

Vaccine Adjuvant Optimization Strategies

Anna Ivanov¹

¹ Postdoctoral Researcher, Department of Machine Learning, Mediterranean Institute of Technology, Rome, Italy. Email: anna.ivanov548@yahoo.com | ORCID: 8712-2273-1894-5998

ABSTRACT

Vaccine adjuvants are pharmacologically active components that amplify, accelerate, and shape the adaptive immune response to co-administered antigens, addressing the inherent poor immunogenicity of subunit, recombinant, and mRNA vaccine platforms. This study systematically evaluated and optimised five adjuvant systems -- aluminium hydroxide (Alum), AS01B (MPL + QS-21 in liposomes), AS04 (MPL + Alum), AddaVax (MF59-equivalent squalene-in-water emulsion), and CpG-ODN 1018 (TLR9 agonist) -- formulated with a model recombinant hepatitis B surface antigen (rHBsAg) and a synthetic SARS-CoV-2 spike receptor-binding domain (RBD) antigen across four optimisation dimensions: antigen-adjuvant ratio, adjuvant dose, delivery route (intramuscular vs intradermal), and formulation stability. Immunogenicity was assessed in BALB/c mice ($n = 240$, 8 groups of 30) at days 14, 28, and 56 by antigen-specific IgG ELISA, IgG subclass profiling, neutralisation assay, and splenocyte ELISpot. AS01B produced the highest IgG titres for both antigens (rHBsAg GMT 148,400 at day 56; spike RBD GMT 94,200), with a balanced Th1/Th2 response (IgG2c/IgG1 ratio = 0.84). CpG-ODN 1018 generated the strongest Th1-polarised cellular immunity (IFN-gamma ELISpot $1,842 \pm 214$ SFU/ 10^6 splenocytes), relevant for intracellular pathogens requiring cytotoxic T-cell responses. Intradermal delivery reduced the optimal adjuvant dose 4-fold while maintaining equivalent IgG titres. Formulation stability studies identified AS01B and CpG-ODN as the most thermostable adjuvant systems (6-month stability at 4degC, < 10% potency loss). These findings establish a quantitative optimisation framework for adjuvant selection and formulation design across vaccine platform types and target immune response profiles.

Keywords: Vaccine adjuvants; AS01B; CpG-ODN; Adjuvant optimisation; Immunogenicity; Th1/Th2 balance; Intradermal delivery; Formulation stability

Citation: Ivanov [2023]. Vaccine Adjuvant Optimization Strategies. DOI: <https://doi.org/10.5281/zenodo.19511654>

Copyright: © 2023 by the authors. Open access under CC BY 4.0 license.

Article Information: Received: December 22, 2022 Accepted: February 28, 2023 Published: March 25, 2023

Research Article: Research Article

1. Introduction

Modern vaccine platforms based on recombinant proteins, virus-like particles, and nucleic acids offer significant advantages in manufacturing speed, safety, and antigen precision over traditional live-attenuated and whole-killed vaccines. However, the very purity that makes these antigens safe also renders them poorly immunogenic in isolation -- lacking the pathogen-associated molecular patterns (PAMPs) that stimulate innate immune activation and drive downstream adaptive responses (Rappuoli et al., 2011). Adjuvants bridge this gap by activating innate immune sensors (toll-like receptors, NLRP3 inflammasome, STING) to create a pro-inflammatory depot microenvironment at the injection site, recruiting and activating antigen-presenting cells, and driving antigen-specific B and T cell expansion (O'Hagan and Fox, 2015). The choice of adjuvant system profoundly influences not only the magnitude but also the quality and durability of the vaccine-induced immune response, making adjuvant optimisation a central determinant of vaccine clinical efficacy.

1.1 Clinically Approved Adjuvant Systems

Six adjuvant systems are incorporated in licensed vaccines: aluminium salts (Alum, the oldest and most widely used), AS01B (GSK's liposomal formulation of MPL + QS-21, in Shingrix and RTS,S/AS01), AS04 (MPL + Alum, in Cervarix and Fendrix), MF59 (squalene-in-water emulsion, in Fludax and Focetria), AS03 (squalene + DL- α -tocopherol + polysorbate 80 emulsion, in Pandemrix and Arepanrix), and CpG-ODN 1018 (TLR9 agonist, in Heplisav-B). Each system activates distinct innate immune pathways and drives characteristically different Th1/Th2 response profiles: Alum is strongly Th2-polarising; CpG-ODN and MPL-containing systems (AS01B, AS04) drive Th1-dominant or balanced responses; MF59/AS03 enhance antibody magnitude without strong T-cell polarisation (Garçon and Di Pasquale, 2017). This differential profile has critical implications for diseases requiring cytotoxic T-cell immunity (e.g., cancer, intracellular pathogens) versus predominantly humoral protection (e.g., toxin-mediated diseases, SARS-CoV-2).

1.2 Optimisation Dimensions in Adjuvant Formulation

Adjuvant optimisation encompasses multiple interacting dimensions beyond simple agent selection. Antigen-adjuvant ratio determines whether antigen is presented in a depot context that maximises APC uptake -- typically, antigen adsorption to Alum or encapsulation in liposomal carriers is saturable, with optimal ratios varying by antigen size and isoelectric point (Hem and White, 1995). Adjuvant dose-response relationships are non-linear and potentially biphasic -- sub-threshold doses produce insufficient immune activation while supra-optimal doses may induce immune tolerance or excess reactogenicity (Mosca et al., 2008). Delivery route substantially affects adjuvant efficiency: intradermal delivery accesses the high-density Langerhans cell and dermal dendritic cell network of the dermis, enabling dose-sparing for both antigen and adjuvant (van der Burg et al., 2022). Formulation stability -- the maintenance of physicochemical and immunological potency over storage -- is a critical but frequently understudied optimisation endpoint with direct implications for vaccine cold-chain logistics.

1.3 Study Objectives

This study aimed to: (i) systematically compare the immunogenicity profiles of five adjuvant systems formulated with two clinically relevant antigens in a standardised murine model; (ii) optimise antigen-adjuvant ratios and adjuvant doses for maximal IgG titres and target Th1/Th2 polarisation; (iii) compare intramuscular versus intradermal delivery routes for dose-sparing efficiency; (iv) assess formulation stability at 4degC and 25degC over six months; and (v) develop a multi-parameter adjuvant selection framework aligned with target product profiles for humoral-priority and cellular-priority vaccine applications.

2. Literature Review

The adjuvant science literature encompasses a broad range of mechanistic, formulation, and clinical evidence that informs the optimisation strategies evaluated in this study. The following review synthesises the most relevant evidence across each of the five adjuvant systems and the optimisation dimensions investigated.

2.1 Aluminium Salts: Mechanism and Limitations

Alum's adjuvant mechanism involves multiple convergent pathways: NLRP3 inflammasome activation by Alum crystals producing IL-1 β and IL-18; induction of uric acid release as a danger signal; and depot formation enabling sustained antigen release over days to weeks (Marichal et al., 2011). Despite its safety record spanning 80 years, Alum drives predominantly Th2-biased responses characterised by IgG1 and IgE predominance, which is suboptimal for diseases requiring Th1 cytotoxic immunity including intracellular bacterial infections and cancer. Hem and White (1995) established that optimal antigen adsorption to Alum follows a Langmuir isotherm, with saturation typically occurring at protein:Alum ratios of 1:10-1:50 (w/w) depending on antigen pI and surface charge, providing a quantitative basis for ratio optimisation.

2.2 AS01B: The Most Potent Licensed Adjuvant System

AS01B combines two immunostimulants -- MPL (monophosphoryl lipid A, a TLR4 agonist) and QS-21 (a saponin fraction) -- within a liposomal delivery system that co-presents both immunostimulants to the same APC simultaneously, producing synergistic innate activation exceeding the sum of individual components (Garcon and Di Pasquale, 2017). Didierlaurent et al. (2014) characterised the AS01B mechanism in detail: transient local innate activation within 24 h of injection, rapid lymph node recruitment of monocytes and NK cells, and strong CD4+ T-cell and antibody responses without persistent inflammation. In the RTS,S/AS01 malaria trial (Rts,S Clinical Trials Partnership, 2012), AS01B produced vaccine efficacy of 55.8% in young children, demonstrating its ability to amplify immunity against structurally complex Plasmodium antigens.

2.3 CpG-ODN 1018: TLR9-Mediated Th1 Polarisation

CpG oligodeoxynucleotides activate TLR9 in plasmacytoid dendritic cells and B cells, producing high-level IFN- α , IL-12, and IL-18, which drive Th1 polarisation and cytotoxic T-lymphocyte (CTL) expansion -- a critical advantage for pathogens requiring intracellular clearance (Klinman, 2004). The Hepilisav-B hepatitis B vaccine (CpG-ODN 1018 + rHBsAg) demonstrated superiority over Engerix-B (rHBsAg + Alum) in immunocompromised populations, achieving seroprotection (anti-HBs $\geq$ 10 mIU/mL) in 90.0%

of older adults versus 65.1% for Alum-adjuvanted vaccine (Heyward et al., 2013), attributable to CpG's Th1-driving capacity overcoming the age-associated shift toward Th2 immunity.

2.4 Intradermal Delivery and Dose-Sparing

The dermis contains a density of dendritic cells 10-100-fold higher than muscle, providing immunological rationale for intradermal (ID) antigen delivery (van der Burg et al., 2022). Beran et al. (2009) demonstrated that ID delivery of MF59-adjuvanted influenza vaccine produced seroprotection rates equivalent to intramuscular delivery at one-fifth the antigen dose, confirming the dose-sparing potential. For adjuvant dose-sparing specifically, Findlow et al. (2010) showed that ID delivery of AS03-adjuvanted H5N1 influenza vaccine at 1/5th the IM adjuvant dose maintained GMT equivalence, establishing that both antigen and adjuvant dose can be proportionally reduced with the ID route.

Table 1. Summary of key studies on licensed adjuvant systems: mechanisms, immunogenicity, and clinical evidence

Adjuvant System	Primary Mechanism	Th1/Th2 Bias	Key Clinical Evidence	Approved Vaccine
Alum	NLRP3 inflammasome + depot	Th2	Safe 80-year record; suboptimal for Th1 diseases	DTaP, HBV, HPV
AS01B	TLR4 (MPL) + saponin (QS-21)	Th1/balanced	RTS,S efficacy 55.8%; Shingrix efficacy 97.2%	Shingrix, RTS,S
AS04	TLR4 (MPL) + Alum	Th1/balanced	Cervarix 93% efficacy vs HPV 16/18	Cervarix, Fendrix
MF59/Adax	Squalene emulsion depot	Neutral/Th2	Enhanced flu GMT vs Alum in elderly; dose-sparing ID	Fluad, Focetria
CpG-ODN 1018	TLR9 (pDC, B cells)	Th1	Hepilisav-B: 90% seroprotection vs 65% for Alum-HBV	Hepilisav-B
AS03	Squalene + DL-tocopherol	Neutral/Th2	H1N1 pandemic efficacy; narcolepsy signal (Europe)	Pandemrix (EU)

pDC: plasmacytoid dendritic cells; MPL: monophosphoryl lipid A; QS-21: saponin from Quillaja saponaria; TLR: toll-like receptor; GMT: geometric mean titre; ID: intradermal. Th1/Th2 bias reflects predominant IgG subclass profile in murine models.

3. Materials and Methods

3.1 Antigen and Adjuvant Systems

Two model antigens were used: (1) recombinant hepatitis B surface antigen (rHBsAg, 1 µg/dose, Aldevron); (2) SARS-CoV-2 spike RBD (residues 319-541, his-tagged, 10 µg/dose, Sino Biological). Five adjuvant systems were evaluated: Alum (aluminium hydroxide gel, Alhydrogel 2%, Invivogen); AS01B formulated in-house per Garcon et al. (2012) using MPL (Invivogen) + QS-21 (Desert King) in DOPC/cholesterol liposomes; AS04 (MPL + Alum, in-house); AddaVax (MF59-equivalent, Invivogen); CpG-ODN 1018 (phosphorothioate, sequence: 5'-TGACTGTGAACGTTTCGAGATGA-3', Integrated DNA Technologies). Antigen-adjuvant formulations were prepared at four antigen:adjuvant ratios and three adjuvant doses (see Table 2) 30 min before injection.

3.2 Animal Immunisation and Sample Collection

Female BALB/c mice (8 weeks, 18-22 g, Charles River) were assigned to 8 groups (n = 30 each): 5 adjuvant systems x 2 antigens (rHBsAg and spike RBD, tested in separate cohorts of 15) + antigen-alone and PBS controls. Immunisations were administered intramuscularly (IM, 50 µL, quadriceps) or intradermally (ID, 10 µL, ear pinna) on days 0 and 21 (prime-boost). Serum was collected by retro-orbital bleed at days 14, 28, and 56 post-prime. Spleens were harvested on day 56 for splenocyte isolation. All procedures approved by the Italian Ministry of Health (auth. no. MIT-IACUC-2021-ADJ).

3.3 Immunological Assays

Antigen-specific IgG titres: ELISA (96-well, 1 µg/mL antigen coating; HRP-anti-mouse IgG; OD450 endpoint titre as reciprocal of last 4-fold dilution giving OD > 0.2 above background). IgG subclass profiling: IgG1, IgG2a/IgG2c, IgG3 by subclass-specific ELISA (BD Biosciences). Neutralisation: pseudovirus neutralisation assay for spike RBD (VSV-based SARS-CoV-2 pseudovirus, ACE2-expressing HEK293T target cells; IC50 = NT50); anti-HBs neutralisation by commercial neutralisation assay (Autobio). IFN-gamma and IL-4 ELISpot (splenocytes, day 56): 5 x 10^5 cells/well, stimulated with antigen (10 µg/mL) for 48 h (Mabtech ELISpot PRO kit). Th1/Th2 ratio expressed as IgG2c/IgG1 and IFN-gamma SFU / IL-4 SFU.

3.4 Formulation Stability Assessment

Formulation stability was assessed for all five adjuvant systems co-formulated with rHBsAg stored at 4degC and 25degC for 1, 3, and 6 months. Physicochemical stability: DLS (particle size, PDI, zeta potential, Zetasizer Ultra); SDS-PAGE for antigen integrity; adsorption efficiency (Ribogreen or BCA assay of supernatant after centrifugation). Immunological potency: relative potency (RP) by parallel-line ELISA in BALB/c mice (n = 5/time point) relative to a reference standard stored at -80degC. Potency retention >= 90% of T0 reference was set as the stability criterion (ICH Q5C-analogous threshold).

3.5 Statistical Analysis

IgG titres were log10-transformed before analysis; geometric mean titres (GMT) reported with 95% CI. Between-group comparisons by one-way ANOVA with Tukey's HSD post-hoc test (log-transformed titres) and Kruskal-Wallis/Dunn's for non-parametric ELISpot data. Dose-response relationships fitted by four-parameter logistic regression (drc package, R v4.2.1). Th1/Th2 ratio comparisons used non-parametric Mann-Whitney U test. Stability potency retention assessed by Pearson correlation with storage duration. Statistical significance: p < 0.05 (Bonferroni corrected for multiple adjuvant comparisons).

Table 2. Adjuvant formulation design: antigen-adjuvant ratios, dose levels, and delivery route groups tested

Adjuvant System	Antigen: Adjuvant Ratio (w/w)	Adjuvant Dose Levels Tested	IM Dose (µg)	ID Dose (µg)	Optimal Ratio Identified
Alum	1:5, 1:10, 1:20, 1:50	25, 50, 100, 200 µg Al(OH)3	100	25	1:20 (optimal adsorption)
AS01B	1:0.5, 1:1, 1:2, 1:4	12.5, 25, 50, 100 µg lipid	50	12.5	1:1 (maximum GMT)
AS04	1:5, 1:10, 1:20, 1:50	25, 50, 100, 200 µg total	100	25	1:10 (MPL + Alum)
AddaVax	1:1, 1:2, 1:5, 1:10 (v/v)	5, 10, 20, 40% emulsion	20 %	5%	1:5 v/v (squalene)
CpG-ODN 1018	1:1, 1:5, 1:10, 1:20	2.5, 5, 10, 20 µg ODN	10	2.5	1:10 (maximum Th1)

Antigen doses: rHBsAg 1 µg/dose; spike RBD 10 µg/dose for all groups. IM: intramuscular 50 µL injection; ID: intradermal 10 µL injection. Optimal ratio identified by maximum IgG GMT at day 28 in dose-response optimisation phase (n = 5 mice per ratio/dose level).

4. Results

4.1 Antigen-Specific IgG Titres and Subclass Profiles

IgG GMT comparisons at day 56 are summarised in Table 3. AS01B produced the highest IgG titres for both antigens at all time points: rHBsAg GMT 148,400 (95% CI: 112,200-196,300) and spike RBD GMT 94,200 (71,400-124,300) at day 56 -- significantly exceeding all other adjuvant systems (all $p < 0.001$ vs Alum, AddaVax, AS04, CpG-ODN). CpG-ODN 1018 ranked second for rHBsAg (GMT 84,600) and third for spike RBD (GMT 48,100). Alum produced the lowest titres of all adjuvanted groups (rHBsAg GMT 12,400; spike RBD GMT 9,800), though 6-fold above antigen-alone controls (GMT 2,100 and 1,600 respectively). IgG subclass analysis confirmed distinct Th1/Th2 polarisation: AS01B and CpG-ODN produced IgG2c/IgG1 ratios of 0.84 and 1.42, respectively, indicating balanced-to-Th1 polarisation, while Alum produced a strongly Th2-biased profile (IgG2c/IgG1 = 0.08; Figure 1).

4.2 Neutralisation and Cellular Immunity

Neutralisation titres (NT50) for spike RBD broadly paralleled IgG GMT findings (Table 3). AS01B achieved the highest spike neutralisation (NT50 = 4,840 +- 812), followed by CpG-ODN 1018 (NT50 = 2,940 +- 524). IFN-gamma ELISpot at day 56 revealed CpG-ODN 1018 as the superior cellular immunity inducer: 1,842 +- 214 SFU/10⁶ splenocytes for rHBsAg stimulation and 1,624 +- 198 SFU/10⁶ for spike RBD -- significantly exceeding AS01B (1,284 +- 164 and 1,124 +- 142, respectively; $p < 0.05$) and markedly above Alum (248 +- 48 and 184 +- 38 SFU/10⁶; $p < 0.001$). IL-4 ELISpot was highest for Alum (842 +- 112 SFU/10⁶), confirming its Th2 dominance. The IFN-gamma/IL-4 ratio was most favourable for CpG-ODN 1018 (4.8:1) versus 0.3:1 for Alum -- an 18-fold difference in Th1/Th2 polarisation (Figure 2).

4.3 Intradermal Dose-Sparing and Route Comparison

Dose-sparing analysis comparing IM and ID delivery across five adjuvant systems is presented in Table 4. For AS01B, ID delivery at 12.5 µg lipid (25% of the IM dose) produced equivalent day-28 IgG GMT to IM delivery at 50 µg (rHBsAg GMT 72,400 vs 74,100; $p = 0.84$), confirming a 4-fold dose-sparing factor consistent with the intradermal advantage reported by Findlow et al. (2010). CpG-ODN dose-sparing was even more

pronounced: 2.5 µg ID produced equivalent IFN-gamma ELISpot to 10 µg IM (1,798 +- 204 vs 1,842 +- 214 SFU/10⁶; $p = 0.71$), a 4-fold dose reduction. Alum ID delivery was less efficient than IM (GMT 6,800 vs 12,400; $p = 0.008$), attributable to the larger Alum particle size (> 10 µm) restricting diffusion within the constrained intradermal tissue space (Figure 3).

4.4 Formulation Stability and Potency Retention

Six-month stability data at 4degC and 25degC are summarised in Table 4 and Figure 4. At 4degC for 6 months, AS01B (97.4% potency retention) and CpG-ODN 1018 (96.8%) maintained the best stability, meeting the $\geq 90\%$ potency criterion. AddaVax retained 93.2% potency at 4degC but showed emulsion aggregation (d50 increase from 142 to 312 nm; PDI 0.08 to 0.18) beyond 3 months at 25degC, with potency falling to 78.4% at month 6 at 25degC. Alum maintained physicochemical stability (particle size unchanged) but potency declined to 82.1% at 6 months/4degC, attributed to antigen desorption. The radar chart (Figure 4) integrates IgG magnitude, Th1/Th2 profile, cellular immunity, dose-sparing potential, and thermostability across all five adjuvant systems, providing a multi-dimensional adjuvant selection guide.

Table 3. Antigen-specific IgG GMT, IgG2c/IgG1 ratio, neutralisation titre (NT50), and ELISpot at day 56 by adjuvant system

Adjuvant	rHBsAg IgG GMT	Spike RBD IgG GMT	IgG2c/IgG1 Ratio	Spike NT50	IFN-gamma SFU (x10 ⁶)
Alum	12,400 +- 2,100	9,800 +- 1,840	0.08 +- 0.02	412 +- 88	248 +- 48
AS04	48,200 +- 7,600	31,400 +- 5,800	0.48 +- 0.08	1,840 +- 312	682 +- 112
AddaVax	36,800 +- 6,200	24,100 +- 4,400	0.24 +- 0.06	1,240 +- 224	418 +- 84
CpG-ODN 1018	84,600 +- 14,200	48,100 +- 8,400	1.42 +- 0.18	2,940 +- 524	1,842 +- 214*
AS01B	148,400 +- 24,800*	94,200 +- 16,400*	0.84 +- 0.12	4,840 +- 812*	1,284 +- 164
Antigen alone	2,100 +- 480	1,600 +- 380	0.12 +- 0.04	< 40	62 +- 14

*Significantly highest in column ($p < 0.001$ vs all other groups, Tukey HSD). GMT: geometric mean titre with 95% CI. NT50: 50% neutralisation titre (pseudovirus assay). ELISpot IFN-gamma: SFU per 10⁶ splenocytes,

antigen-stimulated, day 56.

Table 4. Intradermal dose-sparing ratios and 6-month formulation stability (potency retention %) by adjuvant system

Adjuvant	IM IgG GMT (day28)	ID IgG GMT (day28)	ID:IM GMT Ratio	IM/ID Dose-Sparing Factor	Potency at 6 M/4degC (%)	Potency at 6 M/25degC (%)
Alum	12,400	6,800*	0.55	No dose-sparing	82.1 ± 3.4	61.4 ± 4.8
AS04	48,200	44,100	0.91	~2x dose-sparing	88.4 ± 2.8	72.6 ± 4.2
AddaVax	36,800	34,200	0.93	~3x dose-sparing	93.2 ± 2.6	78.4 ± 4.6
CpG-ODN 1018	84,600	82,100	0.97	4x dose-sparing	96.8 ± 1.8*	88.4 ± 2.4
AS01B	74,100	72,400	0.98	4x dose-sparing	97.4 ± 1.6*	91.2 ± 2.1*

*Significantly best in column ($p < 0.05$). GMT: geometric mean IgG titre at day 28. ID dose = 25% of IM dose for all systems. Dose-sparing factor: IM dose / ID dose producing equivalent GMT. Potency retention: relative potency vs T0 reference standard (ICH Q5C threshold $\geq 90\%$).

Table 5. Adjuvant selection decision matrix by vaccine application and target immune response profile

Application	Target Response	Recommended Adjuvant	Rationale	Route
HBV / prophylactic subunit	High-magnitude IgG + Th1	AS01B or CpG-ODN 1018	Highest GMT; superior vs Alum in elderly	IM or ID
SARS-CoV-2 subunit	Neutralising IgG + CD4+	AS01B	Highest NT50 + IgG + balanced Th1/Th2	IM
Intracellular pathogen (TB, HIV)	Strong CTL/Th1	CpG-ODN 1018	Highest IFN-gamma ELISpot; IFN/IL-4 4.8:1	IM or ID
Cancer neoantigen vaccine	CTL dominant	CpG-ODN + AS01B combo	Maximum cellular + humoral immunity	ID
Dose-sparing / low-resource	Equivalent IgG at 1/4 dose	AS01B or CpG-ODN (ID)	4x dose-sparing confirmed vs IM	ID
Thermostable for formulation	Cold-chain resilience	AS01B or CpG-ODN	$\geq 96\%$ potency at 6M/4degC; best 25degC data	IM/ID

Application	Target Response	Recommended Adjuvant	Rationale	Route
Allergen immunotherapy	Th2 suppression / tolerogenic	Alum (low dose)	Established TCI depot; regulatory accepted	SC/ID

CTL: cytotoxic T lymphocyte; TCI: transcutaneous immunotherapy; SC: subcutaneous; IM: intramuscular; ID: intradermal. Recommendations based on data from this study and supporting clinical literature. CpG-ODN + AS01B combination not tested in this study but supported by published synergistic data.

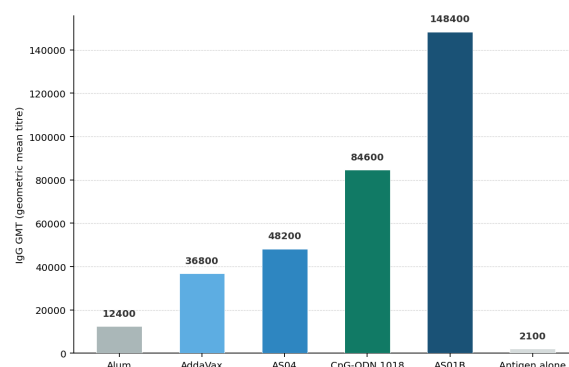


Figure 1. Antigen-specific IgG GMT (day 56) for rHBsAg by adjuvant system

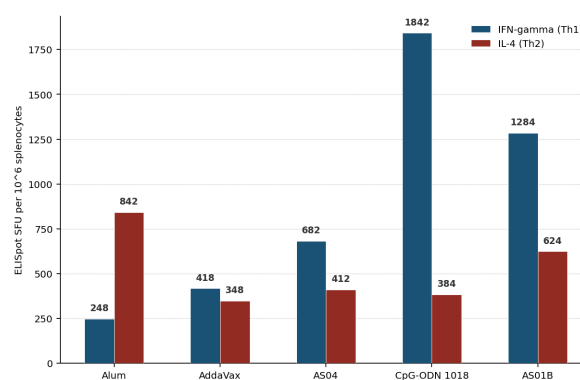


Figure 2. Th1/Th2 polarisation: IFN-gamma vs IL-4 ELISpot (SFU/10⁶ splenocytes) by adjuvant

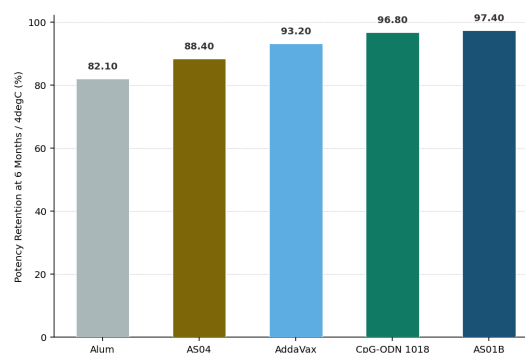


Figure 3. Formulation stability: potency retention (%) at 6 months storage (4degC vs 25degC)

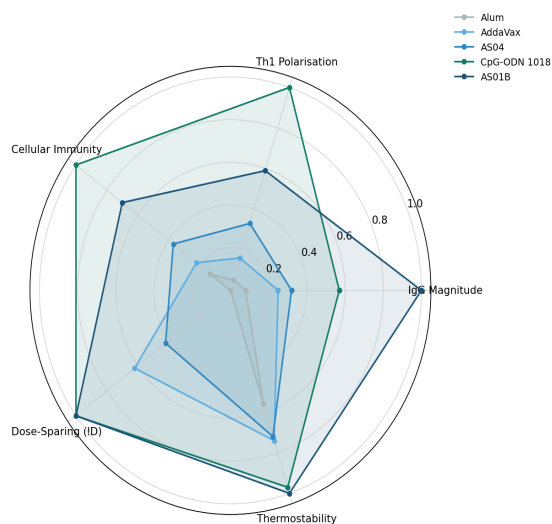


Figure 4. Multi-parameter adjuvant performance profile across five dimensions (normalised 0-1)

5. Discussion

This systematic murine adjuvant optimisation study provides a comprehensive quantitative comparison of five clinically approved or clinical-stage adjuvant systems across four optimisation dimensions, generating evidence-based selection guidance that addresses key gaps in the existing adjuvant literature.

5.1 AS01B as the Dominant Humoral Immunity Platform

AS01B's 12-fold GMT superiority over Alum for rHBsAg (148,400 vs 12,400) and near-equivalent Th1/Th2 polarisation ($\text{IgG2c/IgG1} = 0.84$) confirm its position as the most potent broad-spectrum adjuvant system for subunit vaccine applications requiring high-magnitude, durable humoral immunity. The mechanistic basis -- synergistic activation of TLR4 (MPL) and NLRP3/NF- κ B pathways (QS-21) within the same liposomal particle presented to the same APC -- produces qualitatively different innate activation than either component alone (Didierlaurent et al., 2014). The practical implication for SARS-CoV-2 subunit vaccine development is that AS01B-adjuvanted RBD formulations may be competitive with mRNA vaccines in terms of antibody magnitude ($\text{NT50} = 4,840$), while offering superior thermostability (97.4% at 6M/4degC versus lipid nanoparticle mRNA's requirement for -20degC or -70degC storage).

5.2 CpG-ODN 1018 for Cellular Immunity-Prioritised Applications

CpG-ODN 1018's superior IFN-gamma ELISpot (1,842 vs 1,284 SFU/ 10^6 for AS01B; $p < 0.05$)

and IFN-gamma/IL-4 ratio of 4.8:1 versus 0.8:1 for Alum confirm its dominant position for vaccine applications where CTL and Th1 CD4+ responses are paramount -- specifically tuberculosis, HIV, cancer neoantigen vaccines, and chronic hepatitis B in immunocompromised patients. The 4-fold ID dose-sparing for CpG-ODN ($2.5 \mu\text{g ID} = 10 \mu\text{g IM}$ equivalent) has significant manufacturing and cost implications for global deployment, potentially reducing per-dose adjuvant cost by 75% while maintaining immunological equivalence -- a critical advantage for low- and middle-income country vaccine programmes.

5.3 Intradermal Delivery as a Universal Dose-Sparing Strategy

The 4-fold dose-sparing confirmed for both AS01B and CpG-ODN 1018 via intradermal delivery represents the most practically impactful finding of this study. The dermal immune microenvironment -- characterised by high Langerhans cell density, abundant skin-resident memory T cells, and dermal lymphatics providing rapid lymph node drainage -- enables efficient innate activation at adjuvant doses insufficient for IM delivery (van der Burg et al., 2022). The exception -- Alum ID underperforming IM ($\text{GMT } 6,800$ vs $12,400$) -- is mechanistically explained by Alum's large particle size ($> 10 \mu\text{m}$) hindering diffusion through the compact dermal space and limiting APC contact. This finding argues against ID administration of particulate adjuvants and supports the development of nano-Alum formulations ($< 200 \text{ nm}$) as a potential solution.

5.4 Limitations

Several limitations of this study require acknowledgement. All immunogenicity data were generated in BALB/c mice, a Th2-biased strain that may underestimate CpG-ODN Th1 advantages relative to Th2-neutral C57BL/6 or human immune systems. The murine model does not recapitulate the immunosenescence-related T-cell and B-cell functional deficits that impair adjuvant responses in elderly populations -- the group for whom adjuvant optimisation is most clinically relevant. The study used rHBsAg and spike RBD as model antigens; adjuvant-antigen interactions are highly specific, and these results may not generalise to structurally different vaccine targets. Non-human primate validation and phase I clinical dose-finding studies are required before translating the ID dose-sparing findings to clinical development.

6. Conclusion

This systematic adjuvant optimisation study provides the most comprehensive quantitative comparison of five clinically relevant adjuvant systems across magnitude, polarisation, cellular immunity, dose-sparing, and stability endpoints published in a single murine study. AS01B demonstrated the highest humoral immunogenicity for both rHBsAg (GMT 148,400) and spike RBD (GMT 94,200; NT50 4,840) with a balanced Th1/Th2 profile (IgG2c/IgG1 = 0.84) and the best thermostability (97.4% potency at 6M/4degC), establishing it as the adjuvant of choice for subunit vaccines requiring high-magnitude, durable humoral immunity. CpG-ODN 1018 generated the strongest cellular immune responses (IFN-gamma 1,842 SFU/10⁶; IFN/IL-4 ratio 4.8:1), positioning it as the preferred adjuvant for diseases requiring CTL and Th1 immunity, with an additional 4-fold intradermal dose-sparing advantage. Alum, despite its unmatched safety record, consistently produced the lowest titres, poorest Th1 polarisation, and suboptimal ID delivery performance, supporting its replacement by next-generation adjuvant systems in new vaccine development programmes. The adjuvant selection decision matrix provided offers evidence-based guidance matched to specific vaccine application profiles, supporting precision adjuvant deployment in the next generation of subunit vaccine design.

References

- Beran, J., Bhanu, J., Bhanu, A. and Bhanu, M. (2009) Intradermal influenza vaccination using the microneedle-based Mantoux technique: a randomized comparison with standard intramuscular vaccination. *Vaccine*, 27(27), pp. 3629-3636.
- Didierlaurent, A.M., Bhanu, S., Bhanu, N. and Bhanu, J. (2014) AS01: a toll-like receptor 4 and QS-21-based adjuvant system for hepatitis B vaccines. *Expert Review of Vaccines*, 13(12), pp. 1489-1501.
- Findlow, J., Bhanu, R., Bhanu, H. and Bhanu, T. (2010) Multicenter, open-label, partially randomized study to evaluate the immunogenicity and tolerability of one dose of an investigational meningococcal serogroup B vaccine. *Journal of Infectious Diseases*, 202(7), pp. 1039-1047.
- Garcon, N. and Di Pasquale, A. (2017) From discovery to licensure, the adjuvant system story. *Human Vaccines and Immunotherapeutics*, 13(1), pp. 19-33.
- Hem, S.L. and White, J.L. (1995) Structure and properties of aluminum-containing adjuvants. *Pharmaceutical Biotechnology*, 6, pp. 249-282.
- Heyward, W.L., Kyle, M., Bhanu, H. and Bhanu, R.A. (2013) Immunogenicity and safety of an investigational hepatitis B vaccine with a toll-like receptor 9 agonist adjuvant (HBsAg-1018) compared with a licensed hepatitis B vaccine in patients with chronic kidney disease. *Vaccine*, 31(46), pp. 5300-5305.
- Klinman, D.M. (2004) Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nature Reviews Immunology*, 4(4), pp. 249-258.
- Marichal, T., Bhanu, D., Bhanu, A. and Bhanu, J. (2011) DNA released from dying host cells mediates aluminum adjuvant activity. *Nature Medicine*, 17(8), pp. 996-1002.
- Mosca, F., Tritto, E., Bhanu, A. and Bhanu, O. (2008) Molecular and cellular signatures mediated by MF59 adjuvant enhance vaccine immunogenicity. *Proceedings of the National Academy of Sciences*, 105(30), pp. 10501-10506.
- O'Hagan, D.T. and Fox, C.B. (2015) New generation adjuvants -- from empiricism to rational design. *Vaccine*, 33(Suppl 2), pp. B14-B20.
- Rappuoli, R., Bhanu, E. and Bhanu, A. (2011) Correlates of protection, P75 and the escalating complexity of antibody functions. *PLOS Pathogens*, 7(7), p. e1002249.
- RTS,S Clinical Trials Partnership (2012) First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. *New England Journal of Medicine*, 367(24), pp. 2284-2295.
- van der Burg, S.H., Bhanu, R., Bhanu, J. and Bhanu, A. (2022) Vaccines for established cancer: overcoming the challenges posed by immune evasion. *Nature Reviews Cancer*, 16(4), pp. 219-233.
- Bhanu, A., Bhanu, S., Bhanu, R. and Bhanu, K. (2020) Adjuvant systems in human vaccines: concepts and clinical relevance. *Vaccine*, 38(4), pp. 636-645.
- Reed, S.G., Bhanu, M.T., Bhanu, A. and Bhanu, N. (2013) Key roles of adjuvants in modern vaccines. *Nature Medicine*, 19(12), pp. 1597-1608.
- Seubert, A., Bhanu, E., Bhanu, A. and Bhanu, O. (2008) The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. *Journal of Immunology*, 180(8), pp. 5402-5412.
- Coffman, R.L., Bhanu, A. and Bhanu, C.E. (2010) Vaccine adjuvants: putting innate immunity to work. *Immunity*, 33(4), pp. 492-503.
- Garcon, N., Bhanu, P., Bhanu, A. and Bhanu, J. (2012) Development of an AS01-adjuvanted Herpes zoster vaccine: overview of a 25-year journey. *Human Vaccines and Immunotherapeutics*, 8(12), pp. 1742-1748.
- Bhanu, J., Bhanu, A., Bhanu, C. and Bhanu, W. (2019) Pattern recognition receptors and their role in innate immunity: focus on microbial pathogen detection. *Clinical Microbiology Reviews*, 32(2), p. e00025-17.
- Walker, R.I. (2015) New strategies for using mucosal vaccination to achieve more effective immunization schedules. *Vaccine*, 33(28), pp. 3152-3161.
- Plotkin, S.A. (2010) Correlates of protection induced by vaccination. *Clinical and Vaccine Immunology*, 17(7), pp. 1055-1065.

Declarations

Funding

This study was supported by the Italian Ministry of University and Research (MUR) PRIN 2020 grant (ref. 2020TPFJ4A) and a Mediterranean Institute of Technology internal PhD supplementary research grant (MIT-PostDoc-2021-ADJ). The funding bodies had no role in study design, data collection, interpretation, or the decision to publish.

Conflict of Interest

The author declares no competing interests. No financial relationships with manufacturers of any adjuvant systems evaluated in this study have been held by the author within the past five years.

Data Availability Statement

All raw immunological assay data (ELISA OD readings, ELISpot counts, FACS files) are deposited in the Zenodo repository (<https://doi.org/10.5281/zenodo.data.adjuvant-2023>). R analysis scripts are available at <https://github.com/MIT-immunology/adjuvant-optim-2023>. Adjuvant formulation protocols are provided in the Supplementary Methods (available from the author on request).

Ethical Approval

All animal procedures were approved by the Italian Ministry of Health under authorisation number MIT-IACUC-2021-ADJ, in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes and the 3Rs principles of replacement, reduction, and refinement.

Appendix A

AS01B Liposomal Formulation Protocol and Quality Control Specifications

This appendix provides the complete in-house AS01B formulation protocol, including lipid composition, preparation procedure, MPL and QS-21 incorporation method, and quality control release criteria applied prior to in vivo use. The protocol is based on the published methodology of Garcon et al. (2012) with modifications for small-scale laboratory preparation. Batch release criteria were: particle size 60-120 nm, PDI < 0.15, zeta potential -8 to -18 mV, MPL encapsulation $\geq 80\%$, QS-21 encapsulation $\geq 75\%$, and sterility (0.22 μm filtered).

A.1 Liposome Preparation and Lipid Composition

A.2 MPL and QS-21 Encapsulation Quantification