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Review Article

Vaccine Adjuvants: From Empirical to a More Rational Drug Design

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Abstract

Like other drugs, adjuvants are ligands with distinct structures that interact with specific cell receptors, modulating the immune response. This definition excludes formulations and delivery systems. New adjuvants may be discovered using methods based on a ligand and its receptor's structural and functional traits, a process known as rational drug design. This strategy requires detailed information about both the receptors and their ligands. Such information is obtained using techniques like X-ray crystallography and 2D-nuclear magnetic resonance (NMR) to establish the spatial interactions between a ligand's functional groups and its receptor. This data is necessary to establish reliable structure-activity relationships, which, when applied to computer-aided drug design, facilitate the creation of better adjuvants as an empirical strategy. Since Quillaja saponin adjuvants likely act separately on innate and adaptive immune cells via specific functional groups and unidentified cell receptors, it is crucial to identify these receptors. This task may be achieved using bioorthogonal chemistry and proteomic methods to identify and isolate the receptors. Initially focusing on those unidentified receptors where chemical modifications of these glycosides, such as the aldehyde group and fucose residue, cause drastic changes in adjuvanticity. The isolated receptor(s) can then be characterized by *X*-ray crystallography and/or 2D-NMR; this information can be applied to computer-aided drug design to rationally design new derivatives. This methodology will prevent the proposition of dubious structure-activity relationships based on incomplete immunological data, unknown receptors, and unsuspected physical factors, providing essential information for designing new adjuvants and elucidating these compounds' mechanisms of action.

Introduction

Vaccines are among the oldest and most effective methods to prevent infectious diseases. Variolation, the precursor of vaccination, started in China and likely India around the 16th century. Variolation involved delivering materials from the scabs of smallpox victims nasally or through skin lacerations to induce lifelong immunity against the disease. Ancient reports describe some efforts to "attenuate" the live virus by aging or warming up the preparations in water suspensions.[1] In 1796, variolation was replaced by Jenner's invention of the cowpox virus vaccine, a safer way to elicit protection against smallpox.[1] The success of the cowpox vaccine sparked interest in infectious disease vaccines, with the French chemist Louis Pasteur being their foremost champion. In the late 1800s, the recognition that chemically inactivated diphtheria toxins (or toxoids) and killed bacteria induced protective immunity created interest in these safer vaccines. However, these vaccines elicited a weaker immune response than the non-inactivated ones, which affected the quality of the anti-diphtheria serum produced by horses immunized with diphtheria toxoid. Gaston Ramon from the Pasteur Institute investigated this shortcoming. In 1925, Ramon reported that the inflammatory response against the toxoid was increased by adding materials like starch, saponins, and lecithin, which he called adjuvants,[2] a word derived from Latin meaning "to aid". In 1926, the British scientist Alexander Glenny found that diphtheria toxoid prepared using potassium aluminum sulfate induced a better antibody response. Due to its safety, alum became the most common vaccine adjuvant.[3]

Rational adjuvant design: A new phase

While Ramon and Glenny established the immune-stimulatory properties of adjuvants, they could not explain their mechanisms of action (MOA). For some time, it was believed that the MOA was due to physical reasons, such as the depot effect, where the antigen, aided by the adjuvant, was released slowly over time.

Keywords: Immunopotentiator; Saponins; Toll-like receptors; Drug design; Immunoactivators; TLR agonists; Pharmacophore; Molecular docking.

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This explanation ignored the then-unknown biochemical reactions induced by the adjuvant, which led to the activation of the immune system. Despite current information about the structural and functional properties of these immunomodulators, they are often classified solely based on their physical properties, which are usually incidental. This issue is further complicated by the fact that mixtures of well-defined individual adjuvants are often regarded as novel adjuvants rather than formulations. This is significant because mixtures of adjuvants can exhibit synergistic, antagonistic, or additive effects.[4] Such outcomes can only be determined after a thorough evaluation of the immunological properties of the individual adjuvants and their combinations. The routine consideration of delivery and carrier systems, like liposomes, as new adjuvants add further confusion to the research of these immunopharmacologically active compounds. Due to the nature of immunological work, the search for new adjuvants has traditionally been based on trial and error. This strategy has led to the discovery of some new, safe and well-defined adjuvants, besides alum, such as monophosphoryl lipid A (MPL), CpG, and QS-21. MPL, for instance, resulted from screening lipid A derivatives with specific chemical modifications. In 1989, immunologist Charles A. Janeway expressed his frustrations with adjuvant research, referring to them as the "immunologist's dirty little secret".[5] However, he provided a novel framework for their research by postulating that adjuvants must deliver signals to the host's immune cells, inducing an immune response against an antigen. He correctly hypothesized that certain adjuvants exerted their immune-stimulatory effects by interacting with some unknown cell receptors, which he called pattern recognition receptors (PRRs), and that adjuvants were compounds often found in pathogens such as lipid A. This hypothesis provided a starting point for adjuvant research to move from an empirical to a more rational approach. Janeway's theories gained factual support in 1996 when Jules Hoffman reported that in *Drosophila* flies, the family of Toll genes played a major role in protection against fungal infection.[6] In 1997, Ruslan Medzhitov, from Janeway's laboratory, cloned and characterized the first human homolog of a Toll gene from *Drosophila*, Toll-like receptor 4 (TLR4).[6] In 1998, Bruce Beutler et al. showed that TLR4 was the receptor for lipid A, its natural ligand.[6] These discoveries concerning innate immunity's cell receptors and their ligands transformed adjuvant research into a multidisciplinary field, including immunology, medicinal chemistry, pharmacology, and biochemistry. Janeway's "dirty little secrets" revealed themselves as complex, well-defined compounds found in pathogens. The understanding that adjuvants are the agonistic ligands of some immune cell receptors, capable of inducing cellular signals, largely explains their immunological activities. Consequently, since the information concerning adjuvants shows that they are well-defined chemical compounds with specific cellular receptors, like TLRs, and immune pharmacological activities,[7,8] the notion of adjuvants should be limited to those molecular entities. This excludes adjuvant mixtures, alone or in delivery systems, which should be considered formulations. This change is necessary for the rational discovery of new adjuvants and the elucidation of their MOA at the biochemical level. An approach similar to pharmacognosy, which has delivered many useful drugs from natural sources and, combined with organic chemistry, provided the basis for medicinal chemistry should be applied.[8] An examination of the literature concerning adjuvant research, using several searchable databases, shows that it is evolving towards a more logical approach, where empirical observations are being connected to structural and functional information at the molecular level.

Adjuvant research: Synergism between immunology and medicinal chemistry

The adjuvant discovery was initially conducted largely by comparing antibody responses in animals against an antigen in the absence and presence of the compound under scrutiny. While this method is simple and hints at the stimulatory effects on adaptive immunity, it is limited to humoral immunity, also known as B-cell immunity. However, a sole antibody response or the IgG2a/IgG1 ratio in mice, where IgG2a expression is enhanced by the pro-inflammatory cytokine interferon-γ, cannot accurately indicate T-cell or cell-mediated immunity. Therefore, an unambiguous characterization of the adaptive immune response requires, besides antibody titers, the cytokine profiles, production of cytotoxic T lymphocytes (CTL), and T-cell phenotype profiles at a minimum.[9] Regrettably, it is common practice to use only antibody titers to propose dubious structure-activity relationships (SARs) that often do not agree with those attained by systematic immunological studies.[10] For instance, although the TLR4 ligand MPL elicits a Th1 inflammatory immunity with the corresponding cytokine profile, it does not induce CTL production.[11] This response, critical for defense against viral infections and cancer, can only be detected by specific methods.[12] Additionally, when designing animal studies, it is important to consider the possibility of animal models behaving differently from humans. For decades, alum was deemed a Th2 adjuvant in humans and mice. However, new studies show that in humans, alum elicits a pro-inflammatory immunity, which may explain its efficacy as an adjuvant in many infectious disease vaccines.[13] The fact that non-lipid A-like compounds, which are structurally different from lipid A, such as the opioids morphine and fentanyl, can bind to TLR4, triggering agonistic and antagonistic effects,[14] raises questions about the roles of TLR4 and other TLRs. This situation may be explained by the fact that TLR4 requires an accessory protein, myeloid differentiation factor 2 (MD-2), to respond.[14] The TLR4/MD-2 complex allows the binding of certain drugs of abuse, like opioids, triggering signals to the central nervous system. These non-lipid A-like compounds' hydrophobic regions, hydrogen bonding, ionic groups, and steric characteristics, *i.e.*, pharmacophore features, are serendipitously those needed to ensure binding to the MD-2 receptor site and induce conformational changes that stabilize the TLR4/MD-2 complex,[15] potentially producing neuro-immunological signals. Thus, considering the diversity of immune responses that some ligands can induce by interacting with their receptors, studies proposing adjuvants' SARs or MOA must be supported by explicit immunological data. While these studies may be less problematic for adjuvants with well-known receptors, like some TLRs, where the interactions with their ligands are usually known,[7,16] this is not the case for saponin adjuvants like QS-21 and GPI-0100.[17,18] These are important because QS-21 has enabled the development of effective vaccines for malaria, shingles, and tuberculosis thus far. Despite QS-21's many synthetic analogs with specific chemical modifications, there is still no reliable information about their SARs or MOA, likely due to their incomplete immunological characterization. This situation is aggravated by the fact that saponin adjuvants, like those from *Quillaja saponaria*, such as QS-7, QS-21, and their derivatives, are often classified as either particulate or surfactant adjuvants.[19] This mistake likely arises from the fact that these amphipathic glycosides form micelles: colloidal particles made by the aggregation of amphiphilic molecules in water. However, Q. saponin adjuvants frequently show their adjuvanticity as monomers, below their critical micellar concentrations, and not as micelles.[12] In fact, the first commercial subunit vaccine containing QS-21 for feline leukemia virus has QS-21 at concentrations below its critical micellar concentrations.[20] Thus,

there is no need to invoke nanoparticles to explain their adjuvanticity. That there are over 30,000 known saponins, all forming micelles, but only a few showing adjuvanticity, shows the liabilities of using physical rather than functional properties as factors for adjuvants' classification. The confusion is further compounded by the fact that QS-21, when mixed in liposomes with adjuvants as MPL, the resulting products are usually regarded as new adjuvants rather than delivery systems and/or formulations.[21] Indeed, the addition of MPL and/or QS-21 to the liposomes introduces two active adjuvants, resulting in a new "adjuvant formulation", rather than a new adjuvant. Additionally, due to the intercalation of QS-21 into the liposome carrier's lipid bilayer, the rate of deacylation process is decreased, resulting in a more stable formulation. Hence, the approach of considering "formulations" as new adjuvants may overlook the agonistic and antagonistic interactions among the various adjuvants and the liposomes' protective effects on these saponins' degradation in an aqueous milieu, which results from a new "formulation", rather than a new adjuvant.

Although Q. saponin adjuvants are structurally different from TLR ligands, they share the capacity to modulate immunity through still unclear MOA. However, it is evident that they act on T-cells by providing an alternative co-stimulatory signal and, depending on their acylation state, on the innate immune cell C-type lectin receptor "dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin" or dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN).[12] Thus, some of the strategies used to elucidate TLR ligands' immunomodulatory effects may be applied to investigating this new family of adjuvants. Indeed, Kensil *et al*.[22] showed that modifications of the Q. saponins' C4-aldehyde group caused a loss of their capacity to induce Th1 immunity, confirming reports that the aldehyde group delivers an alternative co-stimulatory signal to T-cells, needed to avert anergy.[23] This finding agrees with Bomford's observation that only those saponins with an aldehyde on their triterpene nucleus had adjuvanticity,[24] although later publications questioned this without providing reliable data concerning T-cell immunity.[10] The fact that these glycosides' proposed SARs, based on systematic chemical and immunological studies, are largely ignored may explain the lack of success in finding new saponin adjuvants and elucidating their MOA. In fact, common structures found in some adjuvants may reveal the functional groups responsible for adjuvanticity, as in the case of the aldehyde group.[22,25] The unknown cellular receptors for these glycosidic adjuvants indicate the need for deliberate strategies to identify them. Identification of the cell receptor target of the Q. saponins' aldehyde group may be attained using chemical proteomics with bioorthogonal ligation chemistry,[26] as shown in Figure 1. A strategy where the reactivity of the aldehyde group to form imines with the amino groups from an unknown receptor on cultured immune cells can be used. Selective stabilization of the imines by reductive amination with sodium cyanoborohydride will allow the formation of a stable conjugate made of the protein receptor covalently bound to the Q. saponin.[22] The Q. saponin, covalently tagged with a fluorochrome like a dansyl group, will allow the detection of the receptor-saponin conjugate separated by a method like polyacrylamide gel electrophoresis.[26] The results would be compared to those obtained with inactivated Q. saponins, where the aldehyde group has been reduced with sodium borohydride to an alcohol. Because many proteins can react with the aldehyde group, studies must be conducted under conditions designed to minimize reactivity with other proteins, like serum proteins. The receptor's nature will be established from its amino acid sequence after proteolysis and validated by using chemical or genetic knockouts of the putative receptor(s).[26] The intricacies of Q. saponins and related glycosides are highlighted by the fact that their capacity to elicit a Th1 pro-inflammatory or Th2 anti-inflammatory immunity depends on the acylation or de-acylation of their fucose residue,[27] respectively. This dependency indicates the crucial role of fucose in the adjuvanticity of these saponins.

Q. saponins' role in biasing T-cells' responses towards Th1 or Th2 immunity

The linkage of fucose to a Th2 anti-inflammatory immunity led to the identification of LNFP-III, an oligosaccharide that binds to DC-SIGN by its terminal fucose and acts as a sole Th2 adjuvant.[28] This finding was later applied to other oligosaccharides bearing fucose, a sugar that in animal-derived products mainly forms the terminal head group of sugar chains. Hence, it is well established that the binding of fucose to the lectin receptor DC-SIGN biases the response towards a sole Th2 immunity.[29] Consequently, it has been hypothesized that deacylated Q. saponins act as anti-inflammatory Th2 adjuvants because of their fucose residue,[30] which has the crucial C-3 and C-4 hydroxyl groups (Fig. 2) free and available to bind DC-SIGN by forming coordination bonds with this receptor's Ca^{2+} ion.[31] However, unlike in animals, plants' oligosaccharides can have fucose as an internal sugar residue, raising some concerns. Nevertheless, the DC-SIGN receptor's binding site has been shown to be highly promiscuous, accepting various sugars and yielding different immunological results.[31] Support for the notion that fucose in Q. saponins, such as QS-21 and closely related compounds, is responsible for their binding to DC-SIGN and its immunomodulatory properties is provided by several facts. Acetylation of the fucose's C-3 and C-4 hydroxyl groups, which interferes with the formation of the coordination bonds needed for binding to DC-SIGN, restores these saponins' capacity to elicit Th1 immunity with a concomitant loss of their ability to elicit a sole Th2 immunity.[32] This fact has been largely ignored. Paradoxically, despite many studies with synthetic Q. saponin analogs where fucose has been substituted by sugars like galactose,[33] it has never been determined what type of immunity these new derivatives will elicit if deacylated. This finding could confirm the proposed role of fucose in biasing the response toward an anti-inflammatory Th2 immunity, which is induced by several saponins carrying this sugar. Indeed, saponin analogs carrying galactose instead of fucose should be unable to induce a Th2 immunity, regardless of their acylation state. An unpredictable finding was that the changes in Q. saponins' adjuvanticity caused by deacylation can be modified by N-acylation of their glucuronic acid residue (Fig. 2), in a manner dependent on the length of the added alkyl chain.[34] In fact, N-acylation of deacylated Q. saponins with increasingly longer alkyl chains results in a shift of the immune response from Th2 to Th1, which reverses to a Th2 immunity with highly lipophilic alkyl chains. This shift depends on the balance between the hydrophilic groups (*i.e.*, sugar residues) and lipophilic groups (*i.e.*, triterpene nucleus and alkyl chain) of each saponin, a property known as the hydrophilic-lipophilic balance.[34] This immunity shift hints at the likely effects of these compounds' conformation and aggregation state on the accessibility to DC-SIGN of their fucose pharmacophore, presumably responsible for biasing the response to Th1 or Th2 immunity.[30] Thus, conclusive proof that these fucosylated glycosides bind to DC-SIGN may be attained by X-ray crystallography and solution nuclear magnetic resonance (NMR),[31,35] aided by molecular modeling.[26] Strategies combined with studies using *in vitro* cell cultures and *in vivo* animals will elucidate these adjuvants' MOA, *i.e.*, how they elicit either an in-

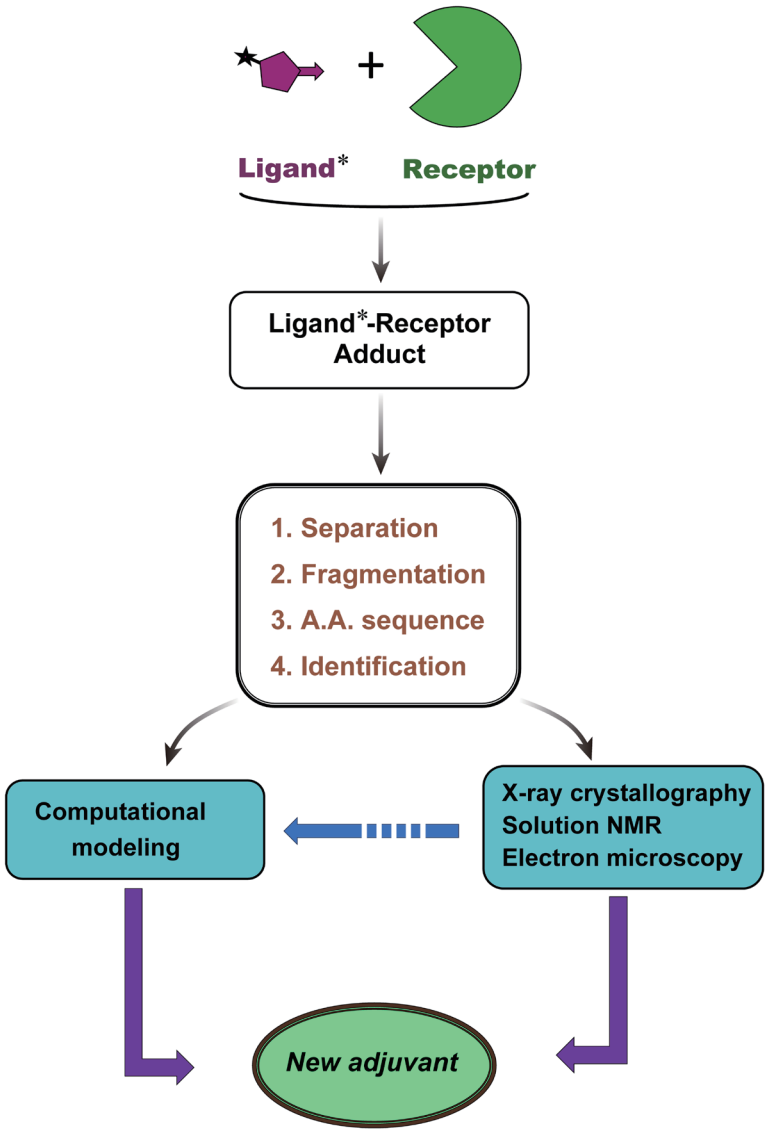

Fig. 1. Chemical proteomics approach to identify the receptor for a Q. saponin showing adjuvanticity. Adjuvants like Q. saponins deliver a co-stimulatory signal critical for the activation of T-cells and stimulation of pro-inflammatory Th1 immunity. This process, in the case of Q. saponins, certainly involves the formation of imines between the aldehyde group of these glycosides and an unknown receptor on T cells. Identification of that receptor may be achieved by exposing these cells to Q. saponins labeled with a fluorescent marker like dansyl (Ligand*), and selectively reducing the imine with Na cyanoborohydride to form a stable Ligand*-Receptor adduct. The fluorescence-labeled adduct, after cell fractionation, may be isolated by methods such as polyacrylamide gel electrophoresis. The recovered adduct will then be treated with proteases, and the peptides sequenced. This process will establish the amino acid sequence of the protein receptor and its identity. The amino acid sequence may also allow the prediction of its secondary and tertiary structure using computer modeling. This information, combined with the sequence for the peptide covalently linked to the glycoside (Ligand*), could pinpoint the binding site on the protein receptor. Direct application of computer modeling can provide a virtual image of the receptor-ligand complex and their interactions, which can be used to design new compounds (purple arrow). Identification and isolation of a receptor would allow the determination of its actual structure using X-ray crystallography and other techniques such as electron microscopy and solution nuclear magnetic resonance (NMR). This information may be used to design new derivatives (purple arrow) and optimize the computer modeling approach (blue arrow), facilitating the design of new saponin analogs with improved adjuvanticity.

flammatory immunity, Th1 and/or Th17 with CTL production or a sole anti-inflammatory Th2. These studies will also define if the adjuvants induce and maintain immune homeostasis,[36] which is a key function to prevent autoimmunity.

A problem hindering the development of sole anti-inflammatory Th2 adjuvants is that immunotolerance is frequently mistaken for damaging global immunosuppression.[37] Sole Th2 adjuvants elicit an anti-inflammatory immunity while inhibiting, but not abrogating, pro-inflammatory Th1 immunity,[28] thus averting a harmful pro-inflammatory immune response against self-antigens while sustaining immunotolerance.[38] In contrast, global immunosuppression completely blocks the immune system's capacity to produce both cell-mediated Th1 and humoral Th2 immunities against any antigen.[37] This total absence of immune response re-

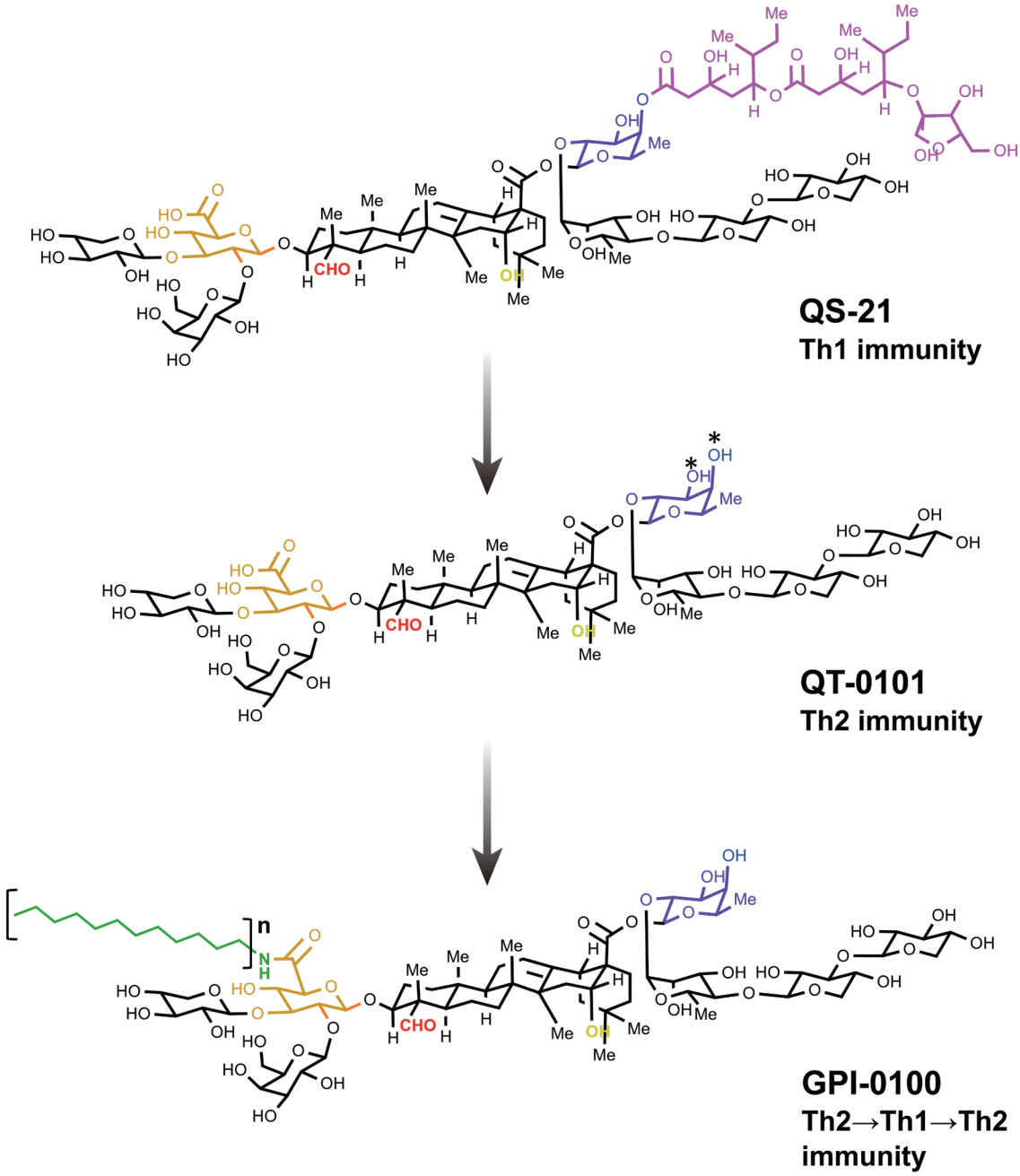

Fig. 2. Structural changes of Q. saponins linked to their immunological properties. The adjuvanticity of Q. saponins, like QS-21, depends on several chemical groups to induce pro-inflammatory Th1 immune responses. (a) Q. saponins have two groups critical for their Th1 adjuvanticity: the C4-aldehyde group (red) on the triterpene nucleus and the fatty acid side-chain with terminal arabinose (fuchsia) bound to the fucose residue (blue). Reduction of the aldehyde group to alcohol results in a loss of adjuvanticity due to the inability to deliver the co-stimulatory signal required for T-cell activation. The fatty acid side-chain bound to the fucose's 3 or 4-hydroxyl groups (*), most likely interferes with the binding of this sugar to the DC-SIGN receptor on dendritic cells (DCs), preventing polarization of DCs towards a Th2 phenotype. It has been claimed that for adjuvanticity, the C4-aldehyde group is irrelevant, while the C16-hydroxyl group (yellow) of the triterpene group is essential, an assumption that ignores the fact that the inactive reduced QS-21, while lacking the C4-aldehyde group, still has the C16-hydroxyl group. Hence, this hydroxyl is practically unnecessary for adjuvanticity. b) Removal of the fatty acid side-chain from the fucose's 3 or 4-hydroxyl groups (*) yields deacylated Q. saponins, QT-0101. This product stimulates solely Th2 anti-inflammatory immunity, likely because the deacylated fucose residue (blue) can bind to DC-SIGN and bias DCs towards a Th2 phenotype. This underscores the critical roles of the fucose's 3 and 4-hydroxyl groups (*) in determining the type of elicited immunity, *i.e.*, Th1 or Th2. c) N-acylation of the Q. saponins' single glucuronic acid residue (orange) by alkyl chains with n = 1 to 14 carbons (green) reverses the capacity of deacylated saponins to elicit Th2 to Th1 immunity; *i.e.*, Th2 → Th1 → Th2. This transition depends on the length of the alkyl chain, with analogs carrying larger alkyl chains reverting to inducing Th2 immunity. This transition is also dependent on the hydrophilic-lipophilic balance value of each saponin derivative. Changes in adjuvanticity are likely due to alterations in both conformational and associative properties of alkylated saponis. DC-SIGN, dendritic cell-specific ICAM-grabbing non-integrin.

sults in broad immunotolerance, with a concomitant increase in cancer and infectious diseases. While adjuvants commonly induce an antigen-specific immune response biased towards either Th1 or Th2 immunity, immunosuppressive agents completely turn off the immune system. This explains why Th2 adjuvants can be useful in safely eliciting an anti-inflammatory response against specific antigens, protecting the host from damaging Th1 pro-inflammatory immunity. This would be the case for vaccines to treat and/or prevent autoimmune conditions, where a sole Th2 immune response against a self-antigen will avert organ damage by inducing immunotolerance without immunosuppression.[37,38] This difference is obvious to transplantation immunologists but apparently not to others. Hence, it is fundamental to differentiate sole anti-inflammatory adjuvants from immunosuppressive drugs. The deep differences between Th1 and Th2 adjuvants are shown by the endless failures in the development of vaccines for Alzheimer's disease (AD). Despite strong support from incidental data, these vaccines have only delivered setbacks for 25 years,[39] likely because all the vaccines used pro-inflammatory adjuvants like QS-21 that induce a Th1 immune response against the antigen amyloid β, triggering a damaging autoimmune response. This was shown by the AN1792 vaccine's clinical studies, which were halted due to the development of meningoencephalitis in some vaccine recipients.[39] This situation is aggravated by the mistaken belief that sole anti-inflammatory Th2 adjuvants in AD vaccines will induce immunosuppression, encouraging the incorrect use of pro-inflammatory adjuvants in vaccines to prevent and/or treat proteopathies like AD to avoid the unfounded fear of immunosuppression. In contrast, vaccines for these conditions must necessarily use anti-inflammatory adjuvants to avert autoimmune responses.[38]

Comprehensive immunological studies, besides accelerating vaccine development, may speed up adjuvant discovery if their findings are correlated with the structures of these immune modulators. This would allow establishing their MOA and designing better adjuvants. Another important issue is the role of carriers like liposomes and nanoparticles, particularly when used with combinations of different adjuvants.[21] Pertinent questions are: Does the carrier alter the intrinsic pharmacological properties of an adjuvant? If so, how? This is relevant as the immunomodulatory properties of N-acylated Q. saponins change with the length of the added inert alkyl chain,[34] strongly indicating that conformational and associative changes affect the availability of certain pharmacophores to bind DC-SIGN and are responsible for variations in adjuvanticity. In the case of combinations of adjuvants, are the outcomes due to synergistic or antagonistic effects? Answers to these questions may allow better vaccine formulations.

Adjuvant discovery and high-throughput screening

The need for a rational approach to adjuvant design becomes essential when using high-throughput screening (HTS) to test large numbers of compounds for adjuvanticity. Since HTS is not amenable to animal testing as the initial step, it is replaced by testing in cultured immune cells, which can be automated. Due to the immune system's intricacies and the diverse responses elicited by different compounds, the chosen biomarkers must be broad enough to detect the immune responses elicited by mixed populations of immune cells, such as primary human peripheral blood mononuclear cells (PBMCs).[40] This strategy has shown that immune responses depend on the donor's age and has allowed the identification of differences between very young and older populations. When the 3D structure of a receptor (such as some TLRs or DC-SIGN) and their ligands are known from X-ray crystallography,[26] electron microscopy, and/or solution NMR, HTS can benefit from the use of computer modeling as the first step to identify potential better adjuvants.[41] This strategy, combined with medicinal chemistry, immunological, and biochemical methods, may deliver superior adjuvants, like some novel TLR7 and 8 agonist adjuvants. For instance, HTS has shown that the targeted lipidation of imidazoquinoline-based compounds can alter their immunomodulatory effects.[42] These changes are linked to their new physicochemical properties, which modify their availability, transfer across cell membranes, and binding properties, usually not anticipated from an adjuvant's SAR information. This situation is similar to the N-acylated saponins discussed earlier.[18,34] These physicochemical properties can be altered by carriers like liposomes or excipients like detergents. Subtle changes in the composition of liposomes can affect the reactogenicity of combinations of MPL and QS-21 and the stability of the Q. saponin.[43] These results emphasize the importance of vaccine formulation, which, while different from rational drug design, is complementary.

While the HTS process is promising for compounds like the TLR agonist ligands, it may not be as effective for adjuvants like Q. saponins and related compounds. The reasons are (i) the largely inadequate immunological data, based solely on limited antibody studies, used to propose broad but unproven SARs[44]; and (ii) the present information implies that these triterpene glycosides act separately but concomitantly on both DCs and T-cells.[12] This situation demands the use of PBMCs as target cells and confirmation of the results using cell lines of the different cells.[40] This strategy, when used with new analogs, could verify the nature of the cell targets, identify new immunomodulatory derivatives, and elucidate these immune modulatory glycosides' SARs. Indeed, like the changes reported for some lipidated imidazoquinoline-based compounds, where adjuvanticity is modified by their new physicochemical properties, some N-acylated derivatives of deacylated Q. saponins also show changes in adjuvanticity. These changes are likely due to alterations in their conformational and associative properties, which may affect the accessibility of pharmacophores relevant for adjuvanticity, an outcome detectable by animal studies. The use of molecular dynamics simulations plus other physicochemical methods may provide information concerning the micellar structure of these N-acylated derivatives.[45] This information may relate to the accessibility of the chemical groups responsible for these glycosides' adjuvanticity, allowing them to interact with their receptors. The fact that a mannosylated saponin analog carrying an aldehyde group induces a Th1 immunity,[46] while similar analogs lacking that active carbonyl group do not,[47] highlights this functional group's role in inducing pro-inflammatory immunity. This crucial co-stimulatory effect extends to other reactive carbonyl groups, like ketones. In saponins, the location of the carbonyl group capable of forming imines seems not to be critical, which could limit the use of computer modeling to predict new and better derivatives. Nonetheless, as in the case of the lipidated imidazoquinolines, carefully designed HTS protocols could assist in the identification of Q. saponin derivatives.

Rational approach to saponin adjuvants' development

From the available information, it is evident that adjuvants must be considered a new family of drugs with unique immunopharmacological properties that depend on specific functional groups and pharmacophores. This conclusion, although evident, is commonly ignored, with exceptions like some TLR agonistic ligands.

The term "adjuvant" includes both well-defined and poorly-defined compounds,[19,31] complicating research in this field. Indeed, until recently, adjuvant research has been phenomenological rather than mechanistic. While the effects on different immune cells and their responses were known, the mechanisms by which these effects occurred were not understood; this is now changing. Some adjuvants, like oil-water emulsions, can elicit effective pro-inflammatory immune responses without immune agonists, implying that their effects are due to physical changes in cells' membranes and organelles. These effects are presumably caused by micelles, which trigger the release of signals, like dangerous ones, that activate a pro-inflammatory response. This effect is shared by other systems like nanoparticles, also known as nano-adjuvants.[48] These systems owe their adjuvanticity to their physical properties, like dimensions, rather than their chemical structure, and are grouped as nano-adjuvants. Thus, it would be advisable to consider these systems, where immune modulation is physically driven, separately from those where effects are triggered by receptor-agonist interactions, *i.e.*, biochemically driven. Although these two systems are mechanistically different, they show cooperative effects, where the pharmacological effects of agonistic ligands are magnified when contained in nanoparticles. These results highlight the importance of formulation in optimizing vaccine formulations, an area that would benefit from knowledge gathered independently for both systems.

Many investigators wrongly believe that saponin adjuvants, like QS-21, owe their adjuvanticity to their particulate nature. This notion ignores the SARs proposed based on the results from chemical modifications of QS-21 and similar saponins, and their effects on T-cell immunity.[12,22] This is evidenced by QS-21's induction of the pro-inflammatory Th1 immunity, characterized by the presence of effector cells Tc CD8 cytotoxic and Th1 CD4 inflammatory T cells. This immune response is always followed by Th2 immunity, considered a reparative mechanism.[49] Complicating this is the evidence that QS-21 acts independently on both T-cells and dendritic cells (DCs), a unique situation for an adjuvant class that exerts its immunological activities on both innate and adaptive immunities.[12] Systematic studies using targeted chemical modifications of Q. saponins conclusively show that their aldehyde group is responsible for activating T-cell immunity.[12,22] Previous drug research has shown that the aldehyde group is crucial for T-cell co-stimulation and preventing anergy.[22,50] An apparent misconception equates the delivery of a T-cell co-stimulatory signal by Q. saponins' aldehyde group to play a role in antigen cross-presentation, which is actually a result of other moieties present in these glycosides.[25] Additionally, various active carbonyl groups, like aldehydes, ketones, and o-quinones, can deliver the co-stimulatory signal, which may explain why some saponins lacking an aldehyde group can still stimulate CTL production.[25] The confusion regarding Q. saponins' aldehyde group is exacerbated by reports suggesting that for immunostimulatory purposes, the triterpene C4-aldehyde group is irrelevant, while the C16-hydroxyl group is important (Fig. 2).[51] This conclusion overlooks the fact that the immunologically inactive reduced QS-21, while lacking the C-4 aldehyde group, still has the C16-hydroxyl group.[12] Thus, there is a need to identify the receptor targeted by the aldehyde and other active carbonyl groups. This can be achieved using bioorthogonal reactions, similar to those used in TLR research. The aldehyde group's ability to readily form imines, which can be stabilized by reductive amination, supports this method. However, this may not apply in situations where the reaction product of a ligand's functional group and its receptor is unstable or short-lived. The receptor identified by bioorthogonal ligation can be confirmed using knockout animal models. In T-cell activation, once the naïve cell receives the co-stimulatory signal to start the activation process, additional co-stimulation will not hasten, reverse, or stop that process. This explains the failure of synthetic saponin analogs with more than one aldehyde group to show additive effects,[51] a failure misinterpreted as an indication that the aldehyde group is irrelevant for T-cell activation.[10,51]

The other proposed pharmacophore, supposedly critical for eliciting an anti-inflammatory Th2 immunity, is the fucose residue, as hypothesized by Marciani.[30] This hypothesis might be confirmed using synthetic QS-21 analogs, where fucose is substituted by mannose. Such a change should result in the induction of pro-inflammatory immunity, as binding mannose to DC-SIGN should bias the DC towards a Th1 phenotype.[52] Since the 3D structure of DC-SIGN, the fucose receptor on DCs, is known,[31,52] computer modeling may indicate if internal fucose could bind to this receptor.[41] Indeed, this calcium-dependent lectin receptor shows glycan-binding promiscuity, yielding different immunological responses depending on the nature of the bound sugar.[53] Experimental support for the hypothesis that fucose plays a role in biasing DCs toward a Th2 phenotype was provided by Wang *et al*.[54,55] They confirmed that acetylation of the fucose's hydroxyl groups 3 and/or 4 abrogated the capacity of deacylated Q. saponins to elicit a sole Th2 immunity.[30] This is due to the acetylated fucose's inability to form coordination bonds with the Ca^{2+} ion from DC-SIGN.[12] Yet, the current prevalent belief is that in Q. saponins and closely related compounds, the fucose residue is just part of a scaffold, with unknown chemical groups responsible for the immunomodulatory properties.[30,33,44] However, as discussed earlier, Q. saponin synthetic analogs with a sugar different from fucose should elicit a Th1 immunity, regardless of their binding or non-binding to DC-SIGN, as long as the compound has a co-stimulatory aldehyde group. In fact, acylation of the fucose in Q. saponins is unrelated to their unforeseen immunomodulatory properties. Yet, serendipitously, this sugar residue plays a pivotal role in Q. saponins biasing the response to a Th1 or Th2 immunity. Based on the available data,[12,27,30] it is possible to expect that many synthetic Q. saponin analogs lacking fucose should elicit a Th1 immune response,[30,33,44] regardless of being acylated or non-acylated. A study on this matter has not been done, likely due to the mistaken notion that fucose is merely a component of a molecular scaffold rather than an active pharmacophore. Studies must have a thorough immune evaluation of the different analogs, not just antibody titers, to be meaningful. In drug discovery, a compound binding its cell receptor triggers a signal initiating an intracellular cascade of biochemical reactions that affect specific physiological functions.[56] Unlike other cells, immune cells can respond to various signals acting simultaneously, establishing cellular interactions with different types of cells.[57] This complex situation can be somewhat reproduced using PBMCs for initial studies of these compounds.[40] Given the intricacies of both the immune system and adjuvants like QS-21, the only acceptable computational method would be structure-based, requiring structural information about the ligand and its receptor.[58] This process will entail identifying the cell receptor, as indicated earlier. It is important to stress that, unlike TLR ligands and their receptors, products of millions of years of natural selection, saponins' adjuvanticity results from serendipity, as plants do not have an immune system or are animal pathogens. Therefore, detailed structural information for both components, the glycoside, and its cell receptor, is essential to benefit from these computational methods. Since Q. saponins and their derivatives act on different types of immune cells, there is also a need for advanced computational models, such as mechanistic computational models.[59] These meth-

ods will benefit from information obtained at the cellular, immunological, and biochemical levels. Hence, for complex adjuvants like Q. saponins, proposing SARs and MOA based on random structural changes without known receptors may not be too helpful. The reason for focusing on the presumed Q. saponins' pharmacophores, the reactive carbonyl group, *i.e.*, the aldehyde and the fucose residue, is that their deliberate chemical modification changed their adjuvanticity in radical ways, showing their potential key role.

Based on the available information, it is evident that explaining the MOA of complex adjuvants like Q. saponins requires identifying the cell receptors that interact with these glycosides' active groups. Once those receptors and their pharmacophores are confirmed, computational methods may establish the interactions of these glycosides with their receptors from different immune cells and optimize their adjuvanticity.[58,60] Therefore, elucidating the MOA of compounds like QS-21, GPI-0100, and QT-0101 should follow a rational methodology, combining different scientific areas. This strategy's benefits may extend beyond adjuvants since these glycosides' immune modulatory properties may allow them to act as drugs themselves, besides being vaccine adjuvants. For autoimmune conditions, inducing targeted anti-inflammatory immunity while maintaining immune homeostasis may prevent a damaging pro-inflammatory response against some organs.[61]

Future directions

From the above discussion, it is possible to conclude that a basic step in the rational design of better adjuvants is the identification of the cellular receptors for their corresponding pharmacophores. This proposition, in the case of Q. saponins, offers unique challenges since they act on both innate and acquired immunities, an uncommon situation. Therefore, research should initially focus on those pharmacophore candidates with the most evidence, *i.e.*, the aldehyde group and the fucose residue. As explained before, the Q. saponins' aldehyde group is just one example of a reactive carbonyl group capable of forming an imine upon reaction with a primary amine. The use of bioorthogonal chemistry to identify the aldehyde group's receptor(s) (Fig. 1) should be complemented with studies using genetically modified animal models, where the receptor candidate has been either knocked out or its amino acid sequence modified to alter the potential binding site in the protein receptor. Information on the 3D structure of the receptor may allow for the design of better aldehyde ligands. Elucidating the DC-SIGN interactions with the Q. saponins' fucose will require X-ray crystallographic studies. This endeavor is facilitated by the commercial availability of the recombinant protein receptor, which may also be used for solution NMR. It would be of interest to assess the effects of these saponins' oligosaccharides on the binding of their fucose residue to the lectin receptor DC-SIGN, a process that may be followed by solution NMR. This information may be correlated with the immunological effects of the different analogs to identify the factors responsible for adjuvanticity, establish accurate SARs, and potentially design better adjuvants. Finally, a new area of adjuvant research is the modulation of the physical properties of certain ligands to alter the immunological properties of certain adjuvants, as in the case of mannans.[62]

Conclusions

New vaccine adjuvants or immunomodulators are well-defined chemical structures that bind to specific cellular receptors, triggering a cascade of biochemical reactions that lead to a series of cellular events culminating in an immunological response. Their binding to a receptor depends on the spatial or 3-D distribution of aggregates of ionic groups, hydrophobic regions, and hydrogen acceptors and donors in the immune agonist—a collection known as the pharmacophore. However, the elicited immune response of an agonist is generally influenced by its overall chemical structure. Typically, adjuvants interact with a single cellular receptor, with the exception of Q. saponins and their derivatives. Glycosides like QS-21 can interact separately with T cells and dendritic cells. T cells are co-stimulated by the glycoside's aldehyde, while dendritic cells are activated by interactions with the triterpene group and fucosyl residue, respectively. Nonetheless, there is evidence that the immunological behavior of some immune agonists can be modified by their conformational and associative properties, emphasizing the need for comprehensive immunological evaluations to detect changes in their immunomodulatory properties. Application of bioorthogonal ligation chemistry to study adjuvants like Q. saponins should allow the identification of the T cell receptor involved in interactions with these glycosides' aldehyde group. Methods such as X-ray crystallography and solution NMR will be required to study the interactions of the fucosyl group with DC-SIGN. This information, combined with molecular modeling, can establish or confirm accurate SARs. For adjuvants with large structures and distinctive conformational and associative properties, physicochemical methods like molecular dynamics simulation may provide insights into the accessibility of chemical groups responsible for these compounds' adjuvanticity and their interactions with receptors, helping to explain unexpected immunological results.

Therefore, the application of chemical proteomics, bioorthogonal ligation chemistry, molecular modeling, and systematic immunological studies should enable the design of novel, safer, and more effective agonists with immune modulatory properties. Modification of their physicochemical properties could also facilitate the creation of compounds that better penetrate biological barriers such as the blood-brain barrier. Ultimately, accurate information about various adjuvants will facilitate the design of new compounds with immune modulatory properties, including both pro- and anti-inflammatory agents, potentially beneficial for treating conditions like autoimmunity.

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Conflict of interest

The author serves as Chief Scientific Officer of Qantu Therapeutics, Inc., a company he co-founded that focuses on developing new adjuvants. He holds equity in the company. Additionally, he has issued and filed patent applications covering the uses of saponins and derivatives as vaccine adjuvants.

Author contributions

DJM is the sole author of the manuscript.

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