# Human in vitro modeling characterizes age-specific mechanism of action of vaccine adjuvant formulations for global open access

# Human *in vitro* modeling characterizes age-specific mechanism of action of vaccine adjuvant formulations for global open access

by

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A dissertation submitted to the faculty of Harvard Medical School

in partial fulfillment of the requirements for the degree of

Master of Medical Sciences in Clinical Investigation

(Translational Investigation track)

Harvard University

Boston, Massachusetts

April 2023

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## Background

Vaccination remains one of the most effective public health strategies to prevent pandemics and epidemics. Successful immunization campaigns have resulted in the eradication of smallpox and significant reduction in the burden of several vaccine-preventable diseases including polio, influenza, hepatitis B, and rotavirus. Despite the progress made, existing barriers hinder translation of advances in vaccinology to global health.

A key factor that hampers development of low-cost vaccines is the restricted access of adjuvant formulations for research due to protection by intellectual property (IP) laws. Though IP rights are critical for advancing discovery and development, patented molecules limit the understanding of their mechanism of action in age-specific settings and their application among vulnerable populations.

Open access adjuvant formulations have a promising role in bridging this gap. Better understanding of mechanistic action may inform development of safe and effective vaccines that are age-specific and tailored to vulnerable populations may have a significant public health impact, particularly in the face of unprecedented events.

**Immune ontogeny and the role of cell-mediated immunity in inducing responses to vaccination:**

Dendritic cells (DCs) activate T cell immune response by functioning as antigen-presenting cells (APCs). DCs identify pathogens via pattern recognition receptors and present the peptide antigens on major histocompatibility complex (MHC) molecules that are detected by T cell receptors (TCRs) expressed on T lymphocytes. TCR diversity provides antigen specificity to T lymphocytes. DCs represent the primary APCs capable of promoting antigen cross-presentation *in vivo* (1).

Naive T cells undergo priming and clonal expansion upon interaction with DCs and the MHC-peptide complex. Intracellular antigens are cleaved into peptides in the DC cytosol/proteasome that get presented on MHC class I molecules, which triggers the activation of CD8 or cytotoxic T cells that kill pathogen-infected host cells by producing lytic proteins (2–4). Extracellular antigens entering the endocytic pathway in the DC are processed and presented by MHC class II molecules that activate CD4 or T helper (Th) cells to eliminate pathogens by secreting cytokines and chemokines that enable recruitment and activation of phagocytic leukocytes such as neutrophils, monocytes, and macrophages (4,5). Additionally, Th lymphocytes are responsible for activating B lymphocytes that proliferate and differentiate into memory B cells and plasma cells which secrete antibodies to neutralize pathogens (5).

Priming of T cells by DCs includes instructions provided by the DC that determine the functionality and phenotype of the T cell. Antigen presentation on MHC is often referred to as the first signal. The second signal refers to the expression of co-stimulatory

receptors such as CD40, CD80, CD83, and CD86 to form an immunological synapse. The third signal is provided by cytokine secretion following this DC-T cell interaction, which determines the fate of Th cell differentiation and resulting immune response type. Dominant transcription factors regulate the expression of these cytokines – T-bet, GATA3, and ROR$\gamma$t drive polarization to Th1, Th2, and Th17 immunity respectively (6–8). Each Th cell subset actively suppresses the expression of transcription factors and production of cytokines by other subsets (9). IL-12 is the principal cytokine responsible for Th1 polarization, IL-4 promotes a Th2 response, and IL-6 and IL-23 drive the response towards Th17 immunity (10,11).

Ontogenetic differences that distinguish immunity in early life from that in adulthood are also influenced by these mechanisms. Factors that contribute to increased susceptibility to infections and poor vaccine responsiveness among infants include an impaired ability to secrete Th1-polarizing cytokines such as IL-12p70, TNF$\alpha$ and IFN$\gamma$, and increased production of the anti-inflammatory cytokine, IL-10 (12–20). Decreased IFN$\gamma$ secretion contributes to increased susceptibility to infections caused by intracellular bacteria, viruses, and protozoa commonly seen among young children. This results from the impaired mechanisms critical for antigen presentation, expression of MHC class I and class II molecules, and B-cell isotype switching (21,22). Promotion of antigen cross-presentation on MHC-I and the induction of antigen-specific $CD8^+$ T cell response remains a challenge in pediatric vaccine design.

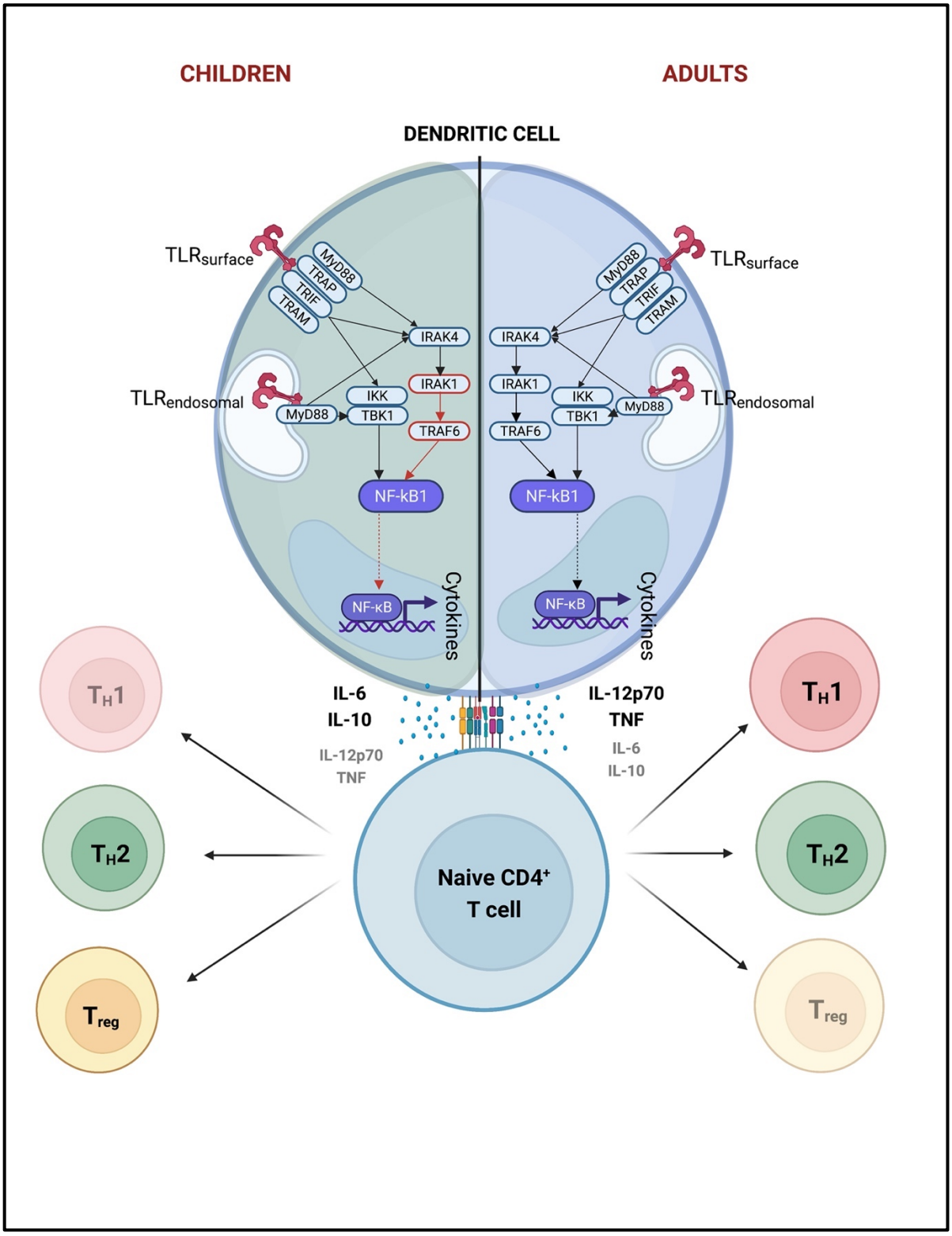


***Figure 1: Age-specific differences in immune responses***

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Primed CD4 T helper (Th) cells differentiate into phenotypically distinct effector cells – Th1, Th2, and Th17 cells – in response to cytokine- and signal transducer and activator of transcription (STAT)-mediated signaling (9,23). Th1 cells secrete IFN$\gamma$, tumor necrosis factor (TNF) and IL-2 to activate macrophages and DCs that kill intracellular bacteria (such as *Mycobacterium tuberculosis*, *Salmonella enterica*), viruses, and protozoa (6,23,24). Th2 cells produce IL-4, IL-5 and IL-13, and play a role in allergic inflammatory diseases (7,25). Th17 cells produce IL-17 and IL-22, and act against extracellular bacteria (such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Corynebacterium diphtheriae*, *Clostridium tetani*) (26–28). T regulatory cells (Tregs) prevent autoimmunity and play an important role in acute and chronic viral infections (29–32).

Upon activation by professional APCs, naive CD8 T cells differentiate into cytotoxic T lymphocytes (CTLs) which are primarily responsible for killing infected host cells (3). This lytic mechanism is activated by MHC class I-dependent antigen presentation and is critical in defense against viral infections as lysis of infected cells inhibits progression of viral replication in the host (2). Antigen-specific CD8 T cells produce IFN$\gamma$ and TNF that activate macrophage- and neutrophil-mediated destruction of microbes.

The hallmark of adaptive T cell immunity is the presence of antigen-specific memory T cells (33). Memory CD4 and CD8 T cells proliferate and survive in response to IL-7 and IL-15 (34–36). Levels of anti-viral CD8 memory T cells remain constant over time, however, that of CD4 memory T cells show a decline (37).

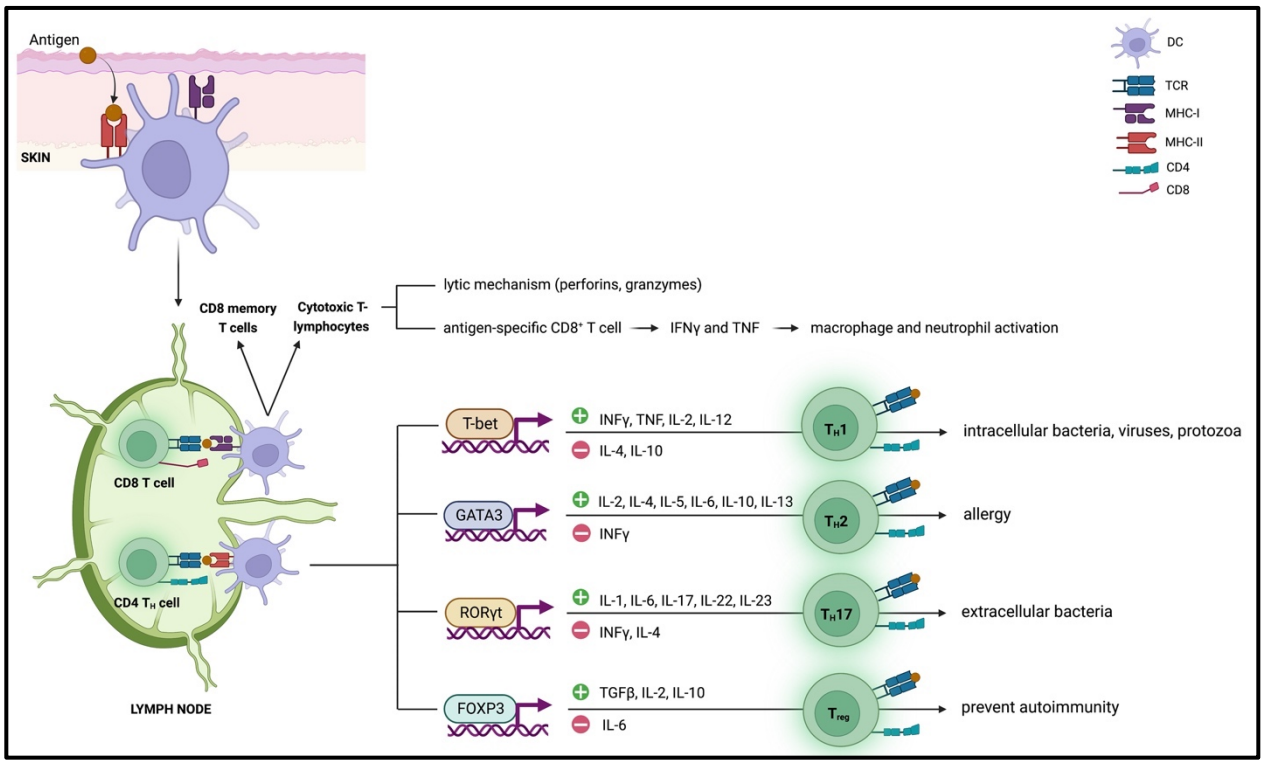


***Figure 2: Events underlying T cell immune response***

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**Developments in vaccinology:**

Since the introduction of the world's first successful vaccine against smallpox by Edward Jenner in 1796, advances in the field of vaccinology contributed to the roll-out of vaccines against SARS-CoV-2 in record time during the COVID-19 pandemic (38). In addition to the messenger RNA (mRNA) technology applied in the development of COVID-19 vaccines, several vaccine types including live-attenuated, inactivated, subunit, recombinant, polysaccharide, conjugate, toxoid, and viral vector vaccines have evolved over the years that overcome limitations of individual types and offer protection against a wide array of pathogens (39–44).

Live attenuated vaccines contain the whole disease-causing pathogen in a weakened form to induce an immune response without causing disease. The resulting immunity is strong and long-lasting due to the combination of adjuvants with the antigen but may not be effective or safe in immunocompromised individuals (45). Examples include measles, mumps, rubella (MMR), varicella, rotavirus, influenza (intranasal), Bacille Calmette-Guérin (BCG), and typhoid (Ty21a) vaccines.

Inactivated vaccines also contain the whole pathogen but are either killed or altered to inhibit replication. Though this approach provides safety in immunocompromised hosts, the immune response generated is not robust or long-lasting – requiring multiple booster doses following priming. Examples include polio, hepatitis A, rabies, and pertussis vaccines. This limitation could be addressed with the addition of adjuvants to stimulate a strong immune response (45).

Subunit vaccines are inactivated and do not contain the whole pathogen. Instead, they consist of a viral or bacterial surface protein that serves as the antigen – eliciting an antigen-specific immune response. Antigens in subunit vaccines could include a protein or a polysaccharide. Though this approach has resulted in the development of safe vaccines, poor immunogenicity affects their efficacy (46,47). With the addition of adjuvants, they are able to induce a strong and long-lasting immunity – improving overall efficacy. Examples include influenza, pneumococcal, hepatitis B, tetanus, and diphtheria vaccines (48). Cross-presentation of the antigen on MHC-I is critical for inducing a cytotoxic $CD8^+$ T cell response. This concept needs consideration while designing subunit vaccines targeted against viruses and intracellular bacteria as the goal is to enhance synergistic action and co-delivery of the exogenous protein antigen and adjuvant to the cytosol of dendritic cells to augment antigen cross-presentation, achieved via bypassing endosomal trafficking.

Recombinant vaccines are a form of inactivated vaccines manufactured by combining DNA from the target pathogen with cells derived from bacteria or yeast to purify the antigenic surface protein. Examples include hepatitis B, human papillomavirus (HPV), and influenza (*Flublok*). They frequently require multiple doses to induce protection, a limitation that could potentially be addressed with the use of adjuvants.

Conjugate vaccines are a form of subunit vaccines developed by combining polysaccharides and proteins to induce an immune response. Their limited ability to stimulate a robust immune response may be overcome with adjuvantation. Examples include *Haemophilus influenzae* type b and pneumococcal conjugate vaccines.

Toxoid vaccines are developed using inactivated bacterial toxins that function like an antigen and trigger a strong immune response. Examples include diphtheria and tetanus toxoid vaccines.

Viral vectored vaccines provide genetic instructions to the host cell via a harmless viral vector to produce protein antigens and induce the desired immune response.

Nucleic acid vaccines also provide genetic instructions necessary for host cells to produce an immune response. RNA vaccines consist of mRNA covered by a lipid membrane that aids cell entry. Inside the host cell, mRNA is translated to the antigenic protein against which immunity is desired. DNA vaccines are more stable compared to mRNA, but on introduction into the host cell, they must first be converted to mRNA which subsequently functions like a RNA vaccine.

***Table 1: Types of vaccines***

| Platform | Component | Features |
|---|---|---|
| Live attenuated | Whole virus/bacteria | ● Reduced pathogenicity with retained immunogenicity<br>● Low safety<br>● Poor stability |
| Inactivated | Inactivated whole virus/bacteria | ● Poor immunogenicity<br>● Multiple booster doses required |
| Subunit | Antigen-specific protein/peptide | ● Improved purity, safety, stability, scalability<br>● Suitable for immunocompromised individuals |
| DNA | Antigen encoded by recombinant plasmid | ● Cheap, stable, safe<br>● Risk of barrier to delivery of DNA to nucleus and insertional mutagenesis |
| mRNA | Protein antigen encoded by mRNA and encapsulated by vector | ● Does not enter nucleus/genome<br>● Intrinsic immune activation effect<br>● Low preservation stability |
| Viral vector | Engineered virus with attenuated gene encoding antigen (example, adenovirus) | ● Intrinsic immune activation effect<br>● Pre-existing immunity to adenovirus in target population |

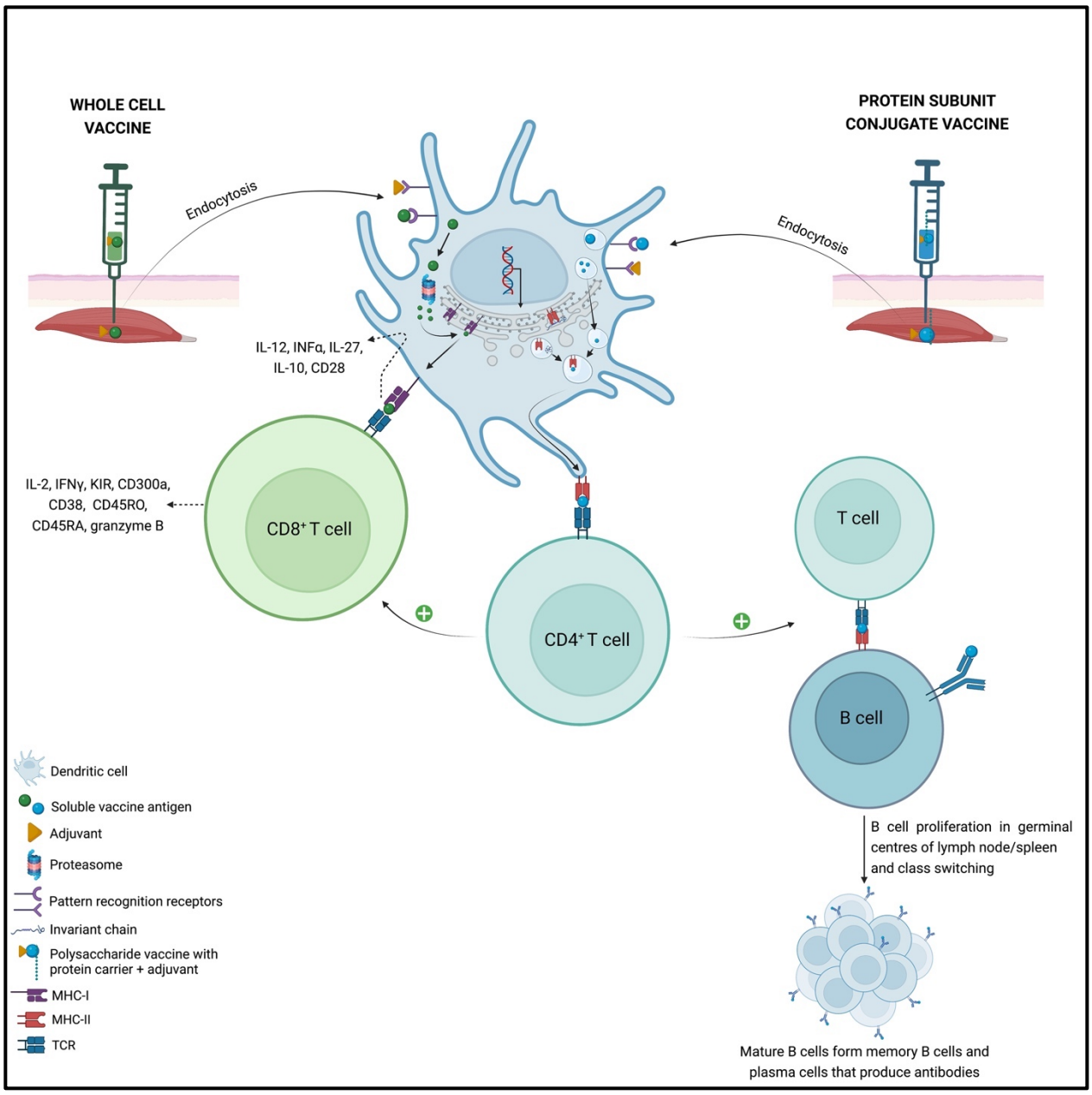


***Figure 3: Immune responses to vaccination*** *(Created with BioRender.com)*

**The evolving scope of adjuvanted vaccines:**

An adjuvant is an ingredient used in vaccine development that enhances magnitude and durability of immunogenicity. Derived from the Latin word *adjuvare* which means 'to help', adjuvants provoke a strong immune response, for instance by contributing to and amplifying the second/co-stimulatory/danger signal that is required in addition to the primary signal provided by the foreign pathogen bound to the MHC molecule to trigger an immune response. Antigen by itself in a vaccine is often incapable of inducing the desired magnitude of immune response; when combined with adjuvants, it succeeds in achieving its immunogenic potential. The interplay between the innate and adaptive immune systems is evident during this process as surveilling innate immune cells send the primary and second signals upon detection of pathogen-associated molecular patterns to activate T lymphocytes in the adaptive wing of immunity. Limitation to immunogenicity of a range of vaccines including live attenuated, inactivated, subunit, recombinant, and conjugate vaccines can be addressed by the addition of adjuvants.

An ideal adjuvant is safe, well tolerated, biodegradable, and stably formulated to enhance a vaccine's immunogenecity and scalability (45). In-depth analysis of risks and benefits of adjuvant formulations will inform their application in healthy individuals in addition to vulnerable groups such as children or those with weak immune systems.

Based on their action, adjuvants can be classified into two groups – 'immune potentiators' that stimulate an immune response by activating immune cells, and 'delivery systems' that promote the uptake of antigens into immune cells (49). Types of immune potentiators include agonists of pathogen recognition receptors (PRRs) such as Toll-like

receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs) that are critical components of innate immunity and are present on APCs (DCs and macrophages) (50,51). These receptors are stimulated via pathogen-associated molecules that serve as their natural ligands such as unmethylated CpG DNA, lipopolysaccharide (LPS), and double-stranded RNA. Activation of PRRs induces upregulation of co-stimulatory receptors, CD80 and CD86, and promotes the production of pro-inflammatory cytokines, IFN$\gamma$, TNF$\alpha$ and IL-6 (50).

Based on their location in APCs, TLRs are of two types – intracellular and extracellular. TLRs 1, 2, 4, 5 and 6 are expressed extracellularly on the cell membrane and recognize microbial elements. TLRs 3, 7, 8 and 9 are expressed intracellularly and function as nucleic acid sensors (52). TLR agonists used as adjuvants in vaccine development include monophosphoryl lipid A (MPL) which is a TLR4 agonist, CpG oligonucleotides which are TLR9 agonists, and resiquimod (a small molecule imidazoquinoline) which is a TLR7 agonist (49,53,54). They function by activating signaling pathways which stimulate innate immunity (55).

While each adjuvant type has a distinct role in immunostimulation, combination adjuvantation systems may be beneficial to vaccine development. For example, combining a TLR agonist with an appropriate delivery system such as alum ensures co-delivery of the antigen and the TLR agonist to the same immune cell, which may enhance potency and efficacy. This approach also reduces non-specific activation of innate immunity, poor tolerability, and potential adverse effects.

Adjuvants approved for use in humans include alum-based adjuvants, emulsion-based adjuvants like MF59 (*Novartis*), AS-03 (*Prepandrix/Arepandrix, GSK*) and AF-03

(*Humenza, Sanofi*), liposomes, microparticles such as poly-lactide-co-glycolide (PLG), particulate systems, and combination adjuvant systems such as AS-04 (*Cervarix, GSK*) consisting of aluminum hydroxide and MPL (45,56).

Alum-based adjuvants are extensively studied and utilized in several commercial vaccines such as hepatitis B, diphtheria, pertussis and tetanus as their safety and potency are well established (57). Alum used in vaccine formulations could be one of three types of salts – aluminum hydroxide, aluminum phosphate, or potassium aluminum sulfate. Studies are necessary to establish similar safety, efficacy, and tolerability as alum in other adjuvant formulations with potential use in human vaccines to improve the breadth of vaccinology.

Emulsions are liquid droplets dispersed in an immiscible liquid. Water-in-oil emulsions are aqueous droplets within oils, and oil-in-water emulsions are oil droplets within water (45). Squalene oil is a commonly used component in several licensed oil-in-water adjuvanted vaccines such as *Fluad* (MF59-adjuvanted seasonal influenza), *Aflunov* (MF59-adjuvanted pre-pandemic influenza), *Focetrea* (MF59-adjuvanted pandemic influenza), *Prepandrix* (AS-03-adjuvanted pre-pandemic influenza), and *Pandremix* (AS-03-adjuvanted pandemic influenza) as it is completely biodegradable.

A liposomal delivery system localizes the immune potentiator via encapsulation in the liposomal core or the lipid membrane, promoting antigen uptake by immune cells and resulting in improved immunogenicity (45). Liposomes are effective for adjuvants such as MPL and CAF01, and AS-01 (*GSK*) remains the most advanced liposomal adjuvant formulation consisting of MPL (lipid-like TLR agonist) and QS-21 (amphiphilic molecule that interacts with cholesterol in the liposomal formulation) (58).

Saponins such as QS-21 activate the intracellular sensor, NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3), that identifies microbial motifs and danger signals to form and activate the NLRP3 inflammasome. This induces production of pro-inflammatory cytokines, IL-1$\beta$ and IL-18 (59). Additionally, saponins lead to lysosomal disruption, enhancing the cytosolic pathway for antigen cross-presentation (60).

Effectiveness of a vaccine is highly dependent on the degree of danger signals produced. Quantitative and qualitative characterization of adjuvant formulations in pre-clinical studies would assist development of safe and effective vaccines by establishing reliability and reproducibility. Measurement of parameters such as particle size, size distribution, and antigen integrity and distribution would ensure transparency of ingredients that constitute vaccine formulations.

**Utilizing human *in vitro* modeling to dissect the mechanism of action of adjuvant formulations:**

In-depth investigation of mechanisms underlying immune responses via *in vitro* modeling enable application of precision medicine approaches to vaccine development. Use of distinct and complementary models assist in characterizing the cellular and molecular functioning of immune cells that inform the ability of vaccine adjuvants to activate innate and adaptive immune cells including DCs and antigen-specific T lymphocytes. As compared to conventional animal immunization studies, human *in vitro* modeling studies can provide crucial information regarding species- or age-specific immunity in addition to

mechanistic insights that inform the rational design of next-generation adjuvants. Isolation of human peripheral blood mononuclear cells (PBMCs) provides the basis for studying responses to vaccine adjuvantation in *in vitro* platforms, results of which inform the optimal dosage and formulation required for inducing the desired immune response through immunization. This approach also allows modeling the immune systems of different populations such as neonates, infants, older adults, immunocompromised individuals and pregnant women, enabling characterization of age- or condition-associated differences in vaccine responsiveness. The use of autologous plasma, a rich source of age-specific immunomodulatory factors, in human *in vitro* platforms enables the study of soluble factors that play a critical role in defining age-specific differences (17). Activity of adjuvant formulations in *in vitro* platforms that are human and age-specific could predict their efficacy and safety *in vivo* as these models allow evaluation of potency and reactogenicity.

Four human *in vitro* platforms were designed to guide the mechanistic studies of the tested adjuvant formulations. The whole blood assay (WBA) identifies the innate immune cell types activated following adjuvant stimulation, in addition to providing insight into the potency and reactogenicity potential of these formulations. The human tissue construct (HTC) model determines the fate of the activated innate immune cells through an unbiased strategy utilized to distinguish cell clusters. With monocytes being the primarily activated cell type in WBA that differentiate into DCs in HTC upon stimulation, the utilization of the monocyte-derived dendritic cell assay informs the mechanism of Th polarization and type of immunity induced by the action of adjuvants. The DC-T cell interface assay enables DCs and T cells to be co-cultured – determining the ability of

adjuvants to enhance antigen cross-presentation and reactivate antigen-specific $CD4^+$ and $CD8^+$ T cells.

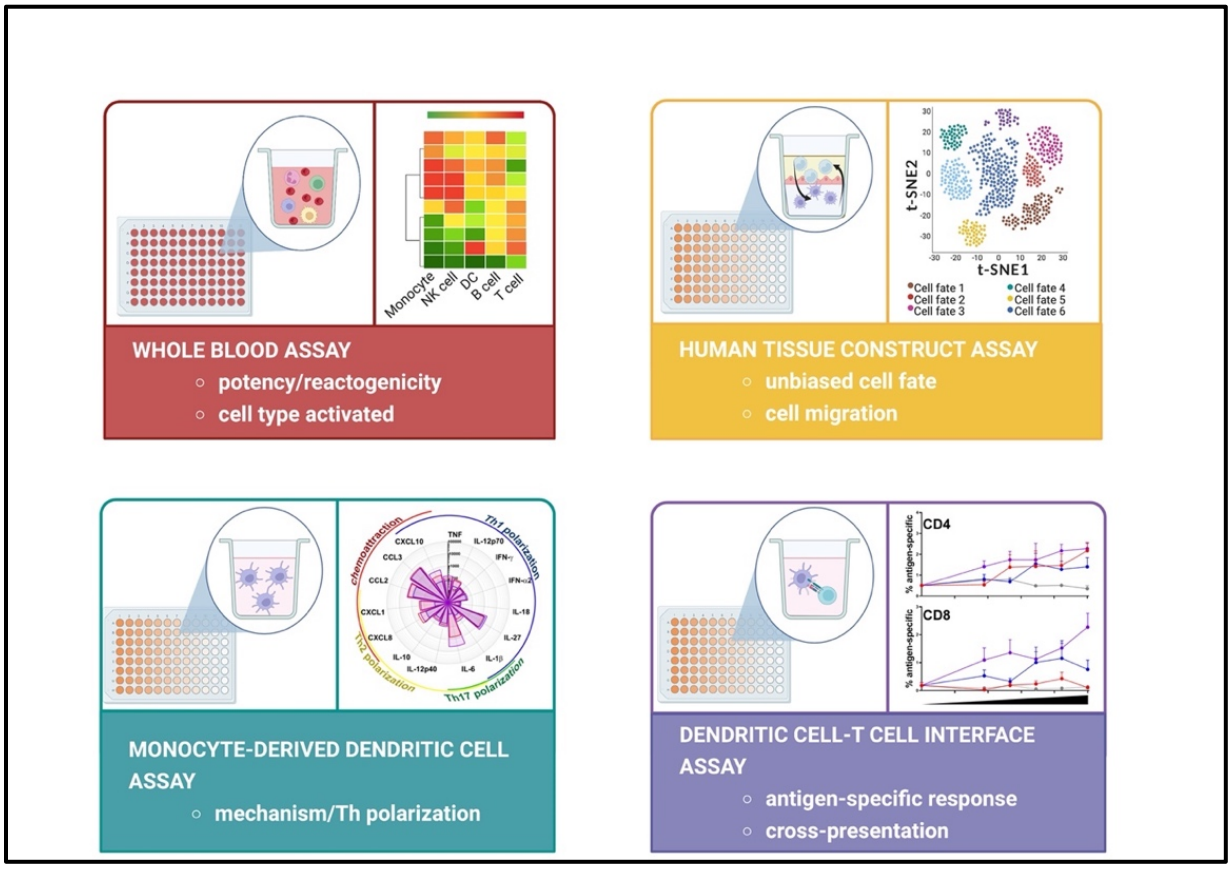


***Figure 4: Distinct and complementary human in vitro models***

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## Project 1

# Human *in vitro* modeling of MPL and QS-21 formulations demonstrates enhancement of dendritic cell and T cell responses to SARS-CoV-2 spike antigen

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**Abstract:**

Adjuvants enhance vaccine immunogenicity but their mechanism of action is often incompletely understood, hampering rapid applicability for pandemic vaccines. Herein, we characterized the cellular and molecular activity of several adjuvant formulations available for pre-clinical evaluation, including several developed for global open access. We applied four complementary human *in vitro* platforms to assess individual and combined adjuvants in soluble, oil-in-water, and liposomal formulations. Of all formulations tested, liposomal co-formulation of MPL and QS-21 was most potent in promoting dendritic cell maturation, selective production of Th1-polarizing cytokines, differentiation of mature dendritic cells in a microphysiological 3D tissue construct, and activation of antigen-specific $CD4^+$ and $CD8^+$ T cells in a SARS-CoV-2 spike antigen-specific co-culture assay. Thus, human *in vitro* modeling provides insight into the mechanism of action of adjuvanted vaccine formulations which may advance public health by accelerating development of affordable and scalable adjuvants for vaccines tailored to vulnerable populations.

**Introduction:**

The impact of infectious diseases and resulting damages recently became evident during the COVID-19 pandemic (61,62). As one of the most successful and cost-effective public health interventions, vaccines prevent around five million deaths globally every year (63). While currently authorized and/or approved SARS-CoV-2 vaccines have demonstrated efficacy and proved lifesaving, their potential is limited by the need for freezing of mRNA formulations, multiple booster dose requirement, and reduced immunogenicity in

vulnerable populations such as children, older adults, and immunocompromised individuals. Thus, there remains an urgent unmet need to develop safe and effective vaccines that can be quickly deployed on a global scale in the event of a pandemic or epidemic (64). Adjuvanted vaccines reduce the number of immunization doses required to induce immune responses, resulting in increased availability for global supply. For example, the formulation of GlaxoSmithKline's *Fendrix* with AS-04 adjuvant and hepatitis B antigen achieved dose sparing, reducing the vaccination series from three to two doses (65,66).

Vaccines comprised solely of purified protein antigens often produce little or no T cell response and do not induce the appropriate antibody (Ab) response with a single dose (67). The need for multiple vaccine doses to achieve adequate Ab response is a challenge which can be overcome by formulating a vaccine with adjuvants to enhance the efficacy of weak antigens and induce desired immune responses. An adjuvant is a component of a vaccine that enhances and/or shapes the magnitude, breadth, and/or durability of antigen-specific immune responses, and has been used in vaccine development since late 19$^{th}$ century (68). Adjuvants currently licensed in the US and/or Europe for use in the development of human vaccines include aluminum salts; oil-in-water emulsions like MF59, AS-03 and AF-03; virosomes; and AS-04, a monophosphoryl lipid A (MPL) preparation with aluminum salt (67). Selection of the adjuvant and its formulation depends on the nature of the antigen used in vaccine development, the desired type of immune response, the age of the target population, and the route of vaccine administration (67).

A major barrier that limits the opportunity to develop low-cost vaccines that would benefit low- and middle-income countries is the restricted access to vaccine- or adjuvant-formulations in part due to protection by intellectual property (IP) laws (69). While IP protection of adjuvants is key to on-going discovery and development of novel adjuvants, it may also limit adjuvant research and reduce global access to adjuvanted vaccines (70).

We studied the cellular and molecular mechanism of action (MOA) of adjuvant formulations developed for global open access as benchmarked to adjuvants currently being evaluated in clinical trials, in four distinct but complementary human *in vitro* platforms – the whole blood assay (WBA), to inform the magnitude of immune activation and specificity towards distinct cell types; the human tissue construct (HTC) assay, to determine the fate of locally activated innate immune cells in a microphysiologic setting (71); the monocyte-derived dendritic cell (MoDC) assay, to assess the ability of adjuvants to promote differentiation of different T-helper subsets; and the dendritic cell-T cell interface (DTI) assay, to measure the ability of adjuvants to boost the processing and presentation of SARS-CoV-2 spike protein and activate antigen-specific $CD4^+$ and $CD8^+$ T cells. Our hypothesis was that studying the MOA of these adjuvants would inform the optimal dosage and formulation needed to induce T-helper cell type 1-mediated immunity and/or CD8-mediated immunity considered favorable for protection against respiratory viral infections. We found that the liposomal co-formulation of MPL and QS-21, comparable to the licensed adjuvant system AS-01 (*GSK*), stimulated the development of T-helper 1 cells and promoted the induction of both $CD4^+$ and $CD8^+$ T cells against SARS-CoV-2.

**Materials and Methods:**

***Adjuvants:***

The following adjuvants were used at the dilutions noted in the figure legends: SWE (squalene oil-in-water (OIW) emulsion, stock 4.1% squalene, SEPPIC, France), MPL (3D-6A PHAD, stock 1 mg/mL in DMSO, Avanti Polar Lipids, Alabaster, AL), QS-21 and QS-7 (stock 1 mg/mL in DMSO, Desert King, San Diego, CA), 3M-052 absorbed to 2 mg/mL alum (AL030, stock 0.12 mg/mL, 3M Corporate Research and Materials Laboratory (St. Paul, MN) and Access to Advanced Health Institute (AAHI, Seattle, Washington)), 3M-052 in 4% OIW (EM128, stock 0.12 mg/mL, 3M/AAHI), and dMLT (stock 1 mg/mL in 700 µg of protein in 42.7 mM sodium phosphate, 10.7 mM potassium phosphate, 82 mM NaCl, 5% lactose, supplied by PATH (Seattle, Washington), manufactured by Walter Reed Army Institute of Research Pilot Bioproduction Facility (Silver Spring, MD)). LM, LQ, LMQ (liposomal (LS) formulations of MPL, QS-21 and MPL+QS-21 respectively), SM, SQ, and SMQ (OIW emulsions of MPL, QS-21 and MPL+QS-21 respectively) were obtained from the Vaccine Formulation Institute (VFI, Geneva, Switzerland). Non-MPL-containing stocks were confirmed to be free of endotoxin at the concentrations used (<1 EU/mL) using a limulus amoebocyte lysate (LAL) assay in accordance with the manufacturer's protocol (Charles River, Wilmington, MA). Unformulated MPL, QS-21, and QS-7 were prepared in DMSO (Fisher Scientific, Hampton, NH), and DMSO was used as the vehicle control. Dynamic light scattering was performed on all formulations using Zetasizer Nano ZS (Malvern Panalytical, Malvern, United Kingdom) to measure size, polydispersity index, and surface zeta potential over time to ensure formulation stability (Supplementary figure 1).

***Blood collection:***

Peripheral blood samples were collected after signed informed consent was received from healthy adult participants (n = 5) who were fully immunized against SARS-CoV-2 (>2 weeks prior) with one of the three FDA Emergency Use Authorized vaccines (Pfizer/BioNTech, Moderna, Janssen) with approval from the Ethics Committee of Boston Children's Hospital, Boston, MA (protocol number X07-05-0223). Samples were de-identified, anti-coagulated with 20 U/mL pyrogen-free heparin sodium (Fresenius Kabi, Bad Homburg, Germany), and processed within 2-4 hours as described below to enable study of adjuvant action across multiple human *in vitro* modules.

***Whole blood assay (WBA):***

We adapted a previously described method to measure adjuvant activity in whole blood (72). Whole blood was mixed 1:1 with sterile RPMI 1640 medium (Gibco, Ward Hill, MA, USA) and 180 µL of this was added to each well of a 96-well U-bottom plate containing 20 µL of freshly prepared adjuvant formulations at 10x the final concentration in RPMI 1640. The 200 µL/well suspensions were gently mixed by pipetting and were incubated at 37°C for 24 hours in a humidified incubator with 5% $CO_2$. After incubation, the plate was centrifuged for 3 minutes at 500x g. Supernatants were collected through pipetting without disturbing the cell pellet and were frozen until cytokine multiplex was run. Partial lysis of erythrocytes was obtained by incubating the cell pellet with BD FACS lysing solution (BD Biosciences, San Jose, CA, USA). Cells were washed three times with

DPBS (Gibco, Ward Hill, MA, USA), fixated in 1% paraformaldehyde (Thermo Fisher Scientific, Ward Hill, MA, USA), and stored at 4°C until flow cytometry analysis.

***Isolation of mononuclear cells, monocytes and T cells, and generation of MoDCs:***

Mononuclear cells and monocytes were isolated as described previously (73). Heparinized blood from adult donors was centrifuged at 500x g for 10 minutes. The upper layer of platelet-rich plasma was removed and further centrifuged at 3000x g for 10 minutes. The platelet-poor plasma was stored at 4°C for use in culture. Blood was restored to its original volume by adding DPBS and was carefully layered onto a Ficoll-Paque gradient (Cytiva, Marlborough, MA, USA). The mononuclear cell fraction was collected after centrifugation for 30 minutes at 1000x g and was washed twice with DPBS. Monocytes were isolated from this cell fraction through positive selection with magnetic CD14 MicroBeads (Miltenyi Biotec, Auburn, CA, USA) in accordance with the manufacturer's instructions. $CD8^+$ and $CD4^+$ T cells were then sequentially isolated from the remaining cell fraction in the same way, using $CD8^+$ and $CD4^+$ MicroBeads in accordance with the manufacturer's instructions. $CD14^+$ monocytes were cultured in a humidified incubator at 5% $CO_2$ at 37°C in 75 $cm^2$ tissue culture flasks at a concentration of $10^6$ cells/mL of RPMI 1640 supplemented with 1% penicillin-streptomycin (Gibco, Ward Hill, MA, USA), 10% FBS, 0.1% IL-4, and 0.1% GM-CSF (Miltenyi Biotec, Bergisch Gladbach, Germany) for five days. $CD8^+$ and $CD4^+$ T cells were frozen in 10% FBS (Gibco, Ward Hill, MA, USA), 10% DMSO and 80% RPMI, and were stored at -80°C until use. After five days of culture, immature monocyte-derived dendritic cells (MoDCs) were harvested by removing the loosely adherent cell fraction through gentle pipetting.

***MoDC assay:***

MoDCs were suspended in fresh MoDC culture medium at a concentration of $1.11x10^6$ cells/mL, and a volume of 180 µL (200,000 cells) was plated into each well of a 96-well U-bottom plate containing 20 µL of freshly prepared adjuvant formulations at 10x the desired final concentration. Suspensions were mixed by gentle pipetting and the cells were then cultured for 24 hours in a humidified incubator at 37°C with 5% $CO_2$. Cells were then centrifuged for 3 minutes at 500x g, and supernatants were harvested and stored at -80°C for use in further functional assays. Cells were washed with DPBS and fixated for flow cytometry.

***MoDC-T cell interface assay (DTI):***

DTI was performed as described previously (74). MoDCs generated as described above were suspended in RPMI 1640 supplemented with 1% penicillin-streptomycin (Gibco, Ward Hill, MA, USA), 10% FBS, 0.1% IL-4, and 0.1% GM-CSF. 25,000 cells were added per well in 90 µL to a 96-well U-bottom plate containing 10 µL of adjuvant formulations at 10x the desired final concentration in the presence or absence of recombinant SARS-CoV-2 spike protein (Thermo Fisher Scientific, Ward Hill, MA, USA). Suspensions were mixed by gentle pipetting and cultured for 24 hours. On the same day, $CD4^+$ and $CD8^+$ T cells were thawed and resuspended in RPMI 1640 with 10% FBS and 1% penicillin-streptomycin at a concentration of $3.33x10^6$ cells/mL. T cells were cultured for 24 hours in a 6-well plate with 3 mL per well, and then harvested by gentle pipetting, resuspended at a concentration of $2.5x10^6$ cells/mL. 100 µL of either $CD4^+$ or $CD8^+$ T cells was added

to each well of the respective MoDC plate, and the co-culture was incubated for four days. Cells were then centrifuged for 3 minutes at 500x g. Cells were washed with DPBS, fixated in 1% PFA, and stored at 4°C for flow cytometry.

***Human tissue construct (HTC):***

A human tissue construct model of monocyte extravasation and in-tissue autonomous differentiation was generated as previously described (71). Endotoxin-free human type 1 collagen cushions were cast in 96-well microtiter plates (Costar round bottom, Thermo Fisher Scientific Inc., Ward Hill, MA, USA). Human type 1 collagen cushion solution was prepared by mixing 10x M199 media, 0.1N NaOH, and human collagen (3 mg/mL) at a proportion of 1:5:8 respectively. 70 µL of this solution was applied to each of the inner 60 wells of a 96-well flat-bottom plate using a repeat pipette, and 200 µL of HBSS was added to the 36 outer wells to act as an evaporation barrier. The plate was incubated at 5% $CO_2$/37°C for 24 hours to congeal. Meanwhile, using trypsin-EDTA and the same M199 media containing 50% FBS and 1% PSG, the 85–90% confluent HUVEC cultures (as assessed with an inverted microscope) were passed to larger (150 $cm^2$) vented cap tissue culture flasks pre-coated with human fibronectin (0.5 mg/mL) and incubated at 5% $CO_2$/37°C. Ten minutes before seeding endothelial cells, collagen cushions were prepared by adding 100 µL of M199 with 1% PSG for 10 minutes before aspirating out and adding 20 µL of 0.5 mg/mL human fibronectin. Typically, one T150 flask of confluent HUVECs contained sufficient cells to coat two cushion plates (120 wells). Prior to seeding, excess fibronectin was aspirated out and cells from one confluent T150 flask were resuspended in approximately 13 mL of media to be dispensed as 100 µL per well (120

wells). Plates were incubated at 5% $CO_2$/37°C until confluency of monolayer was attained. Monolayer integrity, confluence, and morphology of endothelial cells were assessed by inverted microscopy using phase contrast at 4x magnification (Nikon TS100 inverted microscope EVOS XL Core Imaging System (Fisher Scientific, Hampton, NH, USA)). Only 100%-confluent TCs were used for testing. $CD33^+$ cells from each donor were plated on top of autologous platelet-poor plasma. The adjuvant formulations were immediately added to the appropriate wells from a pre-prepared dilution plate. The plates were incubated for 48 hours at 37°C in 5% $CO_2$. After incubation, representative images were taken, and supernatants were collected without disturbing the endothelial layer. The reverse-transmigrated cells were then resuspended in DPBS and transferred to a fresh 96-well U-bottom plate. 10 µL of sample was removed for counting in a hemocytometer after dilution in trypan blue (Gibco, Ward Hill, MA, USA). Remaining cells were washed again in DPBS, fixed in 1% PFA (Fisher Scientific, Hampton, NH, USA), and stored at 4°C for flow cytometry.

***Cytokine multiplex:***

Cytokine profiles of supernatants derived from whole blood assay, MoDC assay, and HTC assay were analyzed using a multianalyte fluorescent bead-based array (Luminex Corp., Austin, TX, USA). Quantification of cytokines was done using a custom built 21-plex kit using the Milliplex HCYTA-60K Human Cytokine/Chemokine/Growth Factor Panel A (Millipore, Merk, Darmstadt, Germany). This customized kit included CXCL8, CXCL10, GRO, IFNα-2, IFN$\gamma$, IL-1$\beta$, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, IL-18, IL-27, MCP-1, MIP-1α and TNF, and was run according to the manufacturer's

instructions. Sample fluorescence data was collected using a Flexmap 3D analyzer running xPONENT software version 4.2, and results were fit to a 5-point log curve and converted into pg/mL values using manufacturer-provided standard solutions and Milliplex Analyst software version 5.1.

***Flow cytometry:***

Flow cytometry was used to identify and characterize cell sub-populations following whole blood assay, MoDC assay, DTI assay, and HTC assay. Flow cytometry was performed using an LSRFortessa flow cytometer (Becton Dickinson) and data analyzed with FlowJo software version 10. Cells were stained for 30 minutes at 4°C in the dark with panels which used the following antibodies: anti-CD14-PE (Clone M5E2), anti-CD56-PerCP-Cy5.5 (Clone B159), anti-CD20-PE-Cy7 (Clone L27), anti-CD86-AF700 (Clone 2331), anti-CD197-PE-Cy7 (Clone 3D12), anti-CD4-V450 (Clone RPA-T4), anti-CD8-V450 (Clone RPA-T8), anti-CD134-PE (Clone L106), anti-CD154-APC (Clone 89-76), anti-CD25-FITC (Clone M-A251), anti-CD16-FITC (Clone B73.1), anti-CD40-FITC (Clone 5C3) (BD Biosciences, East Rutherford, NJ), anti-CD3e-AF647 (Clone UCHT1) (Invitrogen, Waltham, MA), anti-CD123-APC-eFluor780 (Clone 6H6) (eBioscience, San Diego, CA), anti-CD1c-PB (Clone L161), and anti-HLA-DR-BV605 (Clone L243) (Biolegend, San Diego, CA). Cells were then washed and resuspended in DPBS prior to data acquisition. All representative gating strategies are shown in Supplementary figure 3.

***Statistical analyses:***

Statistical analyses were performed using GraphPad Prism version 9.3.1. Dose-response curves were compared using 2-way repeated measures ANOVAs, and multiple comparison testing was performed using either Dunnet's Method when comparing against the vehicle only or Tukey's method when making comparisons across additional groups. A normal 2-way ANOVA was used to analyze the HTC results as comparisons were only made at a single concentration. Synergy was calculated using an adaptation of the Loewe method of additivity described previously (73). D values greater than 1 were considered antagonistic, D values equal to 1 were considered additive, and D values less than 1 were considered synergistic.

**Results:**

***LS formulation attenuates reactogenicity potential of MPL in whole blood assay***

The effects of MPL- and QS-21-containing adjuvants, as well as lipidated TLR7/8 agonist 3M-052, formulated either in an oil-in-water (OIW) emulsion (EM) or with aluminum hydroxide (AL), and double-mutant heat-labile enterotoxin (dMLT), were evaluated *in vitro* using whole blood from five adult donors. MPL and QS-21 were evaluated separately or in combination as these have been reported to act synergistically *in vivo* (75), but not extensively tested *in vitro* and are also used together in licensed vaccine formulations (76,77). To assess the effect of formulation, these adjuvants were also tested in OIW and in liposomes (LS). Whole blood samples were stimulated across a five-point dose-response curve of each formulation tested and the levels of 21 cytokines in the supernatants were quantified. Optimal concentration ranges were determined by assessing cytokine induction versus cytotoxicity across a wide range of concentrations

(Supplementary figure 2). Soluble formulations and OIW emulsions of MPL and QS-21 produced significantly high levels of TNF, IL-12p70, IFN$\gamma$, IL-6, IL-10, CXCL1, CCL2, and CXCL10 as well as decreased levels of CCL3 relative to vehicle controls. Soluble MPL and QS-21 also induced production of IL-12p40 and IL-1$\beta$, while OIW emulsions induced IL-18 production. This broadly immunogenic profile was comparable to that produced by the 3M-052-based adjuvants, EM and AL, as well as to dMLT. In contrast, LS formulations were more selective in inducing cytokines, only increasing production of IL-12p70, IL-6, CXCL8, CCL2, and CXCL10.

Among MPL- and QS-21-containing adjuvants, MPL was the primary driver of activation, with adjuvants containing QS-21 in the absence of MPL inducing increased expression of only IL-12p70, CCL2, and CXCL8. The MPL-driven effect and the impact of formulation type can be clearly observed by TNF for which no increase in expression was seen relative to vehicle controls when whole blood was treated with QS-21-containing adjuvants or LS formulations, while soluble formulations or OIW emulsions which contained MPL induced TNF production.

To identify specific cell types that were activated by the formulations, we measured cell surface markers using flow cytometry. Cells were characterized as B cells ($CD20^+$), mature DCs (mDCs, $CD1c^+$), monocytes ($CD14^+$), NK cells ($CD56^+$), plasmacytoid DCs (pDCs, $CD123^+$), or T cells ($CD3^+$). Activation was quantified based on expression of CD40, CD86, and HLA-DR. We found that most adjuvant formulations primarily activated monocytes, with some activation of pDCs and NK cells. In addition, the 3M-052-containing formulations EM and AL induced higher levels of B cell activation, consistent

with TLR7 expression in these cells. Among monocytes, activation was mainly seen in MPL-containing formulations and was generally lower in LS formulations.

***LS formulations drive monocyte differentiation to a mature dendritic cell-like fate in human tissue construct assay***

To characterize the ability of MPL and QS-21-containing adjuvants to influence cell fate decisions in a microphysiological setting, we utilized a human tissue construct model of monocyte extravasation and in-tissue autonomous differentiation as previously described (71). CD33-expressing monocytes were cultured in autologous plasma on top of a confluent monolayer of endothelial cells grown over collagen cushions. After stimulating these cells for 48 hours with adjuvant formulations, we harvested the reverse-transmigrated cells and characterized them by flow cytometry. Using an unbiased gating strategy utilizing t-distributed stochastic neighbor embedding (t-SNE), we identified four distinct phenotypic clusters among the reverse-transmigrated cells. Based on this approach, we characterized these populations as monocyte-like (CD14pos, CD16neg, HLA-DRlow, CD86low, CCR7neg), macrophage-like (CD14pos, CD16pos, HLA-DRvar, CD86var, CCR7low), immature DC-like (CD14low, CD16low/var, HLA-DRlow, CD86low/var, CCR7low), or mature DC-like (CD14low/var, CD16low, HLA-DRhigh, CD86high, CCR7high).

In the absence of adjuvant stimulation, reverse-transmigrated cells were primarily monocyte-like or macrophage-like. In contrast, stimulation with formulations containing MPL produced a significant increase in immature DC-like phenotype. Furthermore, among both soluble and LS-based formulations, combining MPL and QS-21 also

significantly increased the number of reverse-transmigrated cells with a mature DC-like phenotype.

### *LS-based formulations induce a robust Th1-polarizing cytokine response in human MoDCs*

Having found that MPL- and QS-21-containing adjuvants, and to a lesser degree 3M-052 and dMLT, drive differentiation of monocytes to a DC-like phenotype, we next assessed the effect of these adjuvants on human DCs. We stimulated MoDCs derived from the same donors with adjuvant formulations and cultured them in 10% autologous plasma (v/v). Formulation of MPL and QS-21 in LS or OIW emulsions activated DCs, inducing high levels of MHC-II and CCR7 expression on cell surface. Stimulation with MPL and QS-21 formulated in LS also induced robust expression of CD86, comparable to that induced by 3M-052-based formulations.

To describe the potential to promote differentiation of different T-helper subsets, we measured adjuvant-induced cytokine and chemokine production. While soluble MPL+QS-21 and OIW emulsions induced robust, predominantly Th2-polarizing cytokine responses, MoDCs stimulated with MPL+QS-21 formulated in LS had a more Th1-biased response characterized by lower production of IL-10 and IL-12p40.

Most strikingly, formulations containing only MPL or QS-21 produced between one and three orders of magnitude less IL-1$\beta$ than their co-formulated counterparts. To calculate the magnitude of this synergistic effect, we used a previously described adaptation of the Loewe method to measure the D-value for the dose-response curves. IL-1$\beta$ was synergistically induced by the co-formulation of MPL and QS-21 across

formulation types, with greater degrees of synergy in OIW emulsions and LS than in soluble formulations. Of note, IL-12p40 expression was antagonized by co-formulation in LS, while there was a purely additive effect of co-formulation as soluble adjuvants and in OIW emulsions – further highlighting the Th1-polarizing phenotype of LS formulation.

***Co-formulation of MPL and QS-21 in LS and OIW emulsions enables synergistic induction of a CD8$^+$ T cell response in a dendritic cell-T cell interface assay***

As antigen-presenting cells, DCs are a crucial link between innate and adaptive immunity. To evaluate the effect of the adjuvant formulations on antigen presentation by DCs, we modeled the process of antigen presentation using an *in vitro* DC-T cell interface assay (DTI). We stimulated donor-derived MoDCs with adjuvants for 24 hours in the presence of SARS-CoV-2 spike protein, and then cultured them with autologous CD4$^+$ or CD8$^+$ T cells for four days. We found that DCs from nearly all formulations were able to activate spike-specific CD4$^+$ T cells in a dose-dependent manner but with a different magnitude, with MPL-containing adjuvant formulations resulting in the highest percentage of activated antigen-specific CD4$^+$ T cells.

In contrast, only QS-21-containing OIW emulsions, LS, and AL induced robust activation of CD8$^+$ T cells. Soluble QS-21-containing formulations induced no discernable CD8$^+$ activation, nor did a 3M-052-based emulsion without alum. Furthermore, we observed that relative to formulations with QS21 alone, co-formulation of QS-21 and MPL in OIW and LS induced a greater CD8$^+$ response. The D-values for MPL and QS-21 co-formulation supported this observation, indicating a slight synergistic effect in LS and OIW emulsions.

**Discussion:**

Our findings that the liposomal co-formulation of MPL and QS-21 acted synergistically in multiple human *in vitro* platforms and induced the development of antigen-specific T-helper 1 cells and $CD8^+$ T cells provides an understanding of the underlying MOA of adjuvant formulations that boost immune response through vaccination. We used distinct but complementary human *in vitro* platforms to model innate and adaptive immune responses and made observations that could predict *in vivo* activity and advance the development of safe and effective low-cost vaccines. The formulation type also determines overall reactogenicity and polarity of the innate and adaptive immune responses, and we found LS formulation of adjuvants to be less reactogenic in the whole blood assay platform. QS-21 was the primary adjuvant driving receptor upregulation in the MoDC assay, and the production of Th1-polarizing cytokines was mainly associated with the LS formulation. We also found synergistic induction of IL-1$\beta$ with LS and OIW formulations, and antagonism with Th2-polarizing cytokine IL-12p40 with the LS co-formulation of MPL and QS-21. QS-21-containing formulations promoted antigen processing and cross-presentation on MHC in the DC-T cell interface assay, and QS-21 was the adjuvant responsible for driving the CD8 response. The ability of AS-01 to increase antigen presentation to antigen-specific immune cells enhances vaccine immunogenicity and efficacy, providing durable protection against targeted pathogens (76). The mechanistic and synergistic action of MPL+QS-21 liposomal co-formulation developed for global open access that we describe is comparable to the MOA of AS-01 previously described (78–80). The similarity of MPL+QS-21 liposomal co-formulation to

AS-01 may advance opportunities to develop low-cost vaccines and vaccines for vulnerable populations such as children, older adults, and immunocompromised individuals using adjuvant formulations produced for global open access. This approach could make a significant public health impact globally, especially when we are faced with epidemics and pandemics, by controlling the spread of infections via rapid and equitable distribution of vaccines. The broad cytokine expression profile shown by the adjuvants could potentially be useful in vaccines targeting pathogens displaying antigenic drift, strain variations or both, such as influenza viruses.

Although it featured multiple strengths, our study also has several limitations. One limitation of our study is the small sample size (n = 5 per assay platform), and therefore we did not study demographic differences affecting immune responses. We focused our study on the mechanism of action of individual and combination adjuvants, with each of the participants serving as both the test and control condition in the human *in vitro* platforms. Though a sample size (n) of five in each assay was sufficient to draw statistically significant conclusions on the mechanism of action of the tested adjuvant formulations, an increase in n may likely provide additional insights on cytokine and chemokine production as more analytes might become statistically significant. The current *in vitro* findings could be tested *in vivo* to determine antigen-specific humoral response induced by the adjuvants and safety profile of the liposomal co-formulation of MPL and QS-21, which could then be further confirmed in subsequent clinical trials. Though we used five different concentrations of the adjuvant formulations at single time points to characterize induction of cytokines and chemokines, we did not measure kinetics of production of cytokines such as IL-10 and IL-12p40. It is unclear if these cytokines are

consistently released over time or if the secretion diminishes after the initial production. With the selected concentrations, we were unable to demonstrate a strong response of dMLT that has shown to induce IL-1$\beta$ and other cytokines in previous studies (81–84). We chose concentration ranges that would minimize toxicity and enable *in vitro* study. However, toxicity and pyroptosis could potentially contribute to *in vivo* efficacy.

In sum, we have leveraged a range of innovative human *in vitro* assays to define novel combinatorial effects of adjuvant formulations developed for global open access. The mechanistic understanding of adjuvant activity on immunity that our study provides would inspire further investigations on immune ontogeny and lead to the development of vaccines for global access.

**Acknowledgements:**

We would like to thank all the study participants, phlebotomists, and PVP members for their contributions to this study. We thank the Vaccine Formulation Institute (VFI), SEPPIC, 3M Corporate Research and Materials Laboratory, Access to Advanced Health Institute (AAHI), and PATH for providing us with adjuvant formulations. We thank Dr. Kevin Churchwell and Dr. Gary Fleisher of Boston Children's Hospital for their support for the *Precision Vaccines Program*.

**Conflict of interest statement:**

SvH and OL are named inventors on several patent applications relating to human *in vitro* systems that model the action of adjuvants and vaccines, as well as vaccine adjuvants that were not included in this study. The other authors declare no conflict of interest.

**Data availability:**

The de-identified, quality-assured datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Funding statement:**

This work was supported by the Bill & Melinda Gates Foundation, grant INV-004886 to SvH. Additional support for SvH and OL came from US National Institutes of Health (NIH)/National Institutes of Allergy and Infectious Diseases (NIAID) award Molecular Mechanisms of Combination Adjuvants (1U01AI124284). The *Precision Vaccines Program* is supported in part by the Boston Children's Hospital Department of Pediatrics and Chief Scientific Office.

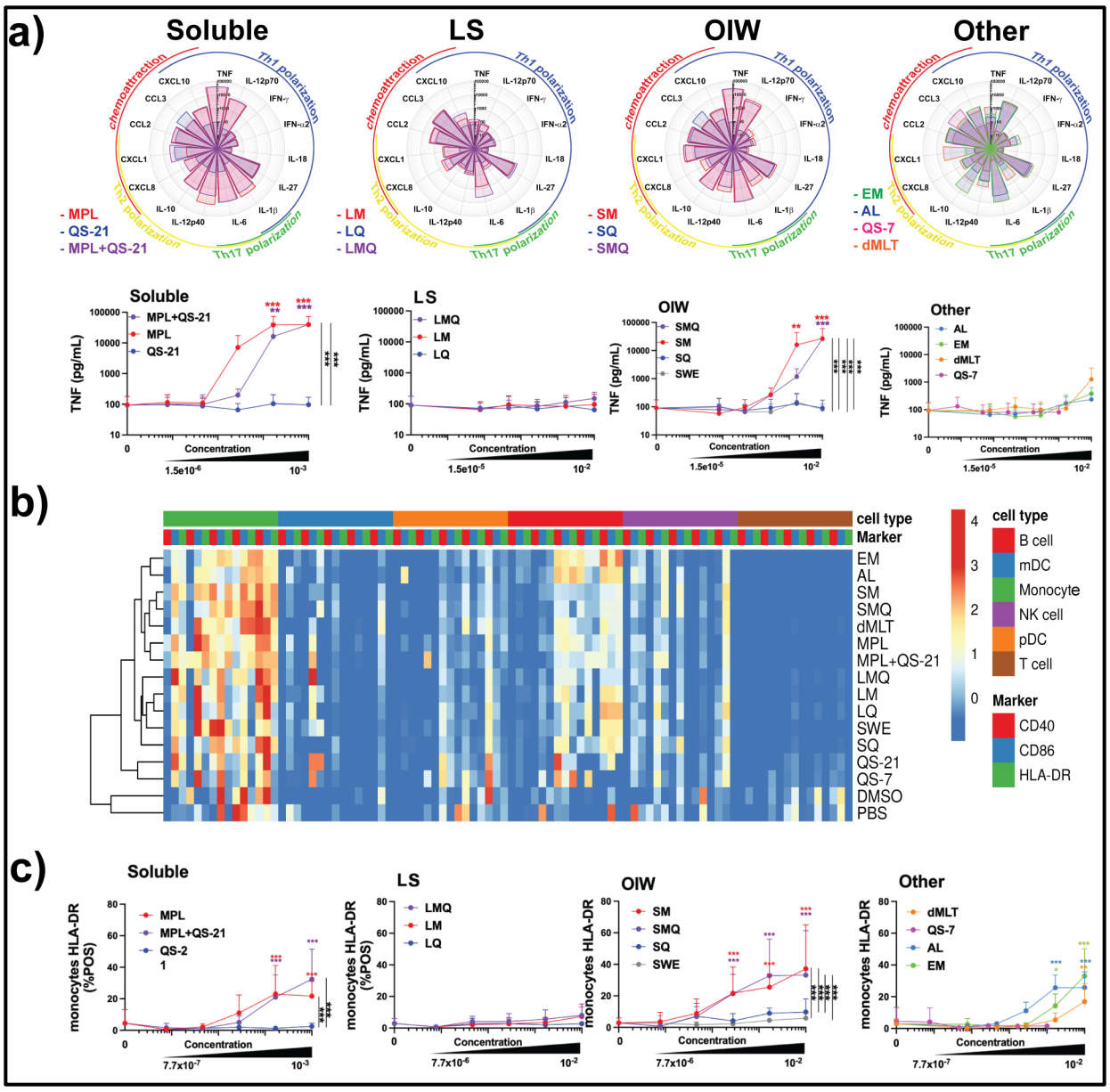



***Figure 5: MPL-containing compounds potently induce innate immune response in whole blood assay.*** *a) Flower plots show expression levels of cytokines in pg/mL induced by treatment of whole blood with adjuvants at a 1:100 dilution for LNP, OIW and other, and 1:1000 dilution for soluble formulations. Dose-response curves indicate TNF production across a five-point dilution series, along with a vehicle control. Top*

*concentrations reflect least diluted. Further dilutions were 1/6 (v/v) in RPMI for all formulations. b) Heatmap indicating log10 mean fluorescence intensity of activation markers across cell types. c) Dose-response curves measuring percentage of HLA-DR$^{+}$ monocytes. Stars above a data point on flower plots and dose-response curves indicate statistical significance relative to vehicle control using a 2-way repeated measures ANOVA and Dunnet's method for multiple comparison testing. Stars adjacent to vertical bars on dose-response curves indicate statistically significant differences between treatment groups at a given concentration using a 2-way repeated measures ANOVA with Tukey's multiple comparison testing. N = 5 for all readouts, except CXCL8 where N = 2. (* p < 0.05, ** p < 0.01, *** p < 0.001)*

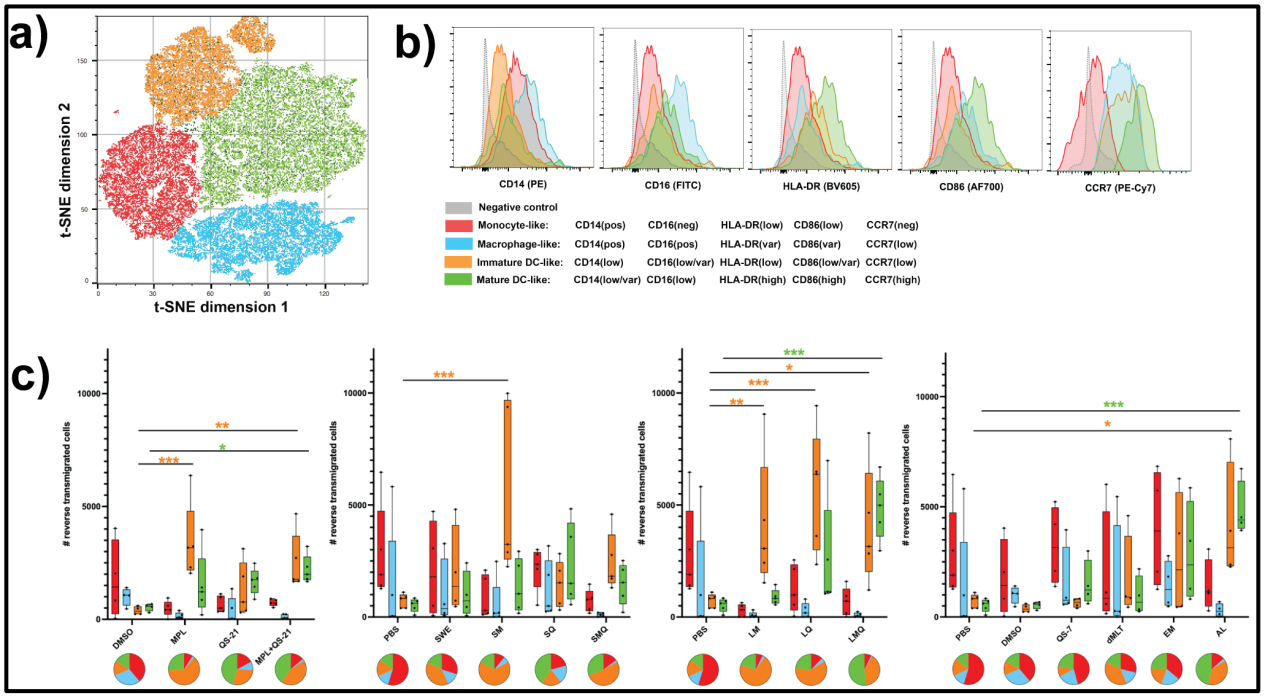


***Figure 6: LS-containing formulations drive monocytes to a DC-like fate in human tissue construct assay.*** *Monocytes were stimulated inside the tissue construct with adjuvants at a 1:100 dilution for LS, OIW and other, and 1:1000 dilution for soluble formulations, before flow cytometric analysis of treatment-induced migrated cells. a) Dimensional reduction by t-distributed stochastic neighbor embedding identifies distinct clusters of phenotypically similar reverse-transmigrated cells. b) Differential expression of surface markers among clustered populations demonstrates similarity to phenotypes of known cell types. c) Box plots indicate reverse-transmigrated cell counts separated by cell phenotype for each treatment condition at its highest concentration. Pie charts for each condition show the fraction of each phenotype within the total population of reverse-transmigrated cells. Stars indicate a statistically significant difference in reverse-transmigrated cells of a given phenotype relative to the vehicle using an ordinary 2-way ANOVA with Dunnet's method for multiple comparisons. N = 5. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)*

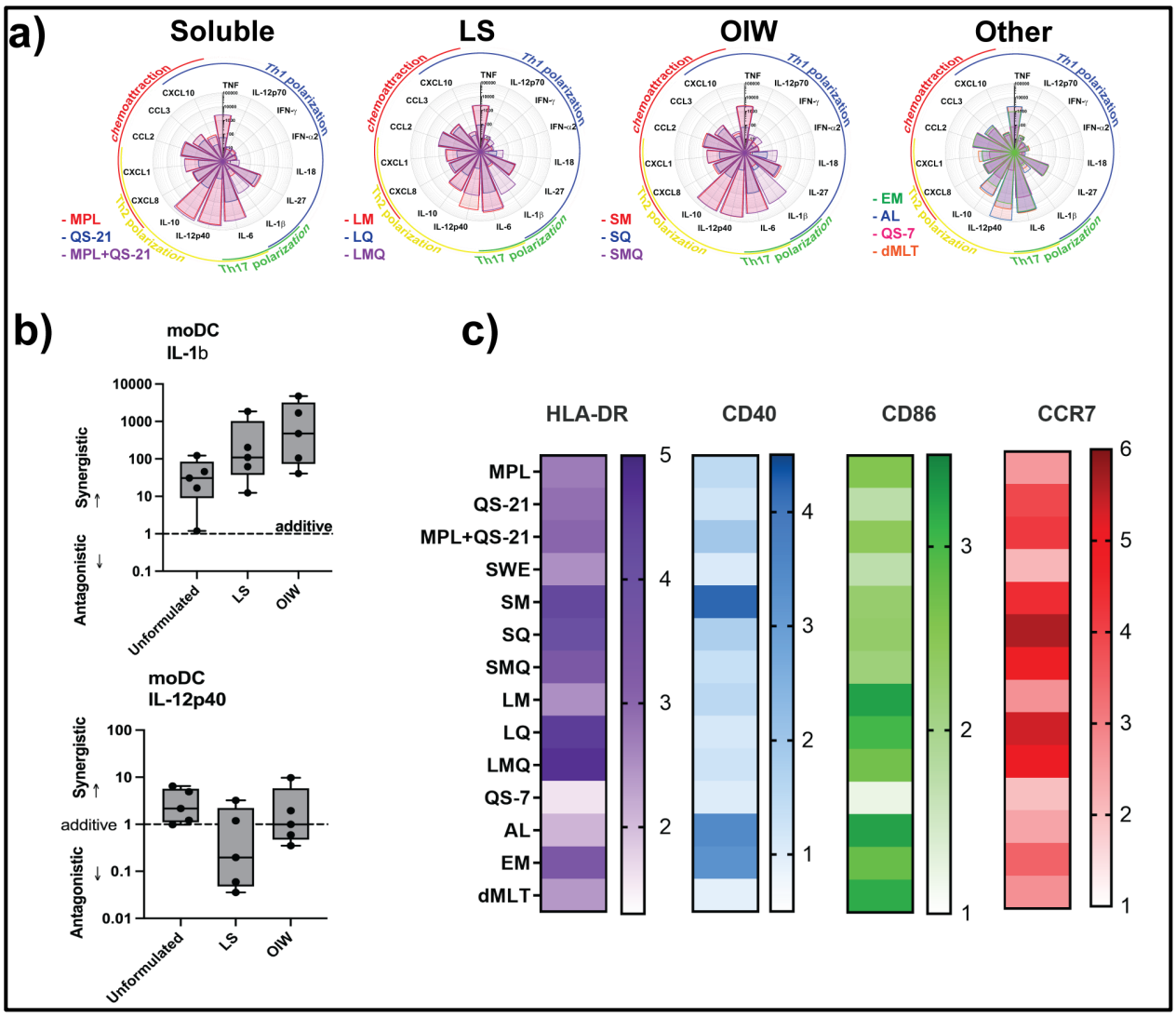



***Figure 7: LS-based MPL, QS-21 or combination formulations induce a Th1-polarizing cytokine response in human monocyte-derived dendritic cells (MoDCs).*** *MoDCs were stimulated with adjuvants at a 1:100 dilution for LS, OIW and other, and 1:1000 dilution for soluble formulations, before flow cytometric analysis of treatment-induced migrated cells and quantification of treatment-induced cytokine production. a) Flower plots depict expression levels of cytokines in pg/mL induced by treatment with adjuvants at their top concentrations. Stars above a data point on flower plots indicate*

*statistical significance relative to vehicle control using a 2-way repeated measures ANOVA and Dunnet's method for multiple comparison testing. b) Calculated D-values quantify the extent of synergy between MPL and QS-21 in each formulation type. D-values >1 indicate antagonism, <1 indicate synergy, and =1 indicate additivity. c) Surface expression of four activation markers based on log10 mean fluorescence intensity. N = 5. (* p < 0.05, ** p < 0.01, *** p < 0.001)*

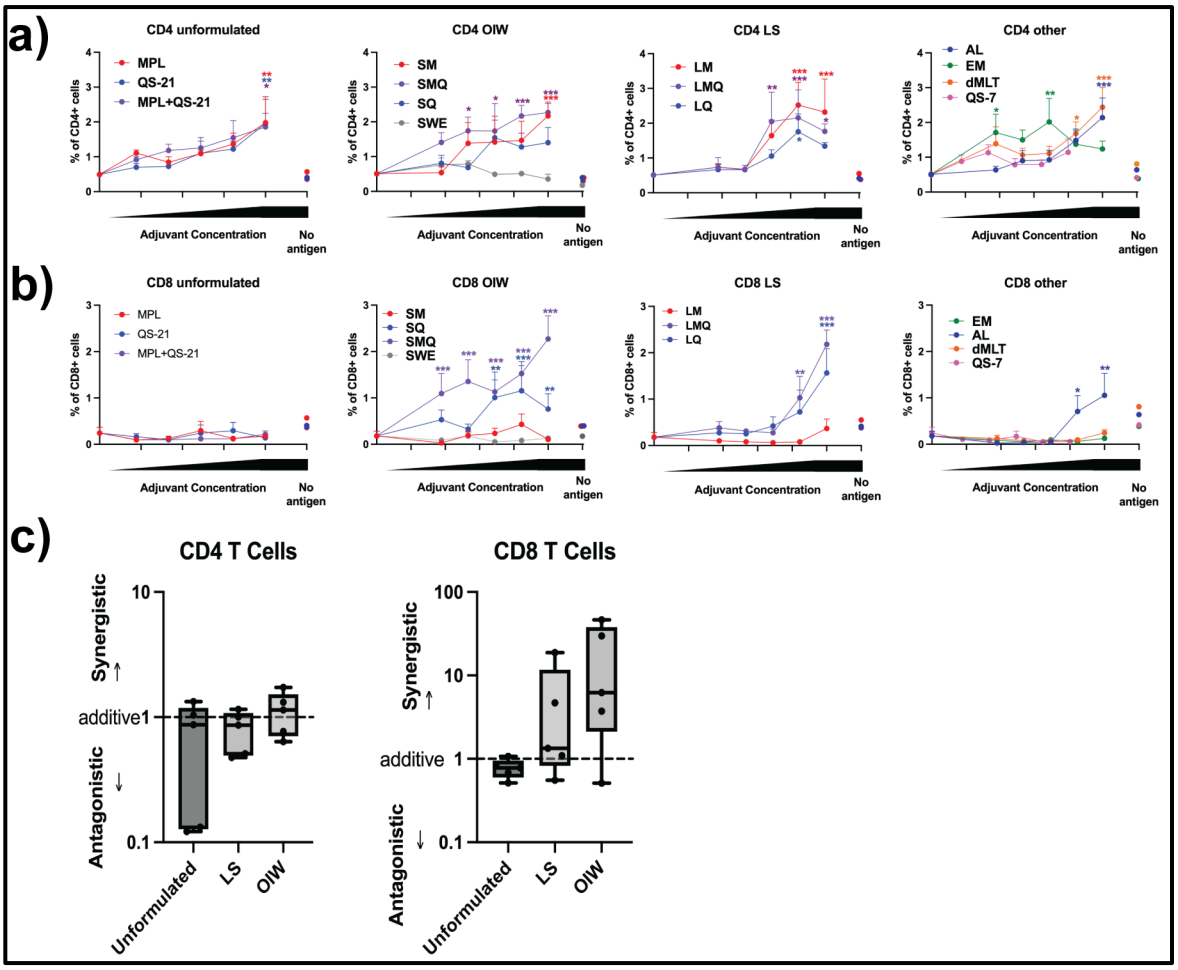
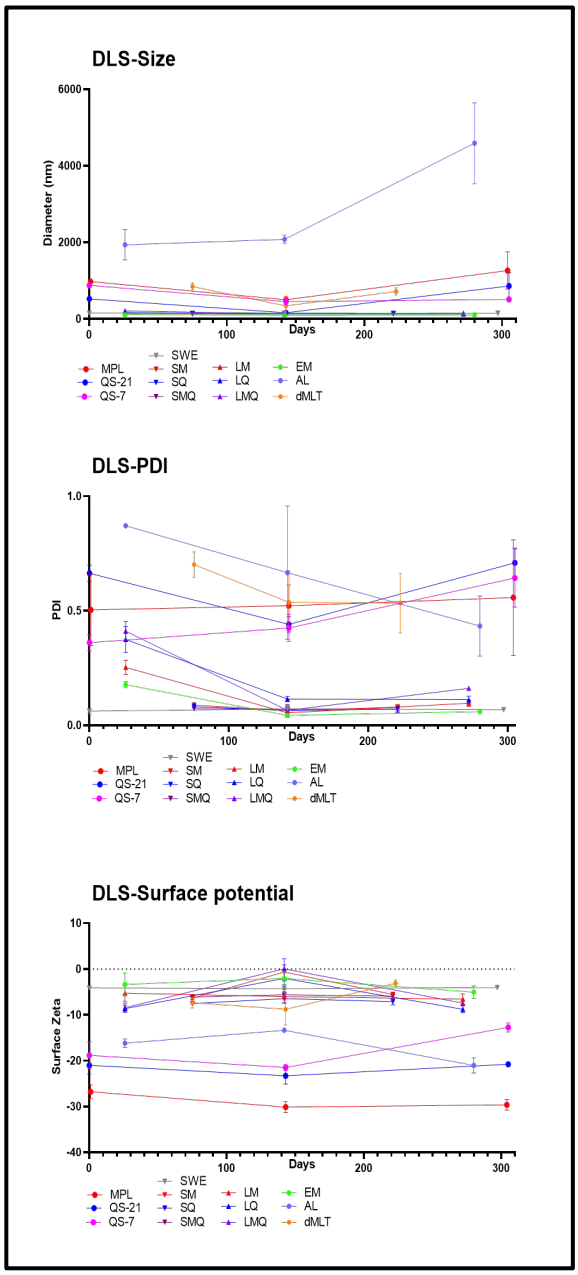


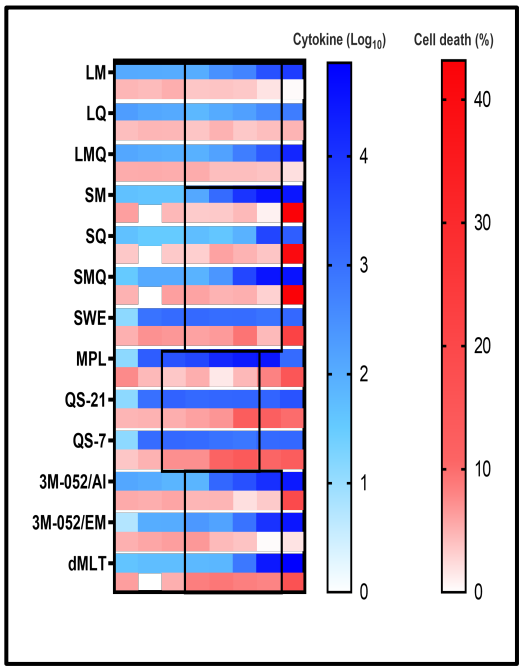
***Figure 8: MoDCs stimulated with co-formulated MPL and QS-21 activate SARS-CoV-2 spike protein-specific T cells in DTI assay.*** *a) and b) MoDCs were stimulated with 5 μg/mL SARS-CoV-2 spike antigen, in the presence or absence of adjuvant formulations at a 1:100 dilution for LS, OIW and other, and 1:1000 dilution for soluble formulations, and subsequent 1/6 (v/v) dilutions for all formulations before co-culture with autologous $CD4^+$ or $CD8^+$ T cells. Activated T cells were defined as $CD4^+$, $CD8^+$, $CD25^+$, $CD134^+$, or $CD154^+$. c) Calculated D-values quantify the extent of adjuvant interaction (i.e., antagonism, additivity, or synergy) between MPL and QS-21 in each formulation type. Three data points were excluded (2 soluble, 1 OIW) because a curve could not be fit using the method described. Stars above a data point on dose-response curves indicate statistical significance relative to vehicle control, stars adjacent to vertical bars on dose-response curves indicate statistically significant differences between treatment groups at a given concentration, and stars above horizontal lines indicate statistically significant differences relative to a control with no antigen. Significance was calculated using a 2-way repeated measures ANOVA with Tukey's multiple comparison testing. N = 5. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)*

## Supplementary figure 1:

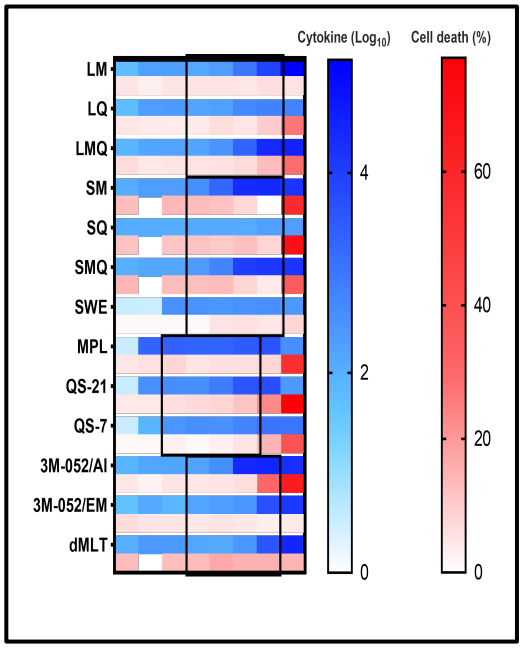


**Supplementary figure 2:**

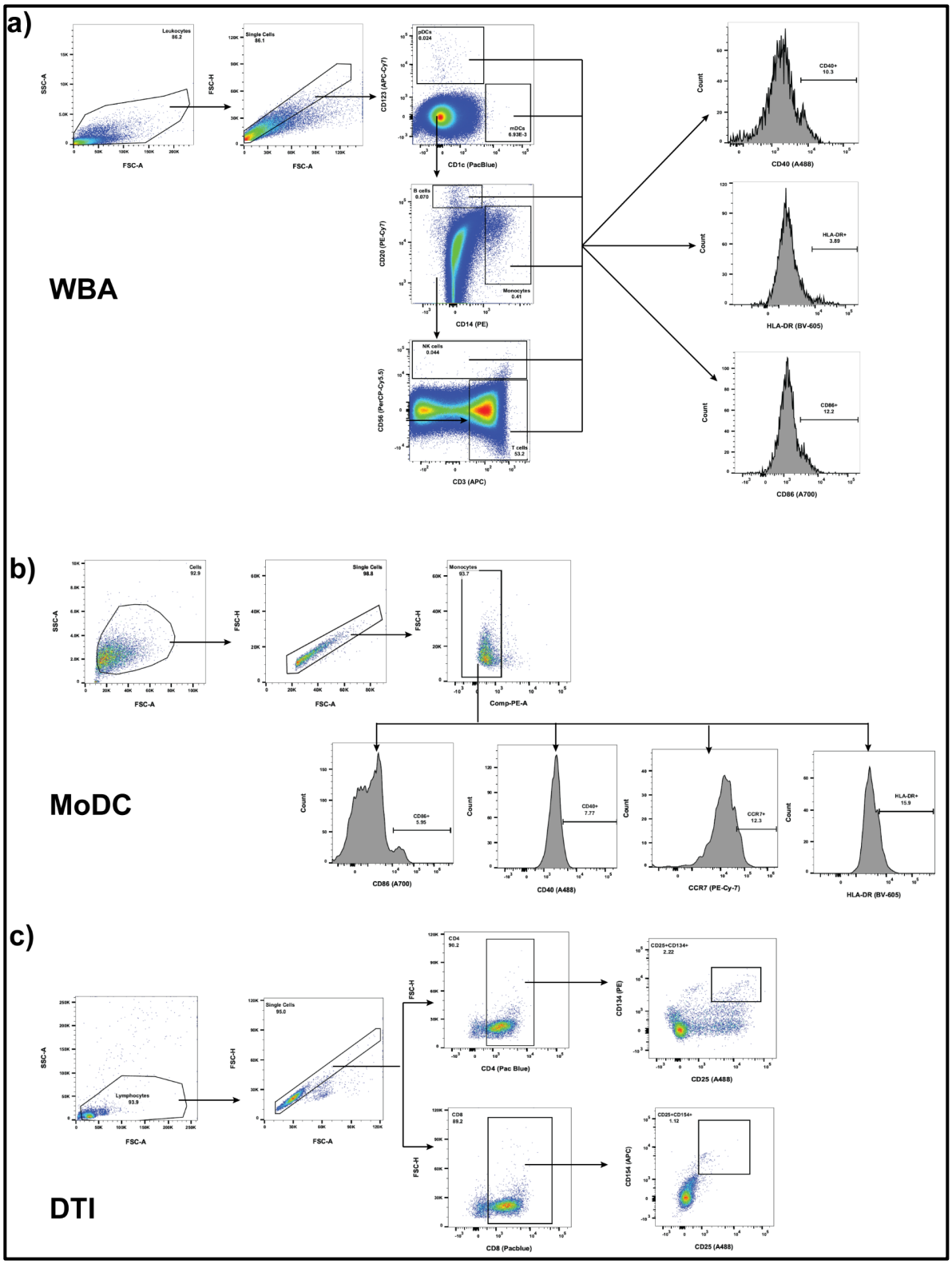
## a) WBA




## b) MoDC



**Supplementary figure 3:**



## Project 2

# Human *in vitro* modeling characterizes mechanism of action of adjuvantation systems defining scalable and affordable precision vaccine formulations for early life

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**Abstract:**

Children demonstrate distinct immunity in early life including diminished Th1-polarizing cytokine production contributing to high susceptibility to respiratory viral infections. This challenge also pertains to pediatric vaccine discovery and development, and could be overcome by developing adjuvantation systems with well characterized mechanisms of action (MOA) that are tailored to enhance age-specific immunogenicity. To this end, we employed novel age-specific human *in vitro* assays to characterize cellular and molecular activities of a selection of adjuvants in children aged between 2-4 years in soluble, oil-in-water and liposomal formulations available for pre-clinical evaluation, including several that were developed for global open access. In a whole blood assay, which predicts the reactogenicity potential of adjuvants, liposomal co-formulation of adjuvants monophosphoryl lipid A (MPL, a TLR4 agonist) and the soap bark tree-derived saponin *Quillaja saponaria* (QS)-21 (LMQ) induced a modest cytokine production compared to soluble and oil-in-water formulations. LMQ particularly enhanced production of CXCL8 and IL-6, markers of Th2 and Th17 responses that distinguish early life immunity from that of adults. Additionally, the double mutant heat-labile enterotoxin (dMLT) induced production of CXCL1 and IL-1$\beta$, and TLR7/8 agonist in oil-in-water (EM) and TLR7/8 agonist + alum (AL) induced production of IFN$\alpha$. In a monocyte-derived dendritic cell assay, which dissects the mechanism by which adjuvants activate differentiation of T helper (Th) cell subsets, LMQ induced a robust TNF response, indicative of Th1 immunity important for anti-viral host defense. In a dendritic cell-T cell interface assay that demonstrates antigen processing and presentation, activation of influenza HA antigen-specific $CD4^+$ T cells was driven by MPL and that of $CD8^+$ T cells was induced by QS-21.

Insight into the MOA of adjuvanted vaccine formulations via human *in vitro* modeling may advance global health by accelerating and de-risking development of affordable and scalable precision-adjuvanted vaccines.

**Introduction:**

The global burden of infectious diseases among under-five children remains high, with respiratory viruses contributing to significant levels of morbidity and mortality (85,86). High risk of infections and sub-optimal vaccine response seen among newborns and infants could partly be attributed to the functionally distinct antigen-presenting cells (APCs) such as monocytes, macrophages and dendritic cells evident in early life (87–89). Additionally, impaired innate and adaptive immune responses induced by the suppressive effects of maternal antibodies and regulatory T cells (Tregs) could sometimes result in poor vaccine responsiveness and production of memory B and T cells (87,90–95).

Naive $CD4^+$ T cells differentiate into functional subsets (such as Th1, Th2, Th17, and Treg cells) following activation (96). Polarization to type 2 immunity that is stimulated by T helper type 2 (Th2) lymphocytes and is predominant in young children, prevents the developing fetus from being seen as an allograft during pregnancy (97). However, this mechanism limits action against pathogens that require a T helper type 1 (Th1)-dominant response characterized by intense phagocytic activity to clear infection (98). Sub-optimal Th1 immunity also diminishes response to vaccination in early life and remains a challenge for pediatric vaccine development.

Th2 immune response includes secretion of anti-inflammatory cytokines such as IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. IL-6 could induce a Th2-polarizing response, or a

Th17-polarizing response in the presence of IL-23 and TGF$\beta$ (99,100). Differentiation into Th17 cells occurs in the presence of IL-1, IL-6, IL-17, IL-22, and IL-23. IL-1$\beta$ has been shown to act synergistically with IL-6 and IL-23 to upregulate the expression of the transcription factor ROR$\gamma$t which is critical for the development of Th17 cells (101). Preterm infants born with a defective Th17 immune response are predisposed to increased susceptibility to extracellular pathogens such as *E. coli* and Candida spp. Reduced Th1 response in children, characterized by low levels of pro-inflammatory cytokines IFN$\gamma$, TNF$\alpha$, IL-1$\beta$, IL-2 and IL-12p40, increases the risk of infections from intracellular pathogens including influenza and respiratory syncytial virus (RSV) (13). Low innate levels of type 1 IFN (IFN-I) impairs the anti-viral response in young children as a result of the compromised apoptotic pathways triggered by IFN$\gamma$-mediated activation of macrophages (102,103). Additionally, low levels of IFN$\gamma$ affects the regulation of MHC-I and MHC-II molecule expression and B-cell isotype switching, resulting in impaired antigen presentation mechanisms as IFN-I has been shown to enhance antigen cross-presentation (21,22,104–106). Impaired cross-presentation mechanism affects activation of $CD8^+$ T cells downstream which is critical for action against viral infections. Diminished IFN$\gamma$ response may also be driven by reduced levels of IL-12p70 – a feature of early life immunity (107). IL-12p70 forms a crucial link between innate and adaptive immunity as its production by activated monocytes, macrophages, neutrophils and DCs is important for activating a Th1 immune response (72,108–110).

Pattern-recognition receptor agonists such as Toll-like receptor (TLR) agonists can activate APCs, enhancing immune responses in the very young. TLRs expressed on APCs trigger the upregulation of co-stimulatory molecules that are critical for inducing an

effective adaptive immune response. Polarization to Th2 and Th17 immunity seen in response to stimulation with most TLRs distinguishes early life immunity from that of adults. Reduced TLR-mediated production of TNF$\alpha$ and enhanced production of IL-6 in response to stimuli such as lipopolysaccharide (TLR4 agonist) were observed in *in vitro* studies employing a whole blood assay among neonates (15,72,111–113). Stimulation with TLR7/8 agonists such as R848, a purine analog, and ssRNA has shown successful induction of neonatal monocytes and monocyte-derived dendritic cells to produce TNF$\alpha$, IL-1$\beta$, and IL-12p70 (109,113,114). Stimulation of TLR7 pathway (for instance, by imidazoquinolines) also enhances production of IFN-I, resulting in improved cross-priming (115–117). TLR4 signaling increases cross-presentation by preventing the fusion of phagosomes and lysosomes, causing delayed antigen degradation (118,119). CpG, a TLR9 agonist, when co-delivered with the antigen to the DC also stimulates cross-presentation, resulting in improved induction of antigen-specific T lymphocytes (120,121). Further in-depth exploration of these mechanisms can expand our current understanding of immune development in early life by providing insights into the role TLRs play in activating Th1-type immune responses that may be important for vaccines protecting against intracellular pathogens such as viruses.

To evaluate the pre-clinical applicability of vaccine adjuvant formulations in a pediatric setting, we hypothesized that the characterization of cellular and molecular immune responses to adjuvant stimulation in human *in vitro* platforms will inform age-specific vaccine development against viral infections. Our prior work has revealed that impaired Th1 response to TLR7/8 stimulation in early life can be overcome by the addition of a second adjuvant, Trehalose-6,6-dibehenate, which, in addition to redirecting

molecular signaling, can also enhance the intracellular delivery of the TLR agonist. As most formulations tested here are comprised of a TLR4 agonist (MPL) and a saponin-based adjuvant capable of enhancing intracellular delivery (QS-21), we also hypothesize that impaired Th1 immunity in children in response to MPL-containing formulations can be overcome by co-formulating it with QS-21.

**Methods:**

***Adjuvant formulations tested:***

The following adjuvants were studied individually and in combination in five concentrations in soluble, liposomal, and oil-in-water emulsion formulations: monophosphoryl lipid A (MPL), a TLR4 agonist; *Quillaja saponaria*-derived, QS-21; *Quillaja saponaria*-derived, QS-7; TLR7/8 agonist + alum (AL); double mutant heat-labile enterotoxin (dMLT); TLR7/8 agonist in oil-in-water (EM); and MF-59-like emulsion, SWE.

Unformulated MPL (3D-6A PHAD, stock 1 mg/mL in DMSO, Avanti Polar Lipids, Alabaster, AL), QS-21 and QS-7 (stock 1 mg/mL in DMSO, Desert King, San Diego, CA), 3M-052 absorbed to 2 mg/mL alum (AL030, stock 0.12 mg/mL, 3M Corporate Research and Materials Laboratory (St. Paul, MN) and Access to Advanced Health Institute (AAHI, Seattle, Washington)), and dMLT (stock 1 mg/mL in 700 µg of protein in 42.7 mM sodium phosphate, 10.7 mM potassium phosphate, 82 mM NaCl, 5% lactose, supplied by PATH (Seattle, Washington), manufactured by Walter Reed Army Institute of Research Pilot Bioproduction Facility (Silver Spring, MD)) were tested with SWE (squalene oil-in-water (OIW) emulsion, stock 4.1% squalene, SEPPIC, France), 3M-052 in 4% OIW (EM128, stock 0.12 mg/mL, 3M/AAHI), liposomal (LS) formulations of MPL, QS-21 and MPL + QS-

21 (LM, LQ and LMQ respectively), and OIW emulsions of MPL, QS-21 and MPL + QS-21 (SM, SQ and SMQ respectively). LM, LQ, LMQ, SM, SQ, and SMQ were obtained from the Vaccine Formulation Institute (VFI, Geneva, Switzerland).

Using a limulus amoebocyte lysate (LAL) assay (Charles River, Wilmington, MA), non-MPL-containing stocks were confirmed to be free of endotoxin at the concentrations used (<1 EU/mL). Unformulated MPL, QS-21, and QS-7 were prepared in DMSO (Fisher Scientific, Hampton, NH). DMSO was used as the vehicle control. Dynamic light scattering was performed on all formulations using Zetasizer Nano ZS (Malvern Panalytical, Malvern, United Kingdom) to measure size, polydispersity index, and surface zeta potential over time to ensure stability of the formulations tested.

***Participant recruitment and blood collection:***

We collaborated with the Children's Hospital Primary Care Center at Boston Children's Hospital (BCH) to enroll eligible children aged between two and four years who were healthy and visiting the clinic for routine immunizations and testing for complete blood count and lead level. Only English-speaking participants were recruited in this study. Children with any co-morbidities were excluded, including those who had a current or recent (within three days of enrollment) fever, cough, rhinorrhea, and/or other viral symptoms. We also excluded patients who were born pre-term, had a first-degree family member with primary immunodeficiencies, or had a sibling who may have been previously recruited in this study. We identified eligible subjects using a schedule of upcoming appointments for the well-child visit. Signed informed consent was obtained prior to blood draw from the parent or caregiver accompanying the child for the well visit. Blood

collection was performed by a phlebotomist trained and certified in drawing pediatric samples in compliance with the protocol approved by the Institutional Review Board of BCH (protocol number P00010750) and participants were monitored for at least 30 minutes for symptoms such as light-headedness. Samples were de-identified to protect the patients' personal information, anti-coagulated with 20 U/mL pyrogen-free heparin sodium (Fresenius Kabi, Bad Homburg, Germany), and processed within 2-4 hours.

We optimized volumes of each assay platform to enable the use of a single blood draw of 20 mL whole blood for multiple assays while maintaining comparability to data generated in the adult population. Optimal concentration ranges were determined by assessing cytokine induction versus cytotoxicity across a wide range of concentrations. Autologous plasma was stored following blood processing to be used downstream in culture as evidence highlights the important role soluble factors in plasma play in shaping immune responses (17).

***Isolation of peripheral blood mononuclear cells, monocytes and T cells, and the generation of monocyte-derived dendritic cells (MoDCs):***

Mononuclear cells and monocytes were isolated following a previously described method (73). Heparinized blood from the pediatric donors was centrifuged at 500x g for 10 minutes. Platelet-rich plasma was separated following this and further centrifuged at 3000x g for 10 minutes to obtain platelet-poor plasma for use in downstream culture. The volume of platelet-rich plasma removed after the initial centrifuge step was restored with DPBS. This was then carefully layered onto Ficoll-Paque (Cytiva, Marlborough, MA, USA)

for density gradient centrifugation at 1000x g for 30 minutes. Following this, the mononuclear cell fraction was collected and washed twice with DPBS.

Magnetic CD14 MicroBeads (Miltenyi Biotec, Auburn, CA, USA) were used to isolate monocytes from this cell pellet. $CD8^+$ and $CD4^+$ T cells were similarly isolated from the remaining cell fraction using $CD8^+$ and $CD4^+$ MicroBeads.

$CD14^+$ monocytes were cultured in a humidified incubator at 5% $CO_2$ at 37°C in 25 $cm^2$ tissue culture flasks at a concentration of $10^6$ cells/mL of RPMI 1640 supplemented with 1% penicillin-streptomycin (Gibco, Ward Hill, MA, USA), 10% FBS, 0.1% IL-4, and 0.1% GM-CSF (Miltenyi Biotec, Bergisch Gladbach, Germany) for five days to obtain monocyte-derived dendritic cells (MoDCs).

***Whole blood assay:***

To quantify the magnitude of immune response to adjuvant stimulation in whole blood, assess the reactogenicity potential of the formulations studied, and identify cellular targets activated in response to adjuvantation, we performed a whole blood assay as previously described (72).

Briefly, whole blood was mixed 1:1 with sterile RPMI 1640 medium (Gibco, Ward Hill, MA, USA) and 135 µL of this was added to each well of a 96-well U-bottom plate containing 15 µL of adjuvant formulations at 10x the final concentration in RPMI 1640. The 150 µL/well suspensions were gently mixed and were incubated at 37°C for 24 hours in a humidified incubator with 5% $CO_2$. Following incubation, the plate was centrifuged at 500x g for 3 minutes. Supernatants were collected for cytokine multiplex analysis. Partial lysis of erythrocytes was achieved by incubating the cell pellet with BD FACS lysing

solution (BD Biosciences, San Jose, CA, USA). Following three washes with DPBS (Gibco, Ward Hill, MA, USA), cells were fixated in 1% paraformaldehyde (Thermo Fisher Scientific, Ward Hill, MA, USA) and stored at 4°C until flow cytometry analysis.

***Monocyte-derived dendritic cell (MoDC) assay:***

We performed the MoDC assay to evaluate the magnitude and type of immune response induced by adjuvant stimulation of MoDCs at a 1:100 dilution for LS, OIW and other, and 1:1000 dilution for soluble formulations. To describe the potential of the tested formulations to promote differentiation of T-helper cell subsets, we quantified the levels of cytokines secreted and cell types activated in response to adjuvantation.

Briefly, MoDCs were suspended in fresh culture medium at a concentration of $1.25x10^6$ cells/mL. A volume of 45 µL (62,500 MoDCs per well) was plated into each well of a 96-well U-bottom plate containing 5 µL of freshly prepared adjuvant formulations at 10x the final concentration. Suspensions were gently mixed, and cells were cultured for 24 hours in a humidified incubator at 37°C with 5% $CO_2$. 10% autologous plasma (v/v) was utilized for culture. Following this, the cells were centrifuged for 3 minutes at 500x g, and supernatants were harvested and stored at -80°C for measuring cytokine levels. Cells were washed with DPBS and fixated for flow cytometry.

***Dendritic cell-T cell interface (DTI) assay:***

To evaluate the potency of adjuvant formulations to promote activation of antigen-specific $CD4^+$ and $CD8^+$ T cells, we performed the dendritic cell-T cell interface assay as previously described (74). Since the study age group was not eligible for vaccination

against SARS-CoV-2 at the time of recruitment, we used the influenza hemagglutinin (HA) antigen (*recombinant Flublok Quadrivalent, season 2022-2023 formulation, Sanofi*) in this assay.

Briefly, MoDCs were suspended in RPMI 1640 supplemented with 1% penicillin-streptomycin, 10% FBS, 0.1% IL-4, and 0.1% GM-CSF. 5,000 cells were added per well in 45 µL to a 96-well U-bottom plate containing 5 µL of adjuvant formulations at 10x the final concentration in the presence or absence of influenza HA. Suspensions were gently mixed and cultured for 24 hours in the presence of influenza HA antigen.

Cryopreserved autologous $CD4^+$ and $CD8^+$ T cells were thawed and resuspended in RPMI 1640 with 10% FBS and 1% penicillin-streptomycin. T cells were cultured for 24 hours and harvested by gentle pipetting, resuspended at a concentration of $0.5x10^6$ cells/mL. 150 µL of either $CD4^+$ or $CD8^+$ T cells was added to each well of the respective plate, and the MoDC-T cell co-culture was incubated for four days. 10% autologous plasma (v/v) was utilized for culture. Following incubation, cells were centrifuged for 3 minutes at 500x g, washed with DPBS, fixated in 1% PFA, and stored at 4°C for flow cytometry.

***Cytokine multiplex:***

Profiles of 20 cytokines and chemokines from supernatants derived from whole blood assay and MoDC assay were quantified and analyzed using a multianalyte fluorescent bead-based array (Luminex Corp., Austin, TX, USA). A custom built 20-plex kit using the Milliplex HCYTA-60K Human Cytokine/Chemokine/Growth Factor Panel A (Millipore, Merk, Darmstadt, Germany) was utilized for this purpose and included TNF, IL-12p70,

IFNα-2, IFN$\gamma$, IL-18, IL-27, IL-1$\beta$, IL-2, IL-3, IL-5, IL-6, IL-12p40, IL-10, IL-13, IL-17, CXCL8, CXCL1, CCL2, CCL3, and CXCL10. Sample fluorescence data was collected using a Flexmap 3D analyzer running xPONENT software version 4.2, and results were fit to a five-point log dose-response curve and converted to pg/mL values using manufacturer-provided standard solutions and Milliplex Analyst software version 5.1.

***Flow cytometry:***

We identified and characterized cell sub-populations following whole blood assay, MoDC assay, and DTI assay using flow cytometry (LSRFortessa flow cytometer (Becton Dickinson)). Data was analyzed with FlowJo software version 10. Cells were stained for 30 minutes at 4°C in the dark with panels which used the following antibodies: anti-CD14-PE (Clone M5E2), anti-CD56-PerCP-Cy5.5 (Clone B159), anti-CD20-PE-Cy7 (Clone L27), anti-CD86-AF700 (Clone 2331), anti-CD197-PE-Cy7 (Clone 3D12), anti-CD4-V450 (Clone RPA-T4), anti-CD8-V450 (Clone RPA-T8), anti-CD134-PE (Clone L106), anti-CD154-APC (Clone 89-76), anti-CD25-FITC (Clone M-A251), anti-CD16-FITC (Clone B73.1), anti-CD40-FITC (Clone 5C3) (BD Biosciences, East Rutherford, NJ), anti-CD3e-AF647 (Clone UCHT1) (Invitrogen, Waltham, MA), anti-CD123-APC-eFluor780 (Clone 6H6) (eBioscience, San Diego, CA), anti-CD1c-PB (Clone L161), and anti-HLA-DR-BV605 (Clone L243) (Biolegend, San Diego, CA). Following staining, cells were washed and resuspended in DPBS prior to data acquisition.

***Statistics:***

Statistical analyses were performed using GraphPad Prism version 9.3.1. Dose-response curves were compared using 2-way repeated measures ANOVAs, and multiple comparison testing was performed using either Dunnet's Method when comparing against the vehicle only or Tukey's method when making comparisons across additional groups. P-values less than 0.05 were considered statistically significant.

**Results:**

***Liposomal formulation potently induces cytokine production and attenuates reactogenicity potential of MPL in whole blood assay***

Evaluation of adjuvants in an *in vitro* assay performed on whole blood samples from six pediatric donors demonstrated activation of cell types that was assessed by measuring surface marker expression of CD40, CD86, and HLA-DR on B cells ($CD20^+$), mature DCs (mDCs, $CD1c^+$), monocytes ($CD14^+$), NK cells ($CD56^+$), plasmacytoid DCs (pDCs, $CD123^+$), and T cells ($CD3^+$). Monocytes showed activation in response to stimulation with most adjuvant formulations. Some activation was also noted in NK cells, B cells, and mDCs. A high degree of variability across donors was observed in these activated cell types.

Robust induction of the Th2-polarizing neutrophil chemokine, CXCL8, with limited IL-12p70 production was observed across the different formulations (soluble, oil-in-water, and liposomal). Soluble formulations of MPL, QS-21 and MPL+QS-21 induced the production of pro-inflammatory cytokines TNF, IL-1$\beta$ and IL-12p40, with MPL primarily driving activation.

Relative to vehicle controls, the liposomal (LS) formulations of MPL (a TLR4 agonist), QS-21 and MPL+QS-21 were more selective in inducing cytokine production. LS formulations induced increased production of IL-6 (a marker of Th17 immune response) and CCL2 (a potent chemokine for monocytes). Production of CXCL10 was possibly induced by the Th1 immunoregulatory cytokine, IFN$\gamma$, which regulates the migration of Th1-polarized effector T cells to sites of inflammation during an adaptive immune response. LS formulations did not appreciably induce production of CCL3, a macrophage inflammatory protein (MIP-1$\alpha$) induced during an inflammatory response.

Similar to the adult cohort, MPL in soluble and oil-in-water formulations upregulated TNF expression in pediatric cells. This effect was attenuated in the LS formulation and by QS-21 – possibly suggesting that when co-formulated with QS-21 in a liposomal formulation type, MPL could be less reactogenic.

The double mutant heat-labile enterotoxin (dMLT) potently induced production of CXCL1 and IL-1$\beta$. The TLR7/8 agonist in oil-in-water, EM, and the TLR7/8 agonist combined with alum, AL, potently induced IFN$\alpha$ production.

***Liposomal co-formulation of MPL and QS-21 induces a robust Th1 immune response in monocyte-derived dendritic cell assay***

Stimulation of monocyte-derived dendritic cells (MoDCs) from five pediatric donors with adjuvant formulations revealed that the LS formulations of MPL and QS-21 induced high levels of expression of cell surface markers, though significant variability was noted between donors.

LS co-formulation of MPL and QS-21 (LMQ) promoted the maturation of MoDCs by inducing TNF production, a critical immunoregulatory cytokine involved in the activation of type 1 (Th1) immune response which defends the host against intracellular pathogens such as viruses. Additionally, there was increased production of CXCL8 (a marker of Th2 immune response) and no IL-12p70 production noted with LS formulations – findings consistent with patterns of cytokine and chemokine production in early life (122,123).

MPL was the primary adjuvant influencing the production of CXCL8 and IL-6 in soluble and OIW formulations. Stimulation of MoDCs with most adjuvant formulations produced low levels of IL-10 and IL-12p40, reflecting a Th1-biased immune response that was particularly amplified by the co-formulated MPL and QS-21.

***MPL and QS-21 drive activation of antigen-specific $CD4^+$ and $CD8^+$ T cells in dendritic cell-T cell interface (DTI) assay***

The DTI assay enabled us to determine the ability of adjuvant formulations to stimulate cross-talk between dendritic cells and T cells to process influenza HA antigen, present it on MHC molecules, and activate antigen-specific T lymphocytes in five pediatric donors.

Dendritic cells could activate antigen-specific T cells in a dose-dependent manner, although high levels of variability between donors was noted as indicated by the error bars in the dose-response curves. We hypothesize that this may reflect pre-existing immunity to respiratory viruses in the study population.

The activation of influenza antigen-specific $CD4^+$ T cells was primarily driven by MPL and that of $CD8^+$ T cells was influenced by QS-21 across formulations. Squalene

oil-in-water emulsion of QS-21 (SQ) particularly activated high levels of antigen-specific $CD4^+$ and $CD8^+$ T cells.

**Discussion:**

Our complementary human *in vitro* models employed to assess the ability of adjuvant formulations to potently induce an antigen-specific type 1 immune response vital for defense against viruses in children provide important insights that could shape the design of next-generation vaccines tailored to the pediatric population which presents unique challenges with respect to immune development. Our whole blood assay predicts the reactogenicity potential of vaccine adjuvant formulations, monocyte-derived dendritic cell assay unravels the mechanism through which these adjuvant formulations activate the differentiation of various T helper cell subsets, and the dendritic cell-T cell interface assay provides insight into antigen cross-presentation and activation of influenza antigen-specific T lymphocytes.

We observed qualitative and quantitative individual variability to adjuvant stimulation in our *in vitro* platforms and hypothesize that this may reflect pre-existing immunity to certain viruses, such as respiratory syncytial virus (RSV), as indicated by the upregulation of Th1- and Th2-specific transcription factors in an immunophenotyping assay.

A limitation of our study is our small sample size of 5-6 participants per assay. Although we generated statistically significant conclusions, an increase in biological replicates may have enabled detection of robust differences as the activity of other cytokines and chemokines may become significant. Additionally, we did not focus on

understanding the varying immune responses to vaccine adjuvantation across demographic cohorts or seek to establish the kinetics of cytokine production in this pediatric cohort. To generate more robust data supporting the current findings, we would continue enrolling eligible children to increase the sample size across the assay platforms and address the donor variability by performing additional assays for immunophenotyping. Additionally, we would perform the human tissue construct assay to study the age-specific mechanism of action of the adjuvant formulations in a microphysiologic model.

We have demonstrated the ability of open access adjuvant formulations to induce robust Th1 immune responses in children via age-specific human *in vitro* modeling. Our observations raise the possibility that these open access adjuvants may benefit pediatric health globally by advancing age-specific vaccine development against infectious diseases with high levels of mortality and morbidity.

**Acknowledgements:**

We would like to thank all the study participants, phlebotomists, PVP members, and collaborators at the Children's Hospital Primary Care Clinic including Dr. Kathleen Conroy, Kristin Karpowicz, Renia Lorjuste and Kayla Lesch for their contributions to this study. We thank the Vaccine Formulation Institute (VFI), SEPPIC, 3M Corporate Research and Materials Laboratory, Access to Advanced Health Institute (AAHI), and PATH for providing us with adjuvant formulations. We thank Dr. Kevin Churchwell and Dr. Gary Fleisher of Boston Children's Hospital for their support for the *Precision Vaccines Program*.

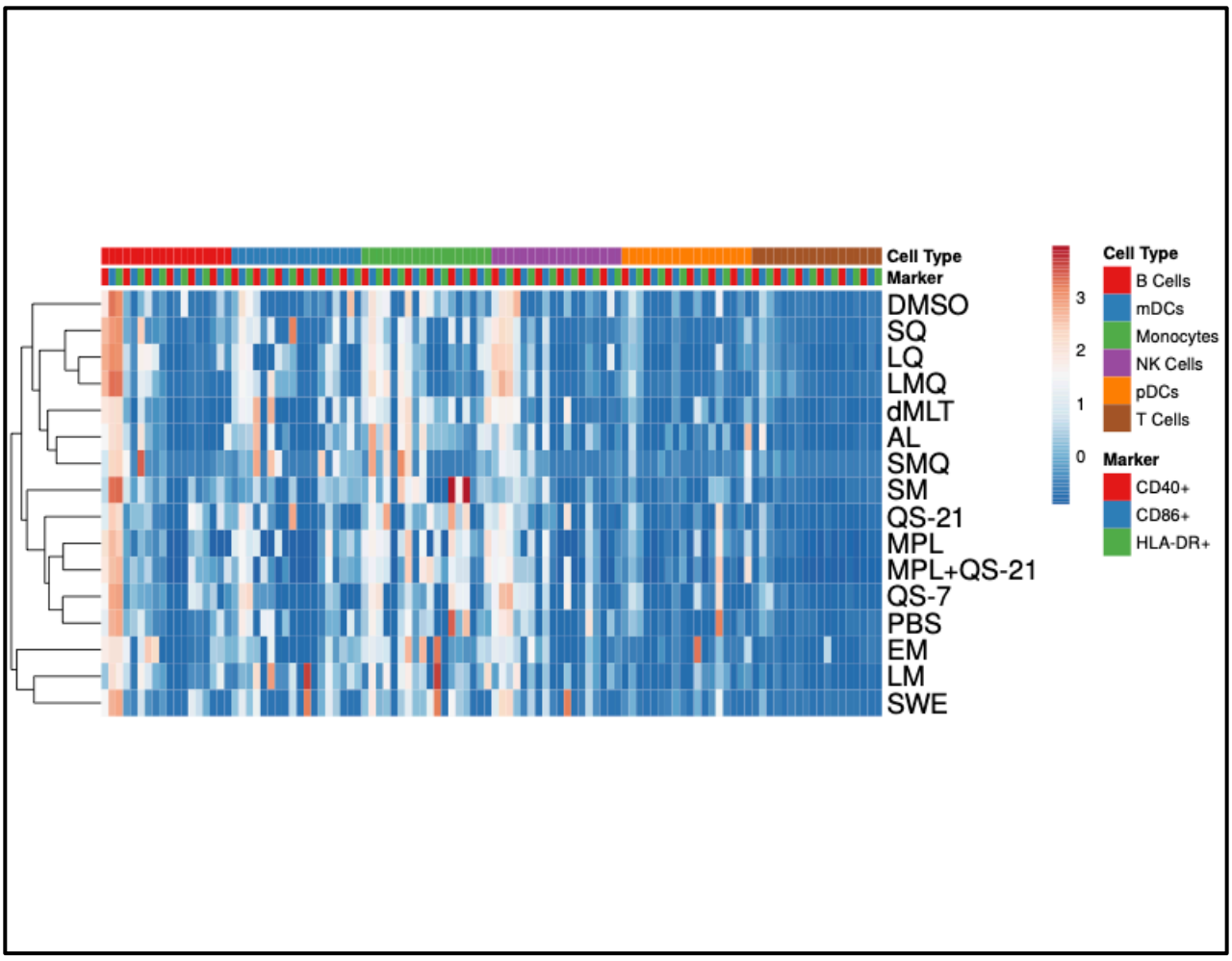
**Conflict of interest statement:**

SvH and OL are named inventors on a patent for a human *in vitro* system that models the action of adjuvants and vaccines, as well as several patent applications related to vaccine adjuvants that were not included in this study. The other authors declare no conflict of interest.

**Funding statement:**

This work was supported by the Bill & Melinda Gates Foundation, grant INV-004886 to SvH. Additional support for SvH and OL came from US National Institutes of Health (NIH)/National Institutes of Allergy and Infectious Diseases (NIAID) award Molecular Mechanisms of Combination Adjuvants (1U01AI124284). The *Precision Vaccines Program* is supported in part by the Boston Children's Hospital Department of Pediatrics and Chief Scientific Office.

**A)**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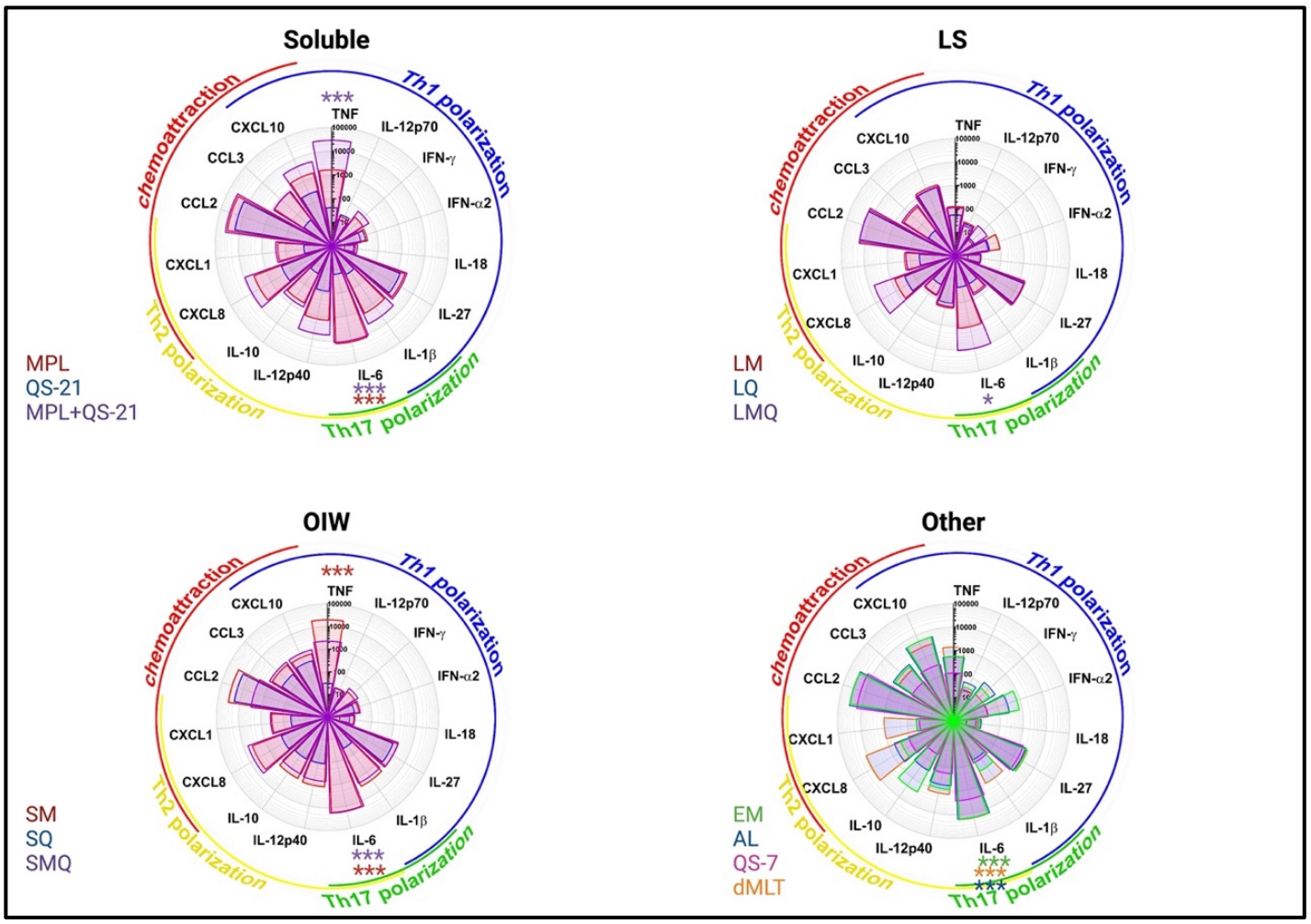
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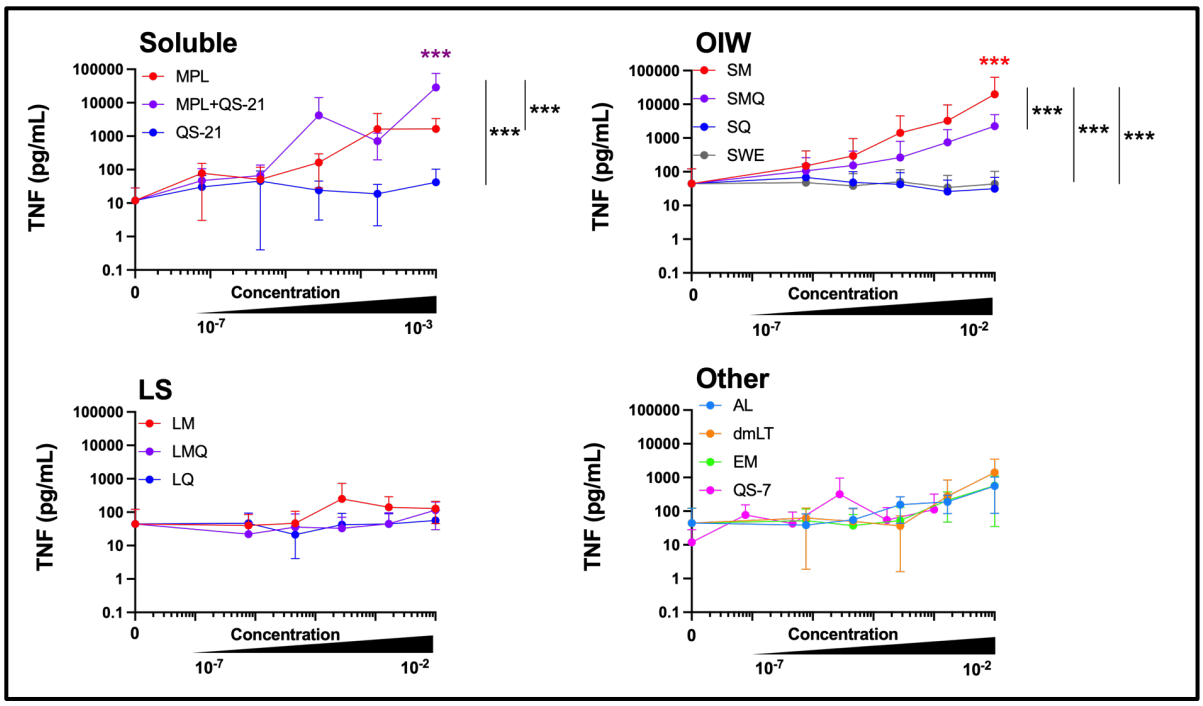
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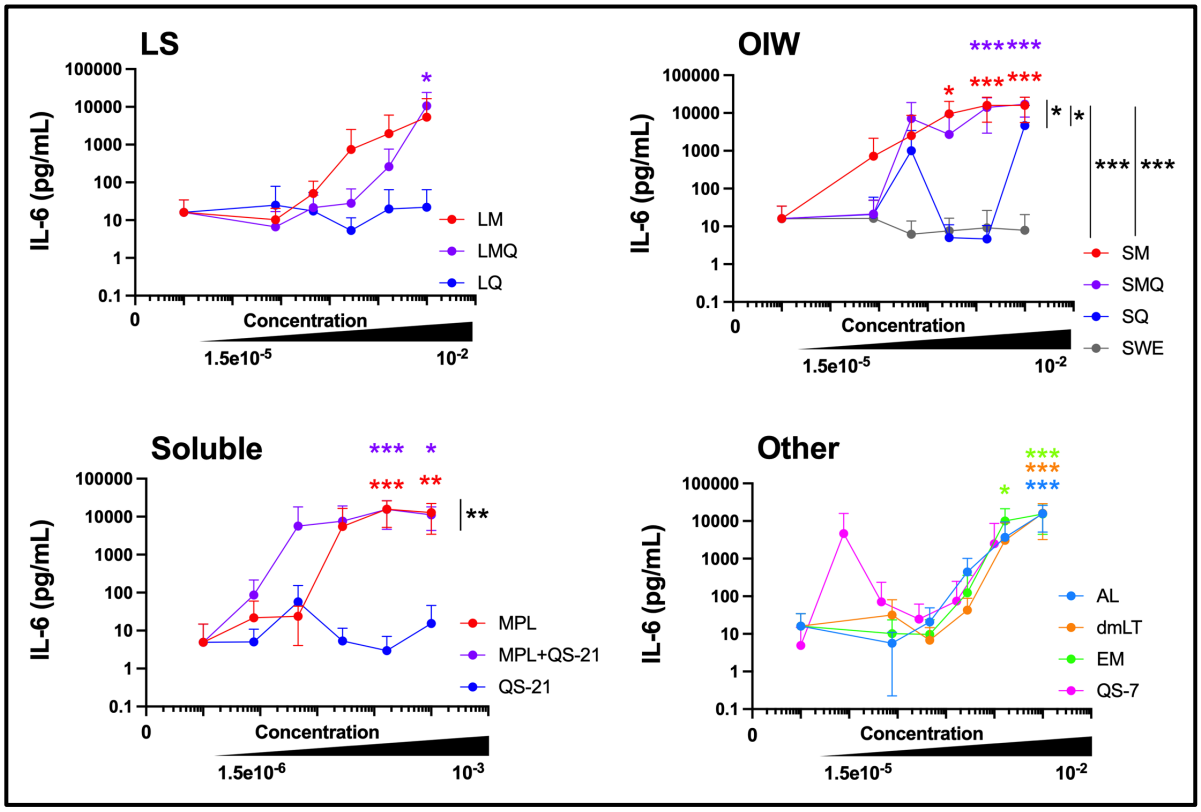
D)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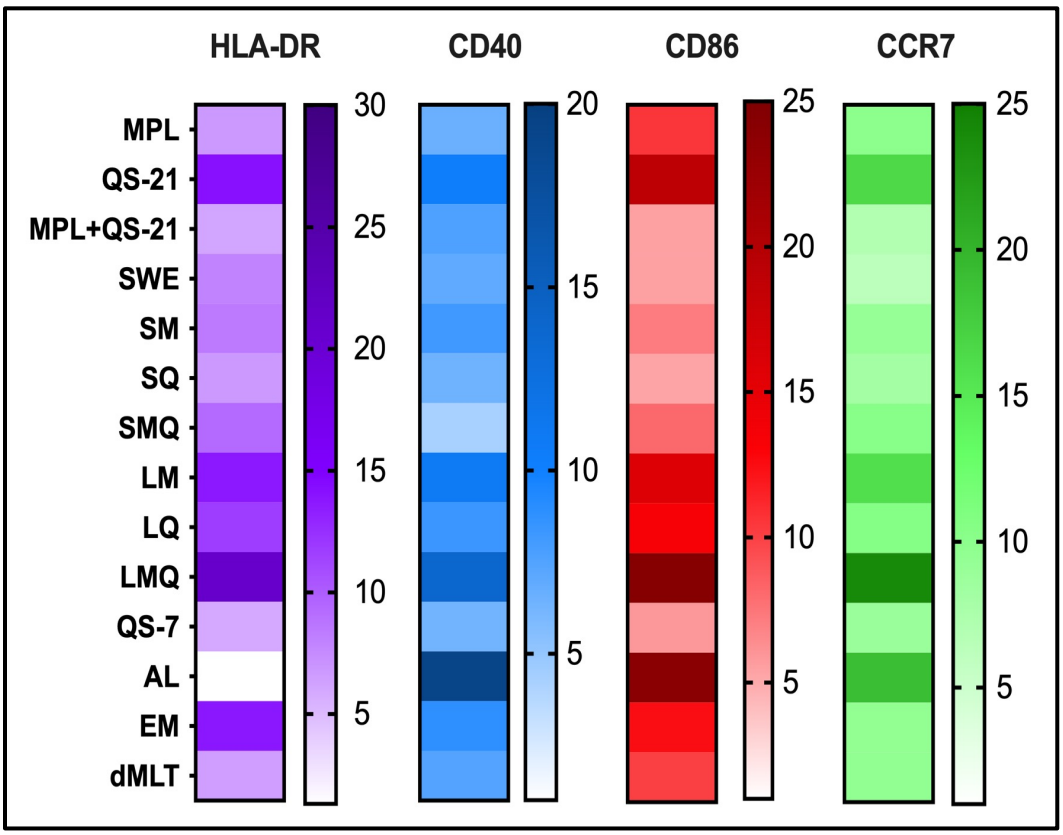
10-3
MPL
MPL+QS-21
QS-21
Other
AL
dmLT
EM
QS-7

***Figure 9: Liposomal formulations induce innate immune response potently and selectively in whole blood assay.*** *A) Heatmap depicting log10 mean fluorescence intensity of activation markers across cell types. B) Radar plots showing expression levels of cytokines in pg/mL induced by treatment of whole blood with adjuvants at a 1:100 dilution for LS, OIW and other, and 1:1000 dilution for soluble formulations. C) and D) Dose-response curves indicating TNF and IL-6 production across a five-point dilution series, along with a vehicle control. Top concentrations are least diluted, and further dilutions were 1/6 (v/v) in RPMI for all formulations. Stars above a data point on dose-response curves indicate statistical significance relative to vehicle control, stars adjacent to vertical bars on dose-response curves indicate statistically significant differences between treatment groups at a given concentration. Significance was calculated using a 2-way repeated measures ANOVA with Tukey's multiple comparison testing. N = 6. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)*

A)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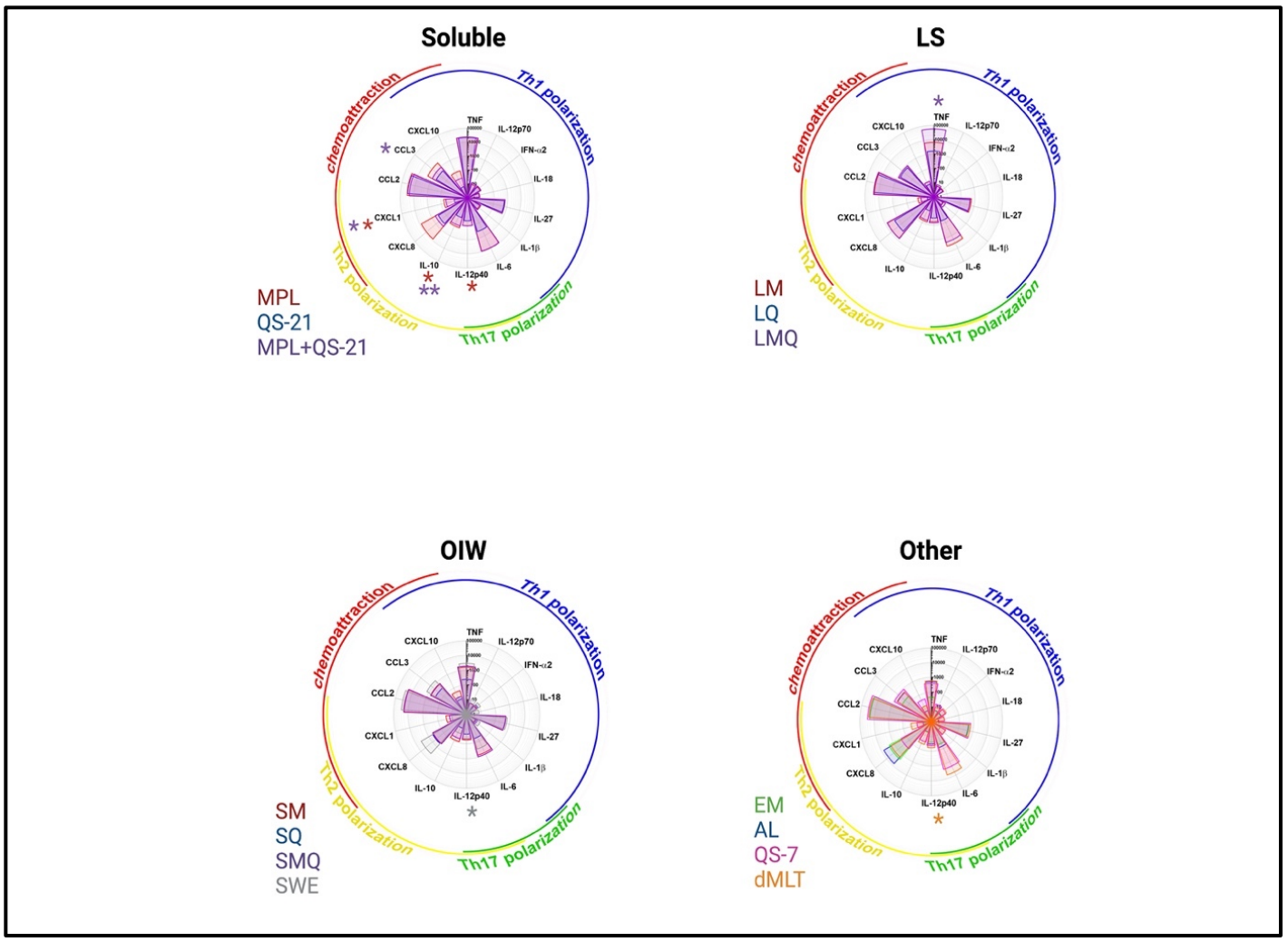
**B)**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CXCL1
CCL2
CCL3
CXCL10
MPL
QS-21
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Other
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AL
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dMLT

C)

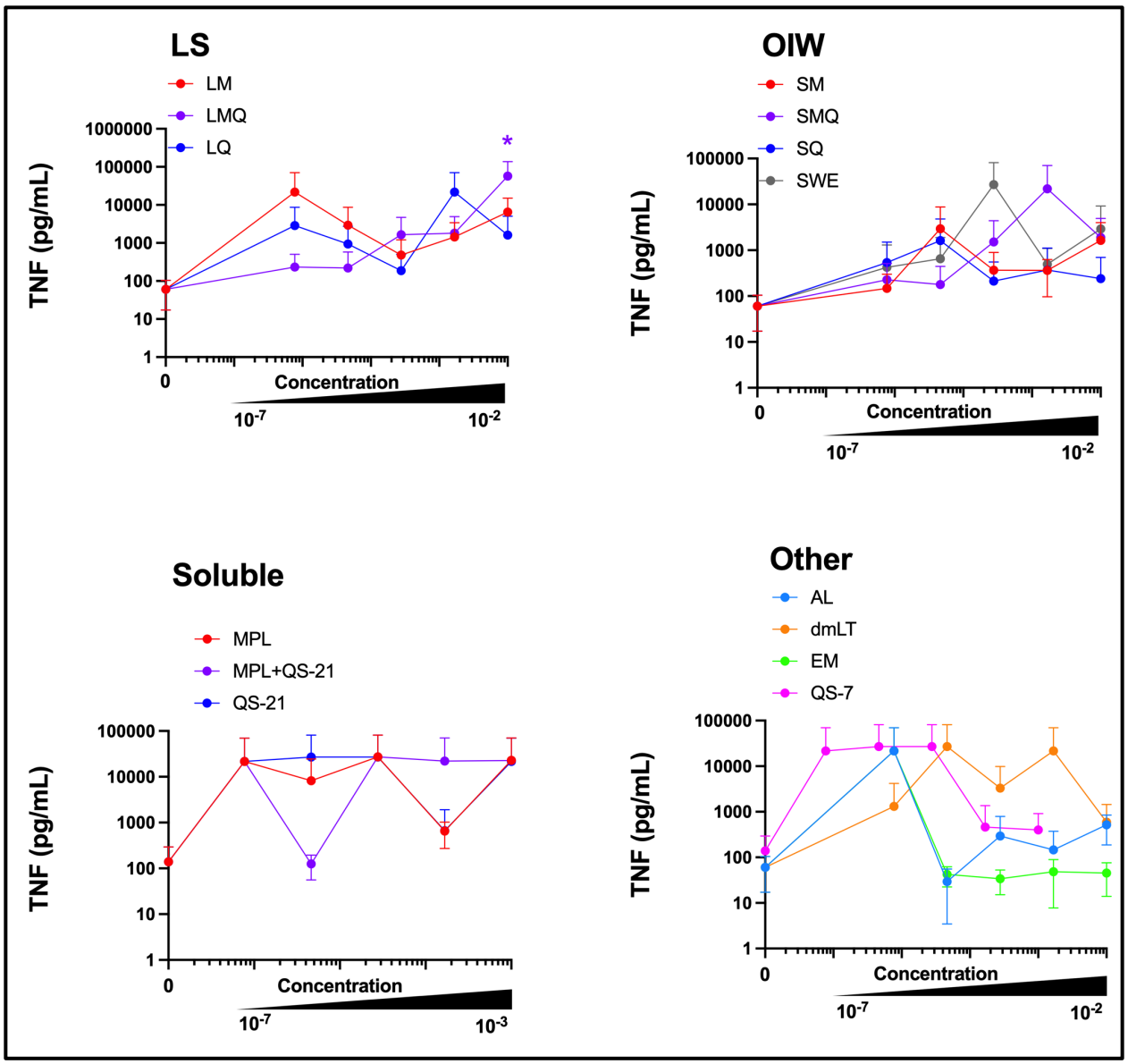


***Figure 10: Liposomal formulations of MPL and QS-21 activate monocyte-derived dendritic cells (MoDC) and promote production of Th1-polarizing cytokines in MoDC assay.*** *A) Surface expression of four activation markers based on log10 mean fluorescence intensity. B) Radar plots depicting expression levels of cytokines in pg/mL induced by adjuvant stimulation. C) Dose-response curves indicating TNF production across a five-point dilution series, along with a vehicle control. Top concentrations are*

*least diluted, and further dilutions were 1/6 (v/v) in RPMI for all formulations. Stars above a data point on dose-response curves indicate statistical significance relative to vehicle control. Significance was calculated using a 2-way repeated measures ANOVA with Tukey's multiple comparison testing. N = 5. (* p < 0.05, ** p < 0.01, *** p < 0.001)*

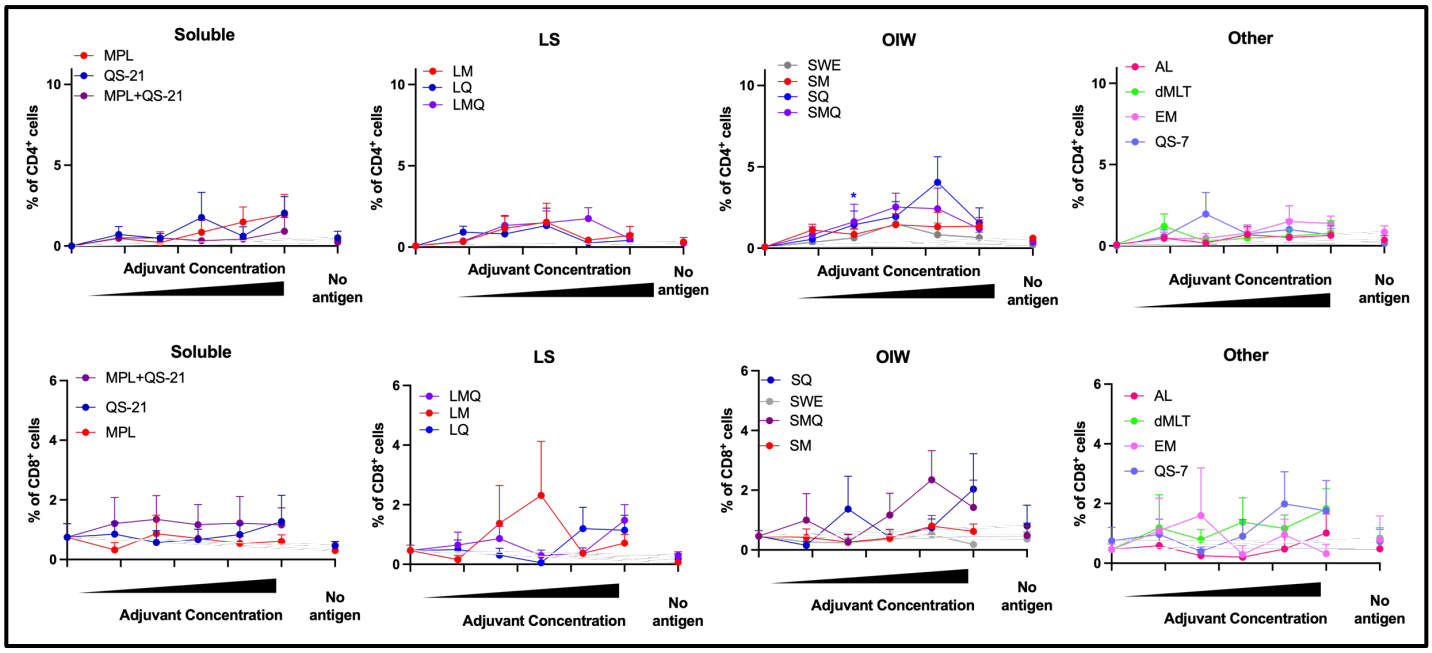


***Figure 11: MPL and QS-21 activate antigen-specific CD4⁺ and CD8⁺ T cells against influenza in dendritic cell-T cell interface assay.*** *MoDCs were stimulated with adjuvant formulations at 1:100 dilution for LS, OIW and other, and 1:1000 dilution for soluble formulations in the presence or absence of 5 µg/mL influenza HA antigen. Subsequent 1/6 (v/v) dilutions for all formulations before co-culture with autologous CD4⁺ or CD8⁺ T*

*cells was performed. Activated T cells were defined as $CD4^+$, $CD8^+$, $CD25^+$, $CD134^+$, or $CD154^+$. Stars above horizontal lines indicate statistically significant differences relative to a control with no antigen. Significance was calculated using a 2-way repeated measures ANOVA with Tukey's multiple comparison testing. N = 5. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)*

## Summary

Utilization of human *in vitro* assays to evaluate the mechanism of action of individual and combination adjuvants in liposomal, oil-in-water, and soluble formulations in age-specific settings inform their immune activation profile and potential application in vaccine development.

In the adult cohort, liposomal co-formulation of MPL and QS-21 (LMQ) was most potent in promoting maturation and differentiation of dendritic cells, production of Th1-polarizing cytokines, and activation of antigen-specific $CD4^+$ and $CD8^+$ T cells. Flow cytometric analysis of activation markers CD40, CD80 and CD86 demonstrated monocytes as the primarily activated cells in whole blood assay, followed by B cells and NK cells. The fate of the activated monocytes towards a mature dendritic cell phenotype was determined in the microphysiologic tissue construct, promoted mainly by LMQ. LMQ synergistically induced the production of Th1 cytokines IL-1$\beta$, IL-12p70 and IL-18 in monocyte-derived dendritic cell assay. Activation of SARS-CoV-2 spike antigen-specific $CD4^+$ and $CD8^+$ T cells was driven by MPL- and QS-21-containing formulations respectively in dendritic cell-T cell co-culture assay.

In the pediatric cohort, cell and volume pre-requisites were downscaled for each *in vitro* platform to maintain comparison with adults and draw age-specific inferences. Compared to adults, monocyte-derived dendritic cells in children did not synergistically induce production of inflammasome-mediated cytokines, IL-1$\beta$ and IL-18. However, MPL and QS-21 acted synergistically in LMQ to enhance production of TNF. MPL in liposomal and oil-in-water formulations drove reactivation of influenza HA antigen-specific $CD4^+$ T

cells, while QS-21 was responsible for driving activation of flu HA antigen-specific $CD8^+$ T cells.

## General discussion

Through human *in vitro* modeling of various adjuvant formulations, including those developed for global open access that may have a similar mechanism of action to formulations that are licensed and protected by intellectual property rights, these projects help unravel the underlying mechanisms that shape immune responses to adjuvanted vaccines. With the knowledge of which formulations proved potent and safe in these *in vitro* assays, we were able to test their effect in age-specific settings and compare results between adult and pediatric populations.

A limitation of these studies is the small sample size (n = 5-6 per assay platform). However, this was sufficient to draw statistically significant conclusions regarding the mechanism of action of the tested adjuvant formulations in age-specific settings. An increase in sample size would likely enable study of demographic differences, kinetics of cytokine production, and measurement of additional analytes that may become statistically significant.

With improved understanding of the cellular and molecular immune responses induced by open access adjuvant formulations in adults and children, application of these adjuvants could be further explored among other populations such as infants, older

adults, immunocompromised individuals, and pregnant women. Promising adjuvant formulations could be tested for other diseases with high burden of mortality and morbidity such as RSV or malaria. The broad immunogenic profile of adjuvants and their ability to successfully induce $CD8^+$ T cell response may improve ongoing efforts to design a universal vaccine against influenza as the cytotoxic effect could result in a less symptomatic infection, and address mutational drift and strain variations (124,125). This cytotoxic mechanism could also have a potential implication in cancer therapeutics (126). The use of lipid-based nanoparticle technology in vaccine design improves particle avidity and size, providing an effective strategy to increase immunogenicity in next-generation vaccines by enhancing antigen uptake by immune cells, lymph node trafficking, and B-cell activation (47). The comparability of open access adjuvant formulations to those that are licensed would allow their unrestricted use for the development of low-cost vaccines in low- and middle-income countries where the burden of vaccine-preventable diseases remains high.

## Acknowledgements

I am indebted to the *Precision Vaccines Program* (PVP) for supporting my work for the past two years. Starting from no bench experience and reaching this point of my thesis would not have been possible without Dr. Simon van Haren's excellent mentorship – I am deeply grateful to him for the opportunities he provided for my learning and growth. For their constant support, I am thankful to all faculty and members of the PVP – especially Dr. Ofer Levy, Dr. Al Ozonoff, and Dr. Joann Arce. I owe special thanks to Kevin Ryff for his efforts and contribution to Project 2, and Simon Doss-Gollin for his work on Project 1.

I sincerely acknowledge the valuable guidance provided by my thesis committee members Dr. Rick Malley and Dr. Suman Srinivasa, and my program directors Dr. Martina McGrath, Dr. Rosalyn Adam, Dr. Finnian McCausland, Dr. Ajay Singh, and Dr. Lourdes Chada. I am incredibly grateful for having the opportunity to learn from all my professors throughout this program.

My classmates and friends made the journey of the last couple of years extremely special – I am grateful for each one of them.

Finally, I can never express enough thanks to my husband, Dr. Stephen Samuel, for always believing in me and supporting my dreams.

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