pathogenic microorganisms,
- purified components of microbial pathogens,
- polysaccharide-carrier protein conjugates
- or recombinant proteins of pathogenic microorganisms [4].

After vaccination, those antigens first activates the host innate immune system, whose products then activate the adaptive immune system [5 -7]. Effective activation of innate immune cells was required for generation of optimum adaptive immune responses. Vaccine antigens that poorly activate the innate immune system could lead to less effective immunity.

Studies have shown that weaker immune responses to vaccine antigens were results of original poor immunogenicity most common with purified and subunit vaccines.

Incorporating relevant adjuvants in the vaccine formulation could enhance the immunogenicity of vaccine antigens [3, 8]. Although vaccines formulated using whole pathogens were highly immunogenic, ineffective inactivation of the pathogens was potentially infective. Consequently, most recent vaccines were being produced as recombinant subunit proteins of the pathogens [9]. For example, hepatitis B virus (HBV) and human papilloma virus (HPV) vaccines were subunit vaccines.

Although subunit vaccines had high purity, they were often poorly immunogenic. Hence, vaccine adjuvants were included in subunit vaccine formulations to enhance immunogenicity and subsequent efficacy [3]. Different vaccines (antigens or antigen-adjuvant combinations) activated specific pathways of the innate immunity, generated varying quantities and profiles of immune mediators that determined the quality of the adaptive immune response.

Understanding the modes (mechanisms) of actions of vaccines and accompanying adjuvants was therefore crucial in designing effective and safe vaccines. The fact that, vaccines and their adjuvants target the innate immunity, it was prudent to identify target cells and specific receptors (pathways) activated by each vaccine antigen-adjuvant combination in order to know the expected immunomudulatory molecules.

Vaccine adjuvants applied in human vaccines

Aluminum compounds (alum) was the earliest and the most widely used adjuvant in human vaccines [10].Alum haS been used in numerous vaccines including *diphtheria-tetanus-pertussis,* human *papilloma* virus and hepatitis B vaccines [11].

Other vaccine adjuvants developed for human use include *monophosphoryl lipid* A (MPLA) [12-14[and MF59 (oil in water emulsion). MPLA was a detoxified form of bacterial cell wall lipid **A** from *Salmonella Minnesota* R595. MPLA adjuvant had been applied in combination with alum to augment immunogenicity of subunit vaccines. The adjuvant activity of MPLA was mediated through activation of TLR4 and was seen to induce Th1 type immune responses [16]. MPLA was used in combination with alum for hepatitis B virus (FENDrix) human *papilloma* virus (Cervarix), [15] and in combination with a water-soluble triterpene glucoside as adjuvants for malaria vaccine trials in human [17].

Further studies on MPLA involved combination with a *mucopolysaccharide chitosan* as a mucosal vaccine with Norwalk norovirus demonstrating induction of antigen-specific antibody responses [18]. MF59 (an oil in water emulsion) had been used to improve the immunogenicity of influenza vaccine and demonstrated induction of protective immunity against influenza virus in humans [19].

One of the significant observation about vaccination outcomes was that, different adjuvants and different routes of vaccine administration could generate different types of immune responses [9, 20]. The immune responses may differ in a number of aspects including Th1 *versus* Th2 type immune profiles or antibody responses versus T cell mediated responses or a combination of these with variations in magnitudes or ratios of responses.

The knowledge about a vaccine was sensitive because certain types of infections (pathogens) could require induction of specific type of immune responses in order to be cleared [21]. That might require unique forms of adjuvant activity which activate specific pathways of innate immunity. The route of vaccine administration might influence the type of cells in the innate immunity activated by an adjuvant. Different sites of vaccine inoculation might differ in the types and distribution of first-line immunity cells such as mast cells, dendritic cells and macrophages.

For instant, some antigen presenting cells could be abundant at mucosal sites while others would be intradermal or subcutaneous affecting their accessibility to vaccine antigens and adjuvants.

Recent developments have identified a variety of potential vaccine adjuvants for possible future application in human vaccines. These include pathogen associated molecular patterns (PAMPs) derived from microbes and their synthetic analogs such as *cytosine* and *guanine* (CpG) *oligodeoxynucleotide* that target toll-like receptors (TLRs) [22] and mast cell activating compound such as compound 48/80 (C48/80) [23, 24].

Objectives

The objective of this review was to identify the current strategies that can be applied in understanding

the mechanisms of action for vaccine adjuvants with regard to:

1. Molecular pathways and physical interactions of the innate immune system.
2. Vaccine and adjuvant doses
3. Vaccination regimens for each vaccine.
4. The current status of knowledge on mechanisms of actions for known adjuvants and those under investigation.
5. Future directions in this important area of biomedical research.

Methodology

Publications on vaccine and adjuvants research and development were identified and downloaded from several data sources that included PubMed (NLM), PubMed Central, Library of congress, LISTA (EBSCO), Google Scholar, Science Direct and Web of Science (TS) databases. Articles were searched using key words as title or subject without restrictions on types of publications that were presented in English language. Since comprehensive searches were conducted on multiple databases as mentioned, no formal assessments for risk of bias were conducted. The type of publications included basic research using experimental animals and clinical research in human.

Literature Review

Strategies Shown to Improve Vaccine Adjuvant Activities

From the literature review there were several strategies that have been applied in the development of effective vaccines in the context of adjuvants incorporated in the vaccine. Some of the key strategies are discussed below:

1. Combination of Vaccine Adjuvants that Activate Different Molecular Pathways Generate Effective Immune Responses

Studies have shown that combination of adjuvants provide superior immune responses compared with individual adjuvants. A recent study using recombinant *hemmaglutinin* (rH5) protein of highly pathogenic avian influenza (HPAI) virus as antigen demonstrated a quicker antibody production, IL-17 and IFN-γ when adjuvant combinations of CpG and nanoemulsion were used compared to nanoemulsion alone [25].

Combination of adjuvants was seen to produce synergistic effect in immune responses and pathogen clearance, but individual adjuvants more often provided a response that was narrow in its effect, being either Th1 or Th2 biased. Cationic molecules such as KLKLLLLLKLK predominantly induce Th2 type immune responses against co administered vaccine antigens [26], while other classes of molecules such as CpG enhance antibody affinity maturation [27] and provide Th1 type immune responses [22, 28].

Despite adjuvants of different molecular properties exhibit distinct immune activation properties when combined, they provide superior immune responses. Studies have shown that, combination of CpG with alum enhanced affinity maturation of anti-hepatitis B vaccine antibody responses [27]. Combination of CpG with cationic peptides has shown to form complexes that facilitate delivery of antigen to APCs [29] and to induce both Th1 and Th2 type antibody responses.

Use of adjuvants was reported to induce diversified immune responses as depicted by combination of cationic peptide HH2 with CpG that induced IgG1 (Th2) and IgG2a (Th1) type antibodies in experimental animals [30]. Consequently, this nature of immune response ends up being more effective than a narrow type of immune response. Due to the different modes of action by vaccine adjuvants, their effects could work in synergy to provide a more diversified and effective immune response.

2. Varying the Dose of Vaccine Antigens and the Interval between Vaccinations Influence Immune Responses.

Some vaccine studies reported that, increasing antigen dose was one strategy used to reduce the number of immunizations while maintaining a desirable immune response. For instance;

> A study conducted in human subjects using inactivated hepatitis **A** antigen induced a significantly higher seroconversion rate at two weeks after the first injection with 100 U of antigen compared to 50 or 25 U of the antigen [31].

However, other studies have demonstrated that this effect is dependent on antigen type. For example;

> *Cytomegalovirus Glycoprotein B* antigen when adjuvanted with MF59 (oil in water emulsion) induced a higher antibody titer using a lower antigen dose compared to antibody titer induced by a higher dose of the same antigen [32].

Those observations demonstrated that, for each antigen type, an optimum dose should be determined through a dose-response study. That was more so with adjuvant included in the vaccine because antigen-adjuvant interactions would lead to formation of secondary complexes that would differ in their capacity to be delivered to activate the immune cells.

The ratio between the antigen and adjuvant in the vaccine would also influence the structure of the final complex formed and its biological activities. Besides the vaccine dose, studies have shown that, intervals between the primary and booster immunizations may influence the magnitude of both antibody and cellular immune responses (33).

This observation suggested that a critical time existed after priming the immune system at which later immunizations effectively activated immune cells to achieve maximum responses. The interval seemed to influence the frequency of antigen-specific memory B and T cells.

> Practically, intranasal *meningococcal* subtype B vaccine induced highly bactericidal immunity with a day 0, 7, 28 and 56 schedules than same vaccine given on days 0, 28 and 56 (34).

The results suggested that the inclusion of a shorter interval between the primary and first booster enhanced the production of immune factors with higher bactericidal activities. It is important to note that the optimum time intervals required between the primary and booster inoculations may vary with different antigen types or types of adjuvants in the vaccine and the respective antigen dose.

Current Understanding of Modes of Actions for Vaccine Adjuvants

To effectively design an adjuvant within a vaccine formulation, there was a need to understand its mechanisms of activity in order to develop a potent, effective and safe vaccine. Aluminum compounds were the longest serving vaccine adjuvants [35]. Although aluminum-based adjuvants have been used for decades with human vaccines, their mechanisms of action have not been fully elucidated [11]. It has been demonstrated that aluminum compounds forms a depot at the site of vaccine inoculation that then releases the antigen in small doses to stimulate the immune cells [36-38]. Recent studies suggest that although alum forms antigen depots, depot formation is not required for its adjuvant activity [36].

Studies have also shown that alum and other pore forming adjuvant molecules activate the inflammasome in macrophages and dendritic cells as a possible intermediate in its adjuvant activity [39]. Inflammasomes are multiprotein complexes in dendritic cells and macrophages activated by danger signals that lead to the recruitment and activation of caspase 1. Caspase 1 activation then leads to processing of pro-inflammatory *cytokines* such as IL-1β and IL-18 that participate in immune responses against insulting pathogens [40]. Although activation of the inflammasome was considered a possible mediator of adjuvant activity by alum [41], similar to the depot formation other studies showed that inflammasome activation of this pathway by alum was not required for antigen specific immunity [39].

Comparably MF59, oil in water emulsion adjuvant was seen to activate the inflammasome in vitro studies. However, that activity was not required for in vivo adjuvant activity because there was no difference between wild type mice and inflammasome deficient mice [42]. The activities of alum and MF59 suggested that adjuvant molecules could activate some molecular pathways or cause changes in the behavior of the antigen in the host but those changes might not be immunologically important. More importantly, it is well documented that those adjuvants augment antigen activation of the innate immune cells lead to enhanced immune responses.

Toll-like receptors (TLRs) were identified among PRRs as important targets for mediating the activity of some vaccine adjuvants.

> Immunization of vaccine antigens combined with TLR agonist monophosphoryl lipid A (MPLA) specifically activates TLR4 to stimulate innate immunity [43]. MPLA has been used

in combination with alum adjuvant (AS04) for human papilloma virus vaccine [15].

This adjuvant system Was approved for use in humans in Europe and USA. model studies displayed the activation signals generated through TLRs involved recruitment and activation of adaptor molecules including MyD88 (44).

MyD88 was found downstream of TLRs, except TLR3, and were critical in transmitting the activation signals that lead to generation of cytokines. Cytokines mediated adjuvant activities on adaptive immune responses [45].Stimulation of MyD88 adaptor protein commonly originate from engagement of TLRs by vaccine adjuvants or microbes or their components. Activation of MyD88 then signals the induction of pro-inflammatory cytokines by innate immune cells that modulate other immune responses.

Early (within hours) cytokine release had also been demonstrated by studies with MF59 adjuvant that was dependent on MyD88 activation in vivo [42]. That could be a non-TLR activation of MyD88. Dependency on MyD88 was demonstrated by significant decrease in the production of IL-5 and G-CSF in MyD88 knockout mice, with a significant reduction in serum antigen-specific IgG antibody titers.

Therefore, understanding the mechanisms of adjuvant activity was important for deciphering their contributions in the induction of immune responses. Apart from MyD88, Mc Lachlan and co-workers have exhibited mast cells activation can enhance induction of immune responses [23]. The immune activation was mediated by release of preformed cytokines within the mast cells including TNF that were released upon mast cell degranulation. This observation had identified mast cell activators as potential vaccine adjuvants, identifying a unique target cell of the innate immunity for vaccine design.

Although mast cells were reported to play a role in undesired allergic reactions, studies have demonstrated that mast cells activation can be induced in a safe manner to provide beneficial immunological functions. Further suggestions that, IL-33 *cytokine* released by *epithelial* cells as a result of cell injury (danger signal) can induce Th2 type cellular immune responses by its activation of innate immunity through MyD88 pathway. The danger signal on epithelial cells may easily be generated by pore forming molecules used as adjuvants in intranasal or oral vaccines that make contact with *epithelial* cells lining mucosal surfaces. Release of IL-33 forms another possible mechanism of adjuvant activity through non-TLR ligands that activate MyD88 [46].

It is postulated that when released by adjuvant or antigen activated *epithelial* cells, IL-33 activates its target cells, CD25+CD44+ *intraepithelial* innate *lymphoid* cells (ILCs) that express the IL-33 receptor ST2/T1. Other target cells for IL -33 include macrophages that were reported to produce IL-5 when activated by IL-33 [47].

Early activation (within hours) of the innate immune system has been demonstrated by studies to induce release of serum cytokines such as IL-5, G-CSF, IL -6 and KC [48] that drive subsequent events leading to adaptive response. These *cytokines* can therefore be used as biomarkers of effective activation of the innate immunity by adjuvants in vaccines.

Studies conducted to understand modes of activation of innate immunity reported that, double stranded DNA (dsDNA) could activate certain *cytosolic* DNA sensors such as stimulator of interferon genes (STING) within innate immune cells including DCs [49].

STING was seen to drive the interferon response factor 3 (IRF3) activation and subsequent generation of IFN *cytokines*. Damaged cells could release double stranded DNA as a danger signal which might be the case with pore forming adjuvants or vaccine molecules. As a possible pathway of adjuvant mechanism activity, dsDNA released after administration of adjuvants that cause local cellular injury activate intracellular STING to activate *cytokine* genes through IRF3.

Host dsDNA released from damaged cells also had the potential to activate innate immune cells including dendritic cells in either TLR9-dependent or TLR9-independent pathways. Generation of dsDNA was a potential mechanism through which localized death-inducing adjuvants could activate MyD88-dependent activity, besides the IL-33 pathway.

The TLR9-independent activity by host dsDNA might account for MyD88-independent activity by some adjuvants and that could involve the activation of

cytosolic sensors of dsDNA such as STING. Although the specific mediator that binds dsDNA to signal IRF3 was not very clear, *mitochondrial* antiviral signaling protein (MAVS) [50] and stimulator of interferon genes (STING) had been proposed [49]. IRF3 could induce interferon response genes that induce transcription and release type I interferon.

That hypothesis showed there were two possible pathways by which damage associated adjuvants could activate MyD88;

1. By induction of IL-33 and subsequent activation of ST2/T1 receptors
2. By induction of dsDNA release that when bound by adjuvants forms a complex and trans-locate into the endosome to activate TLR9 cascading to MyD88 adaptor molecule [29, 51]. immunity [43].

Synthetic cationic peptides inclusive of KLKL5KLK and innate defense regulator (HH2) peptides had demonstrated effective adjuvant activities in mice [26, 29]. In addition to their cationic nature, these peptides possess non-specific cell penetrating properties that might contribute to cell *lysis*. They were active on many cell types and could have had diverse models for adjuvant activity.

Other studies concluded that, inflammasome activation was a possibility to link innate and adaptive immunity induced by adjuvants through activation of *intracytoplasmic* PRRs such as NALP3 [39, 41]. The specific events that lead to inflammasome activation remain unclear. It was postulated that events that lead to pore formation and potassium efflux, generation of Reactive Oxygen Species (ROS), *lysosomal* damage and release of *cathepsin B* could activate the inflammasome.

The fact cationic peptides, penetrated cells leading to pore formation, they could induce generation of ROS [52] and activate the inflammasome. The same mechanism of pore formation in cells of innate immunity might apply to aluminum compounds that were assumed to activate the inflammasome. That theory was further supported by studies using a cationic peptide *melittin* that demonstrated in vitro activation of inflammasome in mice Bone Marrow-Derived Macrophages (BMDM).

In vivo adjuvant activity, similar antigen-specific antibodies in wild-type (WT) and caspase 1-/- (inflammasome-deficient) mice was demonstrated [53]. This showed lack of inflammasome–dependent adjuvant activity in vivo. The striking differences between WT and caspase 1-/- mice was the significantly lower *neutrophi*l infiltration at the site of inoculation in the absence of caspase 1 [53].

In addition to molecular activation, cationic molecules have been shown to delay antigen clearance from the site of inoculation thereby enhancing antigen uptake, processing and presentation. This theory was demonstrated by studies with cationic poly-L-arginine that showed increased retention and absorption of dextran through the *nasal epithelium* in rats [54]. Similar studies using cationic *nanogels, chitosan* and other *nanoconjugate* molecules had demonstrated prolonged antigen residence in the nasal cavity leading to enhanced immune responses in mice [55].

It was reported that, when the vaccine antigen was retained for a longer time at the inoculation site, there was an increase in the induction of antigen-specific immune response. Some vaccine adjuvants function by enhancing antibody avidity for the vaccine antigen. Studies disclosed that, failure to induce sufficiently mature (high avidity) antibodies by a vaccine can lead to lack of protection [56]. Antibodies with high avidity strongly bind to antigenic determinants on the pathogen inducing destructive processes against the pathogen.

On the contrary weakly binding (low avidity) antibodies might lack the capacity to induce neutralization of the pathogen. Antibody avidity could be used to determine pathogen-neutralizing ability of antibodies. Despite in vitro pathogen, neutralization was used as a correlate of protection by an immune response in certain circumstances of pathogen antigenic epitopes which might not have been be available in sufficient amounts on mature virions in vitro to allow neutralization. This may lead to lack of in vitro pathogen neutralization despite the same antibodies having sufficient in vivo pathogen clearance activity.

Another school of thought was that, in vivo, protection could be enhanced by antibody opsonization of the pathogen for clearance by *phagocytic* cells and T cell immune responses. That could make the antibodies with poor pathogen neutralizing capacity in vitro more effective in vivo [57]. From the forgoing literature, it was evident that mechanism studies in vaccine adjuvants was an active area of current and future investigations.

Conclusions and Recommendations

Vaccines (with or without adjuvants) should be safe to the host, and also effective in their functions. The safety and efficacy of vaccines can be predicted by deciphering their mechanisms of actions. The knowledge about the modes of actions for vaccines and any combined adjuvants is useful for designing and developing effective vaccines against particular pathogens. Such knowledge can aid in directing an immune response towards a desired type of protective immunity to effectively eliminate a particular pathogen.

It was reported that, different infectious agents could require different profiles of immunomodulatory molecules for their clearance that might require specific adjuvant activity [21]. In contrast;

> the activity of cholera toxin (CT) from Vibrio cholera and heat labile toxin (LT) from *Escherichia coli* as adjuvants in experimental animals predominantly induced Th2 type T cell immune responses with characteristic CD4+ T cells that secrete IL-4, IL-5, IL-6 and IL-10 [58]. This knowledge was useful for the fact that,

the right choice of an adjuvant can lead to generation of immunological mediators that will clear a particular infection.

Conclusively, understanding how a vaccine (antigen-adjuvant complex) interacts with the immune system can aid in retaining immunogenic components in the vaccine while deleting toxic ones to ensure safety.

Although studies had shown that CT and LT were effective vaccine adjuvants, they were highly toxic to the host animals [59, 60]. It was therefore necessary to retain potent genes in cholera toxin while deleting the virulent ones in order to retain desired adjuvant activity but also increase safety [61, 62]. A similar challenge applied to the potential use of mast cell activating c48/80 adjuvant which was a polymer of many molecules. There was need to isolate the specific monomers that activate mast cells to provide adjuvant activity and removal of nonessential molecules.

In order to separate essential components from redundant deleterious components in most adjuvants one must perform model studies. Mechanism studies will help to identify the target cells and receptors for each component on the larger molecules that are important for effective immune activation so that vaccines are engineered towards the targets while deleting the nonessential ones to minimize toxicity effects. In summary, this review has identified the following key facts:

1. Evidence accrued that, combination of adjuvants with different modes of action was a better strategy to induce a more effective immune response than use of a single adjuvant.

2. Vaccine regimens with fewer doses were more desirable because they ensured high compliance than multiple doses. However, the antigen type, antigen dose and adjuvant activity could influence the number of immunizations and the intervals between vaccine administrations. Optimum doses for each antigen-adjuvant combination could be determined independently through dose-response studies.

3. Studies have demonstrated that, activities of vaccine adjuvants are dependent on a variety of molecular pathways including; MyD88 activation mast cell activation or inflammasome activation (macrophages and dendritic cells). A new class of adjuvants prolonged antigen retention at the site of inoculation to enhance activation of the immune system. Surprisingly, the mechanism of adjuvant activity for the oldest aluminum adjuvants is still under intense investigations. It was clear that an adjuvant molecule might activate multiple pathways but only one or few were relevant to their immunological functions.

4. The recent status of knowledge on mechanisms of action for vaccine adjuvants suggested future studies to be focused on identifying more safe vaccine adjuvants for human use. Previously there were very few options on adjuvants approved for human use that included aluminum compounds MF59 and *monophosphoryl* lipid A.

   The limitation with those adjuvants was that, they had been approved for use with injectable vaccines. There was very limited development of adjuvants that could be safely applied with intranasal vaccines. Intranasal vaccines were desired because they were pain - free and relatively easy to administer. They require less technical training as opposed to injectable vaccines. Injectable vaccines had more risks

associated with use of needles such as safe disposal of needles and possible transmission of blood borne pathogens.

Intranasal adjuvants can be suitable for mass vaccination campaigns especially against respiratory infections such as influenza and CORONA viruses (e.g. SARS Cov-2 (COVID-19)). Therefore, mast cell activating molecules such as c48/80, *mastoparan* peptides and mucoadhesive molecules such as *chitosan* can be explored for use with intranasal vaccines.

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