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Authors

Yokoyama, Yumi

Yao, Shiyin

Cozza, Renna

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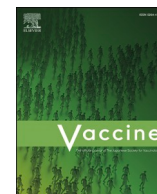
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A novel calcium influx inducer and a TLR7 agonist are synergistic co-adjuvants that enhance cross-reactive immunity against influenza in young and aged mice

Yumi Yokoyama^a, Shiyin Yao^a, Renna Cozza^a, Fernando Gil^a, Ian McLaughlin^a, Paola Anguiano Quiroz^a, Tyler Brown^a, Nikunj M. Shukla^a, Michael Chan^a, Karen Messer^{b,c}, Minya Pu^{b,c}, Maripat Corr^a, Dennis A. Carson^{a,b,*}, Tomoko Hayashi^{a,b,*}

^a Department of Medicine, University of California San Diego, 9500 Gilman Dr, La Jolla, CA 92093-0809, USA

^b Moores Cancer Center, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA. 92093-0809, USA

^c Herbert Wertheim School of Public Health and Human Longevity Science, University of California, San Diego, USA

ABSTRACT

Vaccine adjuvants play a crucial role in the efficacy of vaccines, particularly in immunocompromised populations. Newer agents and combination strategies are needed for adequate defense against emerging pathogens and their evolving variants. Here we report the benefit of a synergistic combination of two adjuvants: a Toll-like receptor 7 agonist, **1V270**, and a calcium influx inducer, **2G272**, that elicit Th1 and Th2 biased immune responses, respectively. *In vitro*, **2G272** significantly enhanced cytokine production induced by **1V270** in human and mouse primary cells, compared to a low activity analog, **2E281**, identified from structure-activity relationship studies. Using A/California/07/2009 (H1N1) inactivated influenza A virus (IIAV) as the antigen, the combination adjuvant (**2G272** + **1V270**) magnified IgG1 and IgG2a responses against hemagglutinin (HA) accompanied by greater hemagglutinin inhibition titers and increased germinal center formation in the draining lymph nodes of immunized BALB/c mice at comparable levels to FDA-approved comparators, MF59 and AS01B. The combination adjuvant also enhanced H1 HA-specific T cell responses, augmenting antigen-specific IFN γ secretion. Importantly, the **2G272** + **1V270** combined adjuvant promoted cross-reactive antibody and cellular immune responses against other HAs in phylogenetic Group 1: H5, H11, and in Group 2: H3. C57BL/6 aged mice immunized with H1N1 IIAV and **2G272** + **1V270** generated significant anti-H1 IgG1 responses and IFN γ splenic T cell responses to H1 which were cross-reactive with H5 and H3. Collectively, our findings suggest that **2G272** + **1V270** may be a versatile vaccine co-adjuvant system for promoting a larger breadth of cross reactive Th1/Th2 immune responses and robust GC B cell formation, including in the elderly population.

1. Introduction

Vaccines against infectious diseases are crucial to improving healthcare and population health management. Influenza virus vaccines can reduce the incidence and severity of infection and prevent flu-related complications such as bacterial pneumonia and organ failure [1]. As a public health measure vaccinating large sectors of the population also promotes herd immunity, which can protect people that cannot be effectively immunized due to immune deficiency [2]. By preventing the spread of pathogens, vaccines also mitigate the emergence of new variants and mutants, thereby promoting overall public

health, with economic benefits [2–4].

Influenza vaccines are used globally but are not completely protective against infection particularly in immunocompromised populations such as older adults and diabetic patients [5]. Improvements are needed to enhance the durability and breadth of immune responses and thus provide protection against a wider range of influenza virus strains with longer-lasting immunity. To effectively address these gaps in immune efficacy vaccine adjuvants have been an intensive area of research. Newer adjuvants can potentially bridge the deficits of classic adjuvants (e.g., alum) by enhancing or extending protective immune responses as single agents or in combination with existing ones like Monophosphoryl

Abbreviations: APCs, Antigen-presenting cells; Ca²⁺, Calcium; DMSO, Dimethyl sulfoxide; FDA, Food and Drug Administration; GPCR, G protein-coupled receptors; HA, Hemagglutinin; HAI, Hemagglutinin inhibition; HTS, High throughput screens; hPBMC, human peripheral blood mononuclear cells; IIAV, Inactivated influenza A virus; IACUC, Institutional Animal Care and Use Committee; MPLA, Monophosphoryl lipid A; mBMDC, murine bone-marrow derived dendritic cells; OVA, Ovalbumin; p-NPP, p-nitrophenyl phosphate substrate; RDE, Receptor destroying enzyme; SAR, Structure activity relationship; TLR, Toll-like receptor; TRBC, Turkey red blood cells.

* Corresponding authors at: Department of Medicine, University of California San Diego, 9500 Gilman Dr, La Jolla, CA 92093-0809, United States.

E-mail addresses: dcarson@health.ucsd.edu (D.A. Carson), thayashi@ucsd.edu (T. Hayashi).

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lipid A (MPLA), MF59 or AS01B [6–10].

Synergistic combinations of adjuvants can also stimulate polarization of alternate types of immune responses which can introduce complexities in outcomes. Current influenza vaccines induce humoral response which targets epitopes in the head domain of hemagglutinin (HA) [11]. These antigenic regions are highly variable and continually undergo antigenic drift [12]. Rational vaccine design is largely focused on the antigens in annual influenza vaccines, but generating a response that is cross reactive to multiple HA antigens that are not present in the vaccine would improve protection against annual variances in HA proteins. Our general approach to improve the protective efficacy of vaccines has been to develop combination adjuvants that activate innate immune cells through complementary signaling receptors or pathways, heightening the efficacy of antigen presenting cells (APCs) such as dendritic cells. One such example is the combination of low molecular weight synthetic adjuvants that activate Toll-like receptor 4 (TLR4) and TLR7 as potent co-adjuvants for influenza viral proteins, inducing rapid and sustained antibody responses that are protective against influenza viruses in homologous and heterologous murine challenge models [13,14].

Continuing our discovery process for novel adjuvants using high throughput screens (HTS) and structure activity relationship (SAR) studies we have recently reported a series of compounds that support APC function by increasing calcium mobilization [15,16]. When the hit compound, **2G272**, was used as a single adjuvant, it resulted in increased antigen-specific antibody responses and skewed toward a Th2-associated immune response [15,16]. Our prior studies with **1V270**, a phospholipid-conjugated TLR7 agonist, indicated that this compound potentially generates Th1 cellular immunity and associated humoral responses [13,14]. In this study, we further examined the adjuvant activities of **2G272** as a single adjuvant and paired with **1V270** *in vitro* and *in vivo* in vaccines targeting A/California/07/2009 (H1N1) inactivated influenza A virus (IIAV) in young and aged mice.

2. Materials and methods

2.1. Chemical synthesis

The lead analog, **2G272**, and a low activity analog, **2E281**, were synthesized in our laboratory. The reagents were purchased as from commercial vendors unless otherwise specified and used without further purification. Solvents were purchased from Fisher Scientific (Waltham, MA) and were either used as purchased or redistilled with an appropriate drying agent. Instrumentation, synthesis, and NMR analysis of **2G272** and **2E281** were performed as previously described [16].

2.2. Animals

Six to eight-week-old female C57BL/6 J (RRID:IMSR_JAX:000664) and BALB/c (RRID:IMSR_JAX:000651) mice were purchased from The Jackson Laboratory. Aged (28-month old) C57BL/6 mice were obtained from the National Institute of Aging. Animal husbandry was performed by the UC San Diego Animal Care Program, including 24-h veterinary care support. All animal experiment protocols in this study received prior approval by the Institutional Animal Care and Use Committee (IACUC) for UC San Diego (approved protocol number S00028).

2.3. Cell culture

Murine bone marrow-derived dendritic cells (mBMDCs) were prepared from bone marrow cells harvested from femurs of BALB/c mice as previously described [17,18]. Human peripheral blood mononuclear cells (hPBMCs) were purchased from STEMCELL Technologies (Kent, WA). Studies were performed in RPMI-1640 supplemented with 10 % heat-inactivated FBS and 100 U/mL Penicillin/Streptomycin (RP-10) in humidified conditions with 5 % CO₂ at 37 °C.

2.4. *In vitro* cytokine ELISA and flow cytometric analysis of CD80 and CD86

mBMDC (5×10^5 cells/mL), and hPBMC (1×10^6 cells/mL) were treated with serially diluted compound **2G272** or **2E281** in RP-10 as detailed in each Figure legend. In the separate experiments, mBMDC and human PBMC were treated with vehicle, **1V270** (10 nM), **2G272** (10 μ M), the combination of **1V270** (10 nM) and **2G272** (10 μ M), or MPLA (0.2 μ g/mL) overnight. Cytokines and chemokines in the culture supernatants were assayed in 96 well plates by capture ELISA using kits with standards and reagents listed in Supplementary Tables S3, S4, and S5. Expression of costimulatory molecules CD80 and CD86 was measured by flow cytometry, and the gating strategy is presented in Supplementary Fig. S5. Antibodies, reagents, and the assay detection ranges are listed in Supplementary Tables S3, S4, and S5.

2.5. Drug combination screening for synergy

mBMDC were plated in 96-well round-bottom tissue culture plates (Corning, Corning, NY) and stimulated with serially diluted concentrations of **2G272** (10 μ M, 5 μ M, 2.5 μ M and 1.25 μ M) and **1V270** (1 μ M, 0.2 μ M, 0.04 μ M, 0.008 μ M, 0.0016 μ M, 0.32 nM and 0.064 nM) in a checkerboard array with vehicle controls (compound = 0 μ M). Supernatants were harvested and tested for levels of IL-12 by ELISA. Synergy for Loewe additivity and BLISS independence were assessed using R-synergyfinder version 3.0 [19].

2.6. Hemagglutination inhibition (HAI) assay

The HAI assay was performed by IBT Bioservices (Rockville, MD). Sera from the animals immunized with IIAV were heat-inactivated and treated with Receptor Destroying Enzyme (RDE) to remove nonspecific inhibitors of hemagglutination. They were then adsorbed to turkey red blood cells (TRBC) to remove nonspecific agglutinin from the serum. The RDE-treated, TRBC-adsorbed serum was tested in the HAI assay starting at a 1:10 dilution with a two-fold serial dilution in duplicate. Plates were scored, and the presence of hemagglutination in each well was individually recorded to calculate the HAI titer of each serum sample, which was reported as the reciprocal of the highest dilution of serum that completely inhibited hemagglutination. The samples were tested within a titer range of 10–1280. HAI titers of naïve sera were < 10 units.

2.7. *In vitro* safety assessment

Genotoxicity assessment by Ames test: The Ames test was performed by Eurofins Discovery (Fremont, CA). The assay used 4 *Salmonella typhimurium* strains with and without rat liver S9 fraction. **2G272** was serially diluted 2-fold for 8 doses for bacterial cytotoxicity and 4 doses for bacterial growth, starting at a concentration of 100 μ M. Wells that displayed bacterial growth due to the reversion of the histidine mutation (as judged by the ratio of OD_{430/570} greater than 1.0) were counted and recorded as positive counts. The significance of the positive counts between the treatment (in the presence of the test compound) and the control (in the absence of the test compound) was calculated using the one-tailed Fisher's exact test.

Off-target assay: Reaction Biology Corp (Malvern, PA) performed the *In Vitro* Evaluation of Safety and Toxicity (InVEST panel) analysis, using 53 clinically relevant targets from 10 target families to assess potential risks. Briefly, **2G272** (10 μ M) or vehicle, 0.1 % DMSO in duplicate were assessed with the InVEST panel, which included 1) G protein-coupled receptors (GPCR; radioligand binding), 2) ion channels (radioligand binding or fluorescence polarization binding), 3) biogenic amine transporters (radioligand binding), 4) nuclear receptors (fluorescence polarization binding), 5) phosphodiesterases (catalytic assay), 6) cytochrome P450 isozymes (catalytic assay), and 7) proteases (catalytic

assay).

2.8. In vivo pharmacodynamic study

BALB/c mice were *i.m.* injected with **2G272** (200 nmol/animal) or vehicle (0.5 % DMSO) in two limbs. MF59 was used as a positive control. The sera samples were collected at 4, 8, 12, 24, 48, and 72 h after injection. IL-6, IL-12, TNF, CXCL1, CCL2, CCL5, CXCL10, and alpha-1 acid glycoprotein levels were analyzed ELISA and Luminex bead assay. ELISAs were performed in 96 well half area plates by capture ELISA, developed with TMB substrate, and read at 405 nm and 570 nm as a reference on a Tecan plate reader (Tecan, Switzerland). Additional measures were done by Luminex bead assay with ProcartaPlex™ Multiplex Immunoassay per the manufacturer's instructions (Thermo-fisher). The detection ranges and assay kit information are provided in Supplementary Tables S3, S4 and S5.

2.9. In vivo immunization protocol

A/California/04/2009 (H1N1)pdm09 IIAV was obtained from BEI Resources Repository (# NR-49450, Manassas, VA, USA). H1N1 IIAV (10 µg/injection) was mixed with compound(s): MPLA (1 µg/animal), Alum (1:1 mixture), MF59 (1:1 mixture) or AS01B (10 µL/animal). Vehicle (0.5 % DMSO) served as a negative control and 50 µL total volume was injected into the gastrocnemius muscle using a 29G insulin syringe on days 0 and 21. Sera were collected on days 28 and 42 and HA protein-specific IgG2a/c and IgG1 titers were measured by ELISA. For antigen-specific splenocyte recall response analysis, spleens were harvested on day 42, and splenocytes (5×10^6 cells/mL) were re-stimulated with 10 µg/mL HA protein for 3–5 days. IL-5, and IFN γ in supernatants were measured by ELISA (specific reagents in Supplementary Table S4).

For GC reaction analysis, draining popliteal and inguinal lymph nodes were harvested on day 28, and a single cell suspension was incubated with anti-mouse CD16/32 antibody blocking Fc receptors in the staining buffer followed by incubation with a cocktail of antibodies against CD3, CD4, CD19 CD11c, CD11b, CD19 CD95, CD138, GL7, PD-1, and CXCL5. Detailed information for antibodies and reagents used in flow cytometry analysis and gating strategies is presented in Supplementary Table S3 and Supplementary Fig. S5, respectively. Data were acquired using MACSQuant® Analyzer 16 and analyzed using FlowJo (version 10.10.0).

2.10. Anti-HA IgG by ELISA

IgG1 and IgG2a/c against HA antigens were measured by ELISA. Assays were performed using half area 96 well plates as previously described [20]. Detailed information for reagents is described in Supplementary Tables S3 and S4. In brief, plates were coated with recombinant HA proteins of A/California/04/2009 (H1N1), blocked, then incubated with serially diluted sera in blocking buffer. After incubation at 4 °C overnight, plates were washed and detecting antibodies were added. After washing and incubation with *p*-nitrophenyl phosphate substrate (*p*-NPP), plates were read at 405 nm and 570 nm as a reference on a Tecan plate reader. We prepared standard sera with known endpoint titers as previously reported [14,20]. Briefly, standard sera were obtained from mice hyper-immunized with H1N1 IIAV. The endpoint titer of pooled standard sera was calculated as a reciprocal of the highest dilution that gave an absorbance reading double the absorbance of the background. Each plate had a titration of the standard sera to generate a standard curve. The titer of the test sample was interpolated from the standard serum titration from the same plate. IgG2a was measured in the sera of BALB/c mice and IgG2c was measured in the sera of C57BL/6 mice [21]. The results are expressed in units per milliliter (arbitrary units). Recombinant HA proteins of heterologous virus strains (H5, H11 and H3) used for coating are presented in Supplementary Table S4. Plates were coated with each protein overnight at

4 °C for IgG and sera were diluted at 1:50 in blocking buffer followed by 4-fold serial dilutions. Geometric means of total IgG titer curves are presented in the results. OD_{405nm} and OD_{570nm} values were measured by a Tecan plate reader. The standard sera prepared above were for measurement of antibodies against homologous strain and thereby are not appropriate to measure cross-reactive antibody titers. Therefore, adjusted optical density (OD)_{405–570nm} values are presented for cross-reactive antibody titers

2.11. Statistical analyses

To compare multiple groups, ANOVA was performed with Dunnett's *post hoc* test for comparisons against a common control group, or Kruskal-Wallis tests with Dunn's *post hoc* test for multiple testing adjustment among selected groups of interest. To examine the pharmacodynamic profile, ANOVA with Bonferroni *post hoc* test was used to compare two groups at each time point. Prism 10 software (GraphPad Software, San Diego, CA) was used. *P* values <0.05 were considered statistically significant.

In addition, to assess the synergistic effect of **2G272** and **1V270** on IL-12 release, IL-12 levels were first normalized to percent response using min-max scaling and the mean of triplicates for each combination of conditions was used in the subsequent analysis. The synergy scoring models, HSA, Loewe, Bliss, and ZIP were used to calculate synergistic scores which were shown using heatmaps and contour plots. Only results from the Loewe and Bliss models were presented since the other two models generated results that were nearly identical. R-synergyfinder version 3.0 [19] was used to perform the analysis.

3. Results

3.1. 2G272 stimulates cytokine release in primary human cells

We previously selected **2G272** as a lead compound from extensive HTS and SAR studies and identified **2E281** as a relatively low-activity analogue of the same chemotype that would serve as a negative control compound (Fig. 1A). The potency of compound **2G272** as an innate immune activator was confirmed by cytokine and chemokine release assays using primary murine and human immune cells (Fig. 1B–I).

2G272 was serially diluted two-fold starting at 10 µM and incubated with mBMDC (BALB/c) or hPBMC overnight. **2E281** and vehicle (0.5 % DMSO) were used as negative controls. MPLA, a U.S. Food and Drug Administration (FDA)-approved vaccine adjuvant [10], served as a comparator. **2G272** stimulated IL-12 release from BALB/c mBMDC in a dose-dependent manner (Fig. 1B). **2G272** also enhanced the immunostimulatory function of a TLR7 ligand, **1V270**, inducing higher IL-12 levels (Fig. 1C). Additionally, **2G272** was active in primary human immune cells, inducing the release of hIL-6 and hIL-8, which are primarily produced by activated innate immune cells, in a dose-dependent fashion (Fig. 1D and F). Similar to mBMDC, **2G272** further increased the release of IL-6 and IL-8 induced by **1V270** (Fig. 1E and G). The combination treatment of **1V270** + **2G272** induced higher levels of IL-2 and IFN γ generally considered to be produced by T cells (Fig. 1H and I). In parallel, we assessed CD80, and CD86 that are costimulatory molecules expressed on APCs for the initial priming and activation of naive T cells. **2G272** significantly enhanced expression of the co-stimulatory molecules, CD80 and CD86, on mBMDC in combination with **1V270** (Fig. 1J, K, L and M).

3.2. In vivo efficacy of 2G272 as a co-adjuvant with the TLR7 ligand, 1V270

Proof-of-principle studies using ovalbumin as a model antigen indicated that **2G272** predominantly enhances Th2-biased immune responses in BALB/c mice [16]. We sought to evaluate whether **2G272** could serve as a co-adjuvant with **1V270**, a TLR7 agonist, which

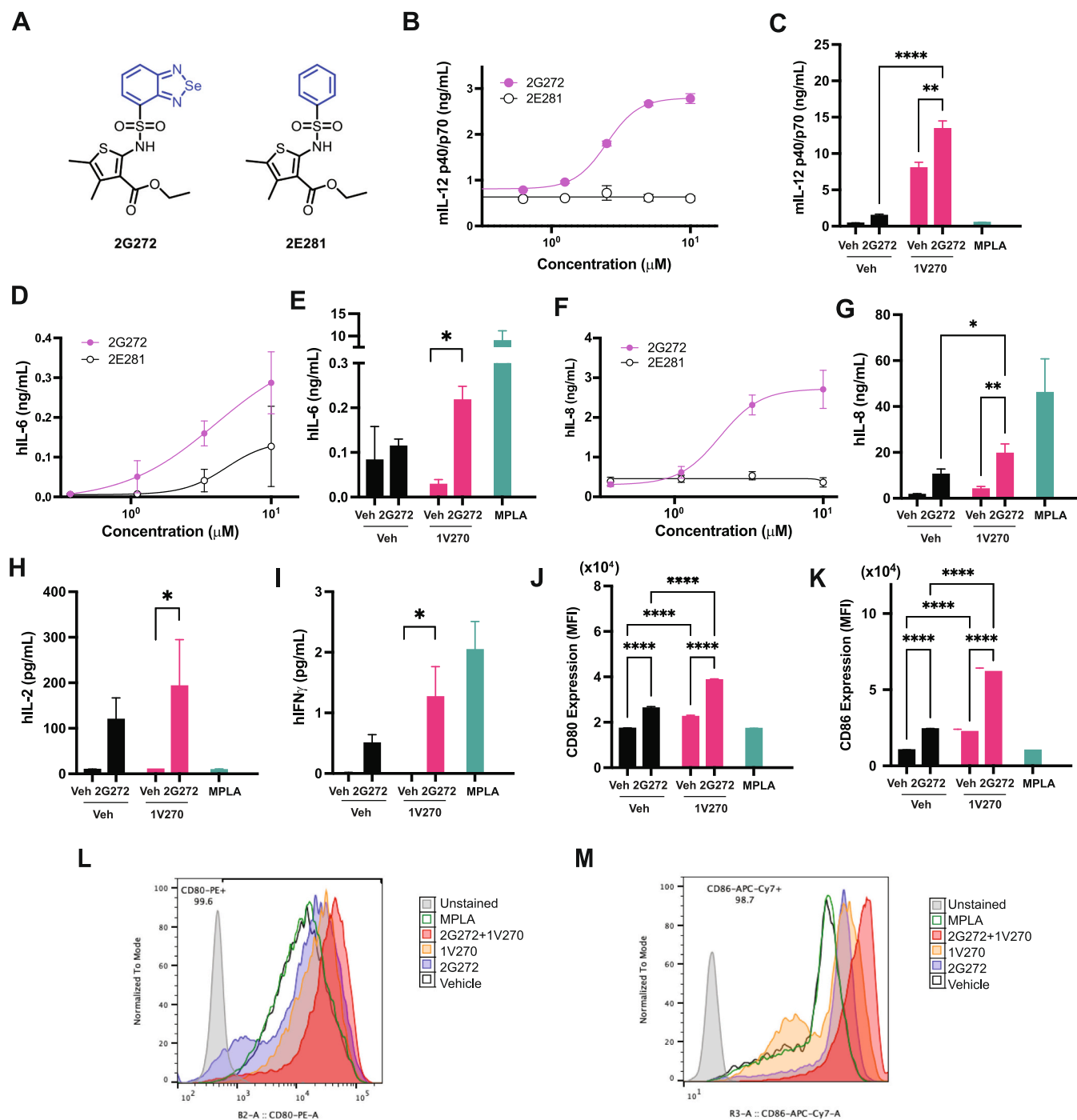


Fig. 1. Compound 2G272 enhanced cytokine production by 1V270 in human and mouse immune cells and expression of co-stimulatory molecules by BMDC. (A) Structures of 2G272 and a low activity analogue 2E281. Mouse (m) BMDCs from BALB/c mice (B) or human (h) PBMCs (D and F, 3 donors) were treated with 2G272, or 2E281 at the indicated concentrations for 24 h. Murine IL-12 (B), human IL-6 (D), human IL-8 (F) levels in the culture supernatants were measured by ELISA. In the separate experiments, mBMDCs from BALB/c (C) mice or hPBMCs (E, G, H, and I, 3 donors) were treated with 1V270 (100 nM) + 2G272 (10 μM) or MPLA (0.2 μg/mL) overnight. mIL-12 (C), hIL-6 (E), hIL-8 (G), hIL-2 (H) or hIFNγ (I) levels in the culture supernatants were measured by ELISA. Expression levels of CD80 and CD86 in BALB/c mBMDC were measured by flow cytometric assay. Data shown are means ± SEM of representative experiments showing similar results. **p* < 0.05, ***p* < 0.01, *****p* < 0.0001, by two-way ANOVA.

consistently induced IgG2c and Th1 responses in prior studies [13,14]. We formally addressed whether there is an additive or synergistic *in vitro* effect on APCs with these two compounds. The compounds were titrated in culture in a checkerboard array with mBMDCs and IL-12 release was measured. Synergy for Loewe additivity (the difference between the observed effect of the combination and the expected additive effect) and BLISS independence (the average excess response across all tested dose

combinations due to the drug interactions, rather than independent drug effects) were assessed and are shown as heat maps and contour plots (Supplementary Fig. 1 A-D). The overall mean Lowe synergy score was 14.5 and the mean Bliss independence score was 13.8. Both scores were > 10, indicating that these two compounds exhibit synergy.

We examined the efficacy of 2G272 and 1V270 as single adjuvant and in combination by immunizing BALB/c mice with adjuvanted IIAV

on days 0 and 21. Sera were collected on day 28, and HA-specific IgG1 and IgG2a antibodies were determined by ELISA. As a single adjuvant with H1N1 IIAV, **2G272** elicited a Th2-associated IgG1 response (Fig. 2A and B; $p < 0.001$) in BALB/c mice. When combined with **1V270**, **2G272** augmented IgG1 ($p < 0.01$) and IgG2a ($p < 0.0001$) responses, suggesting the combination of **1V270** + **2G272** augmented both Th1 and Th2-associated immune responses (Fig. 2A and B). These findings were consistent in a second mouse strain, C57BL/6, which tends to skew toward Th1 immune responses, while BALB/c mice are biased toward Th2 immune responses (Supplementary Fig. S2) [22–24]. In contrast, **2G272** had minimal effects on IgG1 or IgG2a responses when combined with adjuvants in FDA-approved vaccines, alum (Alhydrogel®) or MF59 adjuvants (Supplementary Fig. S3). The antigen-specific IgG1 and IgG2a induced by the **2G272** + **1V270** combined adjuvant were comparable to those induced by MF59 alone or in combination with **2G272** (Supplementary Fig. S3).

The draining lymph nodes from the injection site were harvested on day 28 and assayed by flow cytometry for germinal center sites (GC) B cells and T follicular (Tfh) cells. The increased HA-specific IgG was accompanied by enhanced GC formation, insofar as **2G272** + **1V270** combined adjuvanted IIAV induced significantly higher numbers in GC B cell ($p < 0.001$) and Tfh cells ($p < 0.01$) populations (Fig. 2C and D). The Hemagglutinin inhibition (HAI) assay measures the functional ability of elicited antibodies to block the influenza virus' HA protein from attaching to mucosal epithelial cells, which is associated with the generation of protective antibodies [25,26]. The combined **2G272** + **1V270** adjuvanted IIAV sera contained significantly higher HAI titers compared to vehicle- or **1V270**-adjuvanted IIAV ($p < 0.0001$ and $p < 0.01$, respectively), which were comparable to MF59-adjuvanted

vaccine injected animals (Fig. 2E).

3.3. **2G272** + **1V270** combined adjuvant induces cross-reactive anti-HA antibodies

The induction of cross-reactive antibodies is a crucial attribute to protection against seasonal and pandemic influenza strains [27]. To evaluate the ability of the combined **2G272** + **1V270** adjuvant to elicit cross reactive antibody responses, we assessed antibody titers from mice immunized with IIAV (H1) and the indicated adjuvants against HA subtypes H5, H11, and H3. HA subtypes H1, H5 and H11 are in Group 1 where H5 is the closest HA subtype to H1. In contrast, the H3 subtype belongs to Group 2, the most phylogenetically distant subtype among the three we tested [27,28]. The IgG in the sera from mice vaccinated with IIAV (H1) adjuvanted with the combined **2G272** + **1V270** were highly reactive to H5-, H11-, and H3-HA subtypes at comparable levels to IgG from mice immunized with MF59 and AS01B (Fig. 2F, G, and H; $p < 0.0001$; and Supplementary Fig. S4).

3.4. The combined **2G272** + **1V270** adjuvant promotes cross-reactive T cell responses

The cellular immune responses elicited by influenza vaccination are crucial for eliminating virus-infected cells, preventing severe diseases, and providing broader protection against influenza virus infection. To test whether the combined adjuvant could induce cross-reactive cellular responses, splenocytes were isolated from BALB/c mice immunized with IIAV strain A/California/04/2009 (H1N1) on day 42 and cultured in the presence of recombinant HA from homologous HA antigen (H1), or

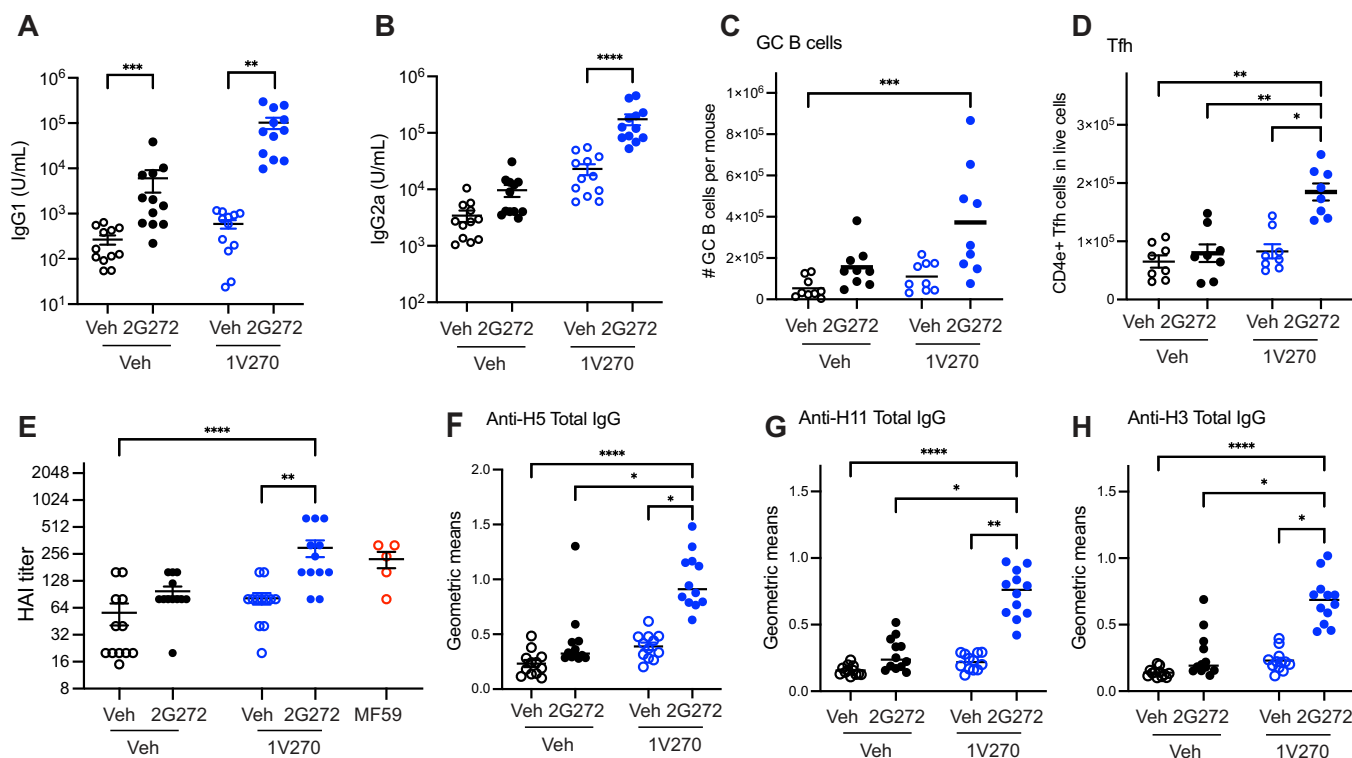


Fig. 2. Assessment of 2G272 as a co-adjuvant. (A and B) **2G272** co-adjuvant activity with **1V270**. BALB/c mice ($n = 12$ /group) were immunized with IIAV adjuvanted with **2G272** (200 nmol/animal) or vehicle (Veh) alone or in combination with **1V270** (1 nmol/animal) on days 0 and 21. Sera were collected on day 28, and HA-specific IgG1 and IgG2a antibodies were determined by ELISA. (C and D) **2G272** + **1V270** combination adjuvant enhanced GC formation. GC B cells (C) and T follicular cells (Tfh) (D) in draining lymph nodes were identified by flow cytometric assay. Gating strategies are shown in Supplemental Fig. 4. (E) HAI titers in the serum samples from mice immunized with IIAV adjuvanted with vehicle, **2G272**, **1V270**, **2G272** + **1V270** or MF59 were measured by IBT Bioservices. (F, G, and H) **2G272** + **1V270** combination adjuvant induced IgG that are cross reactive to HAs from phylogenetically distant influenza virus strains. HA specific total IgG were measured by ELISA and geometric means of the ODs are shown. Data are pooled from 2 to 3 experiments and presented with means ± SEM. Statistical significance was determined by Kruskal-Wallis test followed by Dunn's post-hoc analysis: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

heterologous HA antigens of the H5, H11, and H3 subtypes. IL-5 and IFN γ were quantified in the supernatants by ELISA (Fig. 3A-D). The combined **2G272** + **1V270** adjuvanted H1N1 IIAV significantly enhanced HA-specific IFN γ release ($p < 0.001$; Fig. 3A). Splenocytes from mice that received **2G272** produced detectable Th2-associated cytokine IL-5 which was not significant (Fig. 3C). Furthermore, the combined **2G272** + **1V270** adjuvanted H1N1 IIAV promoted higher Th1 associated cytokine IFN γ release by splenocytes upon re-stimulation with H5-, H11-, and H3-HA proteins, indicating the induction of cross-reactive T cell responses (Fig. 3B and D).

3.5. The combined **2G272** + **1V270** adjuvant promotes cross-reactive T cell responses in aged mice

There is a pressing public health need for more effective influenza vaccines in older adults [5]. To address this, we tested the efficacy of the combined **2G272** + **1V270** adjuvant in the available 28-month-old mice (C57BL/6 males), which are considered aged relative to humans [29]. To examine T cell responses induced by the combined **2G272** + **1V270** adjuvant, release of Th1 and Th2 associated cytokines, IFN γ , and IL-5, respectively, was measured after re-stimulation of splenic T cells with H1, H5, H11, or H3 HA protein on day 42 (Fig. 4A-H). The vaccination with **2G272** + **1V270** adjuvanted H1N1 IIAV elicited elevated splenic IFN γ responses against homologous (H1-HA; $p < 0.05$) and heterologous H5 ($p < 0.01$) and H3 ($p < 0.05$) HAs (Fig. 4). There was also an increase in IgG1 against homologous H1 HA ($p < 0.01$; Fig. 4I), but not in IgG2c (Fig. 4J). The IgG in the sera from mice vaccinated with H1N1 IIAV adjuvanted with the combined **2G272** + **1V270** did not elicit significant levels of cross reactive IgG to H5-, H11- or H3-HA (Fig. 4K-M).

3.6. Safety and pharmacodynamic assessment of **2G272**

Prior to any *in vivo* use, we conducted preliminary toxicity and pharmacodynamic assessments of **2G272** to evaluate its safety profile. The toxicology assessment included an Ames test (Eurofin Discovery) for genotoxicity and screening for potential off-target effects (Reaction Biology Corp.). The Ames test for **2G272** was performed with and without the S9 fraction from rat liver in four *Salmonella typhimurium* strains and showed no evidence of genetic toxicity for **2G272** (Supplementary Table S2). To assess potential off-target effects, we screened **2G272** using an InVEST[®] panel (Reaction Biology Corp.). This panel screens for unexpected binding to 53 clinically relevant targets that could lead to side effects including 1) GPCRs (radioligand binding), 2) ion channels (radioligand binding or fluorescence polarization binding), 3) biogenic amine transporters (radioligand binding), 4) nuclear receptors (fluorescence polarization binding), 5) phosphodiesterases (catalytic assay), 6) cytochrome P450 isozymes (catalytic assay), and 7) proteases (catalytic assay). The results, expressed as relative values compared to the vehicle control (0.1 % DMSO), are presented in Supplementary Table S5. **2G272** (10 μ M) showed significant inhibition (>60 %) of three cytochrome P isozymes (Cyp3A4, Cyp3A5, and Cyp2C9) and moderate inhibition (30–60 %) of two additional targets (Cyp1A2 and D1 dopamine receptor). These findings provide initial insights into the potential safety of **2G272**.

To examine the pharmacodynamic profile, we conducted a comprehensive analysis of serum cytokines and chemokines in BALB/c mice after intramuscular (*i.m.*) injection. **2G272** (200 nmol/animal) was injected into mice and sera were collected at 4, 8, 12, 24, 48, and 72 h post-administration. Sera collected before compound administration served as naïve controls. For a preliminary kinetics study MF59 was used as a comparator. IL-6, IL-12, TNF, CXCL1, CCL2, CCL5, and CXCL10 were measured using ELISA and Luminex bead assay (Fig. 5). None of

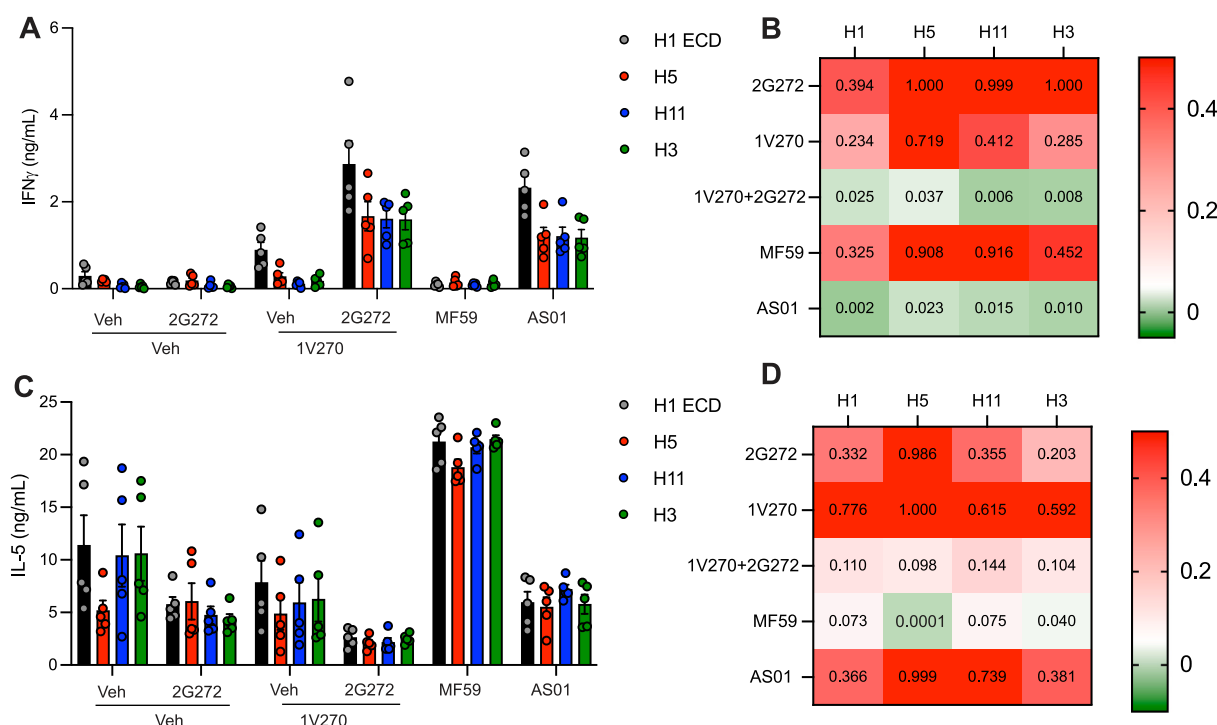


Fig. 3. 1V270 + 2G272 combination adjuvant induced cross-reactive splenic T cell responses.

(A-D) Female BALB/c ($n = 4-8$) were immunized with H1N1 IIAV (10 μ g/animal) adjuvanted with **2G272** (200 nmol/animal), **1V270** (1 nmol/animal), or combination (**2G272** + **1V270**), MPLA (1 μ g/animal) or AS01B (10 μ L/animal) on day 0 and 21. The splenocytes were re-stimulated with H1, H5, H11 and H3 HA for 5 days. (A and C) Antigen specific IFN γ (A), and IL-5 (C) release was measured by ELISA and are presented as means $\pm$ SEM. Each dot indicated individual animals. (B and D) Statistical differences compared to vehicle group were calculated by two-way ANOVA followed by Dunnet's *post hoc* analysis. The p values are summarized in grids with levels represented as heat maps.

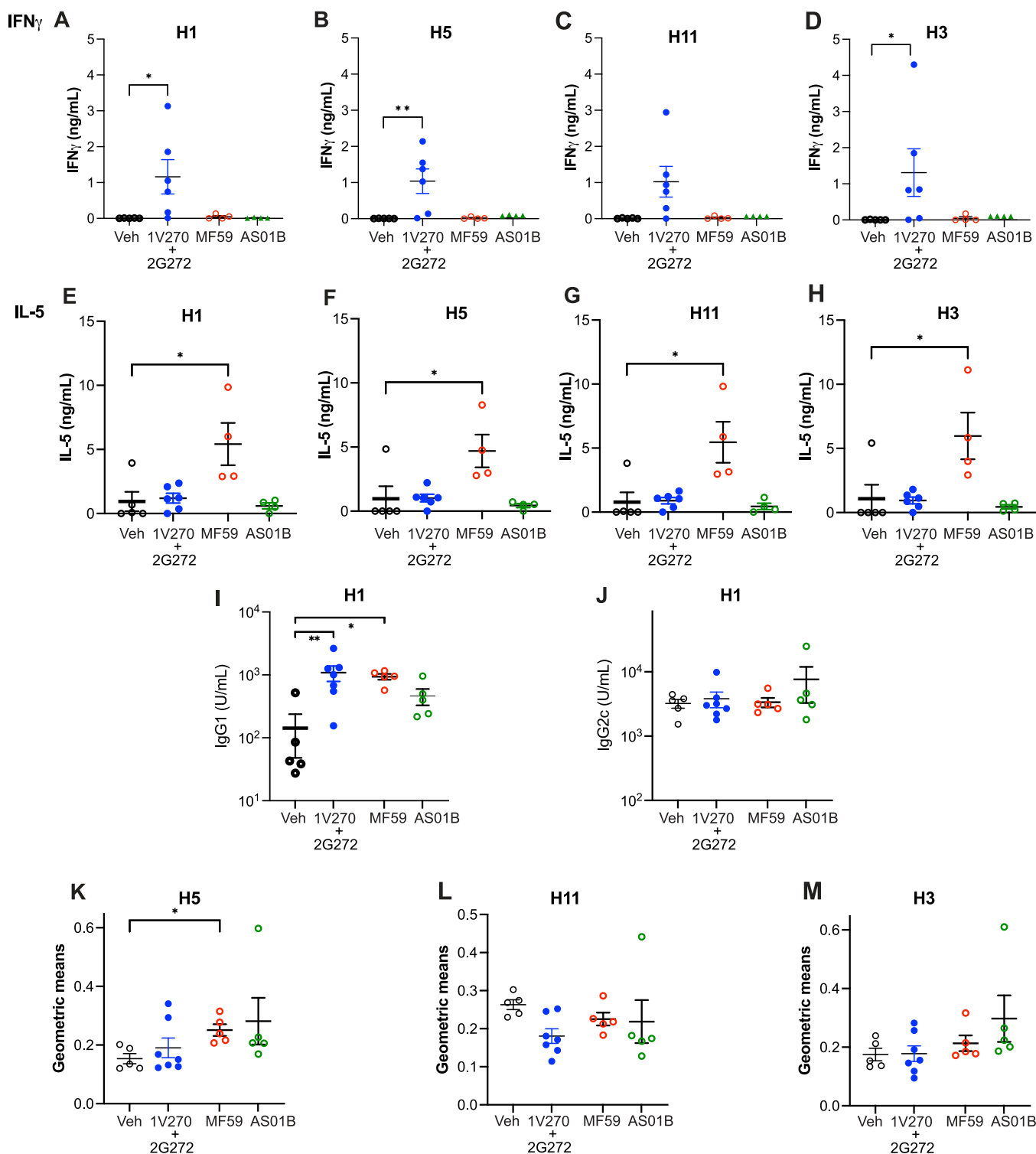


Fig. 4. 1V270 + 2G272 combination adjuvant induced cross-reactive splenic T cell response in aged mice. Aged male C57BL/6 mice ($n = 4-6$) were immunized with H1N1 IIAV (10 μ g/animal) adjuvanted with 1V270 (1 nmol/animal) + 2G272 (200 nmol/animal) or vehicle (10 % DMSO) on day 0 and 21. MF59 (25 μ L/animal) and AS01B (10 μ L/animal), were used for comparisons. H1, H5, H11, and H3 specific IFN- γ (A, B, C, and D) and IL-5 (E, F, G, and H) are presented. H1 HA-specific IgG1 (I), and IgG2a (J) were presented in arbitrary units. Cross reactive immunoglobulins were presented in geometric means (K, L, and M). The experiment was performed once due to limited availability of aged mice. Data are presented as means $\pm$ SEM. Statistical significance was determined by Kruskal-Wallis test followed by Dunn's post-hoc analysis: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

the detectable analytes, IL-6, CXCL1, and CCL2, were elevated in 2G272-injected mice, whereas IL-6, CXCL1, and CCL2 were transiently increased 4 to 12 h post-injection of MF59 (Fig. 5A-C). IL-12, TNF, CCL5, and CXCL10 were under the detection levels. The inflammatory protein,

alpha-1 acid glycoprotein, a well-established murine acute phase protein marker with a normal range of 0.2 mg/mL - 0.4 mg/mL [30,31], gradually increased reaching a peak 48 h after injection, and declining thereafter (Fig. 5D). These findings suggest that 2G272 presents

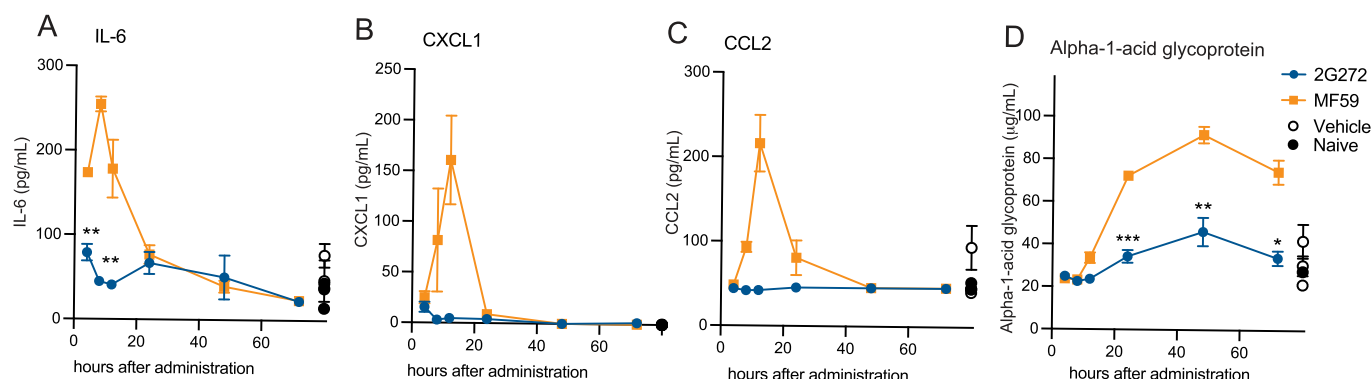


Fig. 5. Time-course analysis of cytokine and chemokine responses in mice administered with 2G272.

Female BALB/c mice ($n = 3-5$) were *i.m.* injected with 2G272 (200 nmol/animal) or vehicle (0.5 % DMSO). MF59 (25 μ L/animal, 50 % in PBS) was used as a positive control. Sera samples were collected 4, 8, 12, 24, 48, and 72 h after injection. Sera collected before the compound administration served as naïve controls. The levels of (A) IL-6, (B) CXCL1, (C) CCL2, and (D) alpha 1 acid glycoprotein were measured in serum samples by ELISA and Luminex bead assays. Serum concentrations of IL-12, TNF, CCL5 and CXCL10 were below detection limits of the ELISAs and Luminex bead assays. Statistical significance was determined by two-way ANOVA with Bonferroni *post hoc* test: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

minimal systemic reactogenicity compared to MF59.

Body weight monitoring is a non-invasive and sensitive method to evaluate the adverse effects in murine models because mice are sensitive to a broad range of stresses [32]. Thus, we monitored the body weights of mice following vaccination with 2G272 + 1V270 adjuvanted H1N1 IIAV. No significant weight loss was observed in animals receiving 2G272 + 1V270 adjuvanted H1N1 IIAV, indicating that the injections were well tolerated (Supplementary Fig. S6). In addition, no behavioral changes were observed in any of the cohorts. These findings collectively indicate that the combined 2G272 + 1V270 adjuvant significantly enhanced protective humoral immune responses when used with IIAV as an antigen with minimal adverse effects.

4. Discussion

In response to the continuous challenge of emerging infectious pathogens to public health, it is necessary to develop modern adjuvants which broaden and strengthen immune response, particularly in vulnerable populations. Most adjuvants function as immunostimulants, acting as danger signal agonists that induce the maturation and activation of APCs by triggering the inflammasome or targeting TLRs and other pattern recognition receptors. In general, this process promotes the production of co-stimulatory signals and proinflammatory cytokine secretion, enhancing adaptive immune responses. In this manuscript, we present a new small molecular weight adjuvant candidate, 2G272, that mobilizes Ca^{2+} and activates APCs independent on TLRs [15,16]. In a vaccination study, A/California/07/2009 (H1N1) (IIAV) adjuvanted with 2G272 exhibited higher Th2-dominant immune responses than those receiving IIAV alone (Fig. 2). Significantly, the combination of 2G272 with a synthetic TLR7 agonist, 1V270, which biases toward Th1 immune responses, induced both Th1 and Th2 antibody responses as evidenced by the B cell isotype switching. This regimen also promoted GC formation, increased HAI titers, and generated cross-reactive antibody and cellular responses.

Influenza remains a persistent public health concern, with vaccine efficacy limited by seasonal antigenic variation [2,33]. Current influenza vaccines primarily elicit humoral immunity, which is associated with HAI responses targeting the immunodominant globular head of the HA protein [34]. Although neutralizing antibodies typically recognize epitopes within this domain, its high variability and ongoing antigenic drift allow the virus to evade immunity conferred by both natural infection and vaccination [34,35]. While rational antigen design has been a major focus for improving vaccine efficacy [36,37], the development of effective adjuvants is increasingly recognized as essential to optimizing vaccine performance [13,38,39].

Seasonal influenza vaccines are formulated based on predictions of circulating strains typically including antigens from H1N1 and H3N2 influenza A viruses and one or two influenza B virus lineages. Despite these annual changes, significant annual mortality persists, particularly among the elderly. In response, the U.S. FDA approved Flud in 2015, the first seasonal influenza vaccine specifically designed for the elderly which incorporates the adjuvant MF59, an oil-in-water emulsion of squalene [40–42].

Unlike previously licensed adjuvants, contemporary strategies increasingly utilize combinations of molecules with complementary mechanisms to optimize immunity. These “combination adjuvants” are registered with several vaccines, such as AS04, approved for human papilloma virus (Cervarix), and AS01B for varicella zoster virus (Shingrix) [8,10,43]. The 2G272 + 1V270 combination adjuvant reported here synergistically induced Th1- and Th2-biased immune responses with broad humoral and cellular cross-reactivity including against a Group 2 HA, H3. Its efficacy for generating cross-reactive T cell responses surpassed that of MF59 in young mice. In aged mice, both MF59 and 2G272 + 1V270 elicited IgG1 but not IgG2c responses. The combination of 2G272 + 1V270 promoted Th1 IFN γ responses whereas MF59 generated Th2 associated IL-5 splenic responses, suggesting direct immunomodulation and reduced cellular senescence.

In the experiment with 28-month-old male C57BL/6 mice which were available from the National Institute of Aging, the combined adjuvant 2G272 + 1V270 induced significantly higher levels of splenic IFN γ , which was cross-reactive to other strains (Fig. 4). In contrast, we did not observe an increase in homologous H1-HA specific IgG2c, although there was an increase in IgG1 against homologous H1-HA (Fig. 4). These data suggested that immune responses in the aged population show a discrepancy in Th cell differentiation and B cell isotype class switching, which may reduce IgG induction by helper T cells and consequently decrease vaccine efficacy [44]. Some reports indicate that aging enhances Th1 and Th17 responses, while others describe increased Th2 immune responses in the aged population [45]. Moreover, aging might also affect immune responses, and increased IFN γ release may worsen age-related chronic inflammation [46,47]. Therefore, further comprehensive safety evaluations of the 1V270 + 2G272 adjuvant combination should be conducted in the aged population.

Although the combined adjuvant system induced antibody responses comparable to those of MF59 there are potential advantages of this system. The combination of 2G272 + 1V270 has less systemic inflammation evidenced by lower alpha-1 acid glycoprotein levels. The maximum serum concentrations, observed after 24–72 h, were significantly lower than those of MF59, supporting a favorable reactogenicity profile. Furthermore, the manufacturing of the small molecules 1V270

+ **2G272** combination adjuvant is fully synthetic and can be produced inexpensively at scale, reducing costs and supply risks. In contrast, MF59 depends on squalene, a naturally derived lipid limited in large-scale, high-purity production due to source availability and complex synthetic routes.

There were some limitations to our study. The use of the combined (**2G272** + **1V270**) adjuvant was slightly more effective in eliciting antibody responses in female BALB/c mice than in C57BL/6 mice. While female BALB/c mice were used in most experiments, comparisons with the data from the aged male C57BL/6 mice are potentially confounded by sex and gender. The use of aged mice was constrained by their natural lifespan limiting their availability by strain, gender and number. In a large mouse longevity study C57BL/6 male mice survived longer than females averaging a ~ 900-day lifespan and BALB/c male mice averaged a shorter (~700 day) lifespan [48]. While the **2G272** + **1V270** adjuvanted vaccine elicited robust immunogenic responses in younger animal models, we sought to evaluate its efficacy in an equivalent to a more vulnerable population. In the advanced age range only male C57BL/6 mice were available in sufficient numbers. Another limitation of our studies is the use of HAI titers as a functional surrogate marker for anti-influenza activity. Studies have shown that higher post vaccination HAI titers were associated with a lower risk of influenza infection and morbidity [25,49–51]. Our data demonstrated that the titers in sera from mice immunized with IIAV adjuvanted with **2G272** + **1V270** were comparable to those observed in animals vaccinated with MF59-adjuvanted IIAV (Supplementary Fig. S4).

Future studies are warranted based on the current data. Although we have independently performed pharmacodynamic experiments on **2G272** and **1V270**, showing minimal innate immune cytokine induction [52], comprehensive pharmacokinetic and pharmacotoxicity studies, along with optimized formulations of the **2G272** + **1V270** adjuvant, remain necessary to confirm safety and efficacy. The off-target assays indicated some cytochrome P enzyme binding, suggesting potential for drug-drug interactions. However, our results underscore the potential of **2G272** + **1V270** + **2G272** as an adjuvant and *in vivo* influenza challenge studies will be required for further development.

The **2G272** + **1V270** combination adjuvant represents a promising strategy for enhancing influenza vaccine efficacy, particularly in aged populations. It induces balanced Th1/Th2 responses, broad cross-reactivity, and reduced reactogenicity compared to MF59. Continued development and optimization of **2G272** as a novel adjuvant and co-adjuvant are warranted.

CRediT authorship contribution statement

Yumi Yokoyama: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Shiyin Yao:** Writing – review & editing, Data curation. **Renna Cozza:** Writing – review & editing, Data curation. **Fernando Gil:** Writing – review & editing, Data curation. **Ian McLaughlin:** Data curation. **Paola Anguiano Quiroz:** Data curation. **Tyler Brown:** Data curation. **Nikunj M. Shukla:** Writing – review & editing, Formal analysis, Conceptualization. **Michael Chan:** Writing – review & editing, Data curation. **Karen Messer:** Writing – review & editing, Formal analysis, Conceptualization. **Minya Pu:** Writing – review & editing, Formal analysis. **Maripat Corr:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Dennis A. Carson:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Tomoko Hayashi:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2025.128189>.

Data availability

All data supporting the findings of this study are available within this article and its Supplementary Information.

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