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Comparison of the effects of different potent adjuvants on enhancing the immunogenicity and cross-protection by influenza virus vaccination in young and aged mice

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Abstract

Vaccination against influenza viruses suffers from low efficacy in conferring homologous and cross-protection, particularly in older adults. Here, we compared the effects of three different adjuvant types (QS-21+MPL, CpG+MPL and bacterial cell wall CWS) on enhancing the immunogenicity and homologous and heterosubtypic protection of influenza vaccination in young adult and aged mouse models. A combination of saponin QS-21 and monophosphoryl lipid A (QS-21+MPL) was most effective in inducing T helper type 1 (Th1) T cell and cross-reactive IgG as well as hemagglutination inhibiting antibody responses to influenza vaccination. Both combination adjuvants (QS-21+MPL and CpG+MPL) exhibited high potency by preventing weight loss and reducing viral loads and enhanced homologous and cross-protection by influenza vaccination in adult and aged mouse models. Bacillus Calmette-Guerin cell-wall skeleton (CWS) displayed substantial adjuvant effects on immune responses to influenza vaccination but lower adjuvant efficacy in inducing Th1 IgG responses, cross-protection in adult mice, and in conferring homologous protection in aged mice. This study has significance in comparing the effects of potent adjuvants on enhancing humoral and cellular immune responses to influenza virus vaccination, inducing homologous and cross-protection in adult and aged populations.

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Declaration of Interest Statement

The authors declare that there is no interest of conflicts in this manuscript

Keywords

Influenza virus; Split vaccine; Adjuvant; Cross-protection

1. Introduction

It is estimated that the influenza epidemic viruses infect 3 to 5 million individuals, causing approximately 250,000–500,000 annual deaths and an enormous economic burden worldwide (Viboud et al., 2010). Annually updated vaccination is recommended to reflect the new antigenic changes due to the high mutation frequencies of influenza viruses. Vaccine effectiveness has been variably low, between 10 and 60%, from 2005 to 2018 seasons (CDC). Older individuals are known to have further lower influenza vaccination efficacy (12–13%), accounting for the majority (90%) of influenza-related deaths (Iuliano et al., 2018; Thompson et al., 2003). Aging-related immunosenescence explains the reduced capacity to generate protective *de novo* B and T cells to vaccination (Frasca and Blomberg, 2020; Kim et al., 2021). Potent vaccine adjuvants would enable enhancing the efficacy of influenza vaccination in older adults.

Aluminum salts (alum) have been the most commonly used adjuvant for licensed vaccine products, promoting T helper type 2 (Th2) antibody responses but with limited efficacy (Young et al., 2015). Since the 1990s, more potent adjuvants have been developed and approved for use in human vaccines that include Squalene oil-in-water emulsion MF59, AS04 (Alum+MPL), AS01 (Saponin QS-21+MPL in liposome), and CpG oligodeoxynucleotides (O'Hagan et al., 2020). MF59 in influenza vaccination significantly enhances vaccine immunogenicity in humans (O'Hagan et al., 2013), although cross-reactive antibodies are relatively limited (Bihari et al., 2012). The discovery of natural and synthetic agonists of pathogen recognition receptors such as Toll-like receptors (TLRs) has contributed to advancing the adjuvant field and understanding the mechanism of adjuvant actions. Particularly, monophosphoryl lipid A (MPL in AS04) and CpG, TLR4, and TLR9 agonists, respectively, are approved for use in licensed vaccines, including Cervarix and Heplisav (O'Hagan et al., 2020). AS01 containing MPL and QS-21 from *Quillaja Saponaria* is included in Shingles vaccination for older individuals (≥ 50 years old) (James et al., 2018; Lal et al., 2015). Bacillus Calmette-Guerin (BCG) vaccination is associated with better clinical outcomes in COVID-19 patients due to immune training rather than specific immune memory (Escobar et al., 2020). Clinical benefits of BCG cell-wall skeleton (CWS) as an innate immune therapy have been demonstrated in cancer patients (Kodama et al., 2009). CWS is known to mediate adjuvant effects via TLR2 and TLR4 signaling pathways (Tsuji et al., 2000). CWS-adjuvanted influenza vaccination significantly enhances the immunogenicity and protective efficacy in infant, adult, and older age BALB/c mouse models (Kim et al., 2021). CpG plus MPL adjuvanted influenza vaccination has been shown to induce enhanced protection against homologous and heterosubtypic viruses in mice (Ko et al., 2018). Despite many studies reporting superior effects of new adjuvants over Alum, parallel comparison to determine the superiority of these potent adjuvants in terms of immunogenicity and protection from challenge remains unknown.

In this study, we compared the effects of three potent adjuvants or combination adjuvants (CWS, QS-21+MPL, CpG+MPL) on enhancing the immunogenicity and homologous and heterosubtypic protection of influenza vaccination in adult C57BL/6 and aged BALB/c mouse models. QS-21+MPL was found to be most effective in inducing Th1 type IgG antibody responses, whereas both CpG+MPL and QS-21+MPL combination adjuvants exhibited similar potency in enhancing vaccination responses leading to increased protection by influenza challenge in adult C57BL/6 and aged BALB/c mouse models.

2. Materials and methods

2.1. Animals, reagents, and viruses

Adult C57BL/6 mice (6- to 8-week-old, female) were purchased from Jackson laboratory (Bar Harbor, ME). Aged BALB/c mice (17- to 20-month-old, female) were provided by the National Institutes of Health (NIH). BALB/c mice at ages of 8 to 10 months, purchased from Taconic Biosciences (Rensselaer, NY), were aged to become 17- to 20-month-old in the animal facility at Georgia State University to supplement the limited supply of aged mice from NIH. All mouse experiments were performed under the guidelines of the approved IACUC protocol (A21004).

Pure *Quillaja* saponin, QS-21, and monophosphoryl lipid A (MPL) adjuvants were purchased from Desert King International (San Diego, CA, USA) and Sigma Aldrich (St. Louis, MO), respectively, and dissolved in dimethyl sulfoxide (DMSO) following the manufacturer's protocol. Mouse-specific oligodeoxynucleotide (ODN) with CpG motifs (ODN1826, 5′-TCC ATG ACG TTC CTG ACG TT-3′) was synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The lyophilized CpG was resuspended in ultra-pure water. Cell wall skeleton (CWS) *from Mycobacterium bovis* BCG (Pasteur 1173P2) was generously provided by Dr. Jo (Chungnam National University, Korea) and prepared as described (Kim et al., 2021). All the adjuvants were aliquoted and saved at −80 °C until use.

A/California/04/2009 (A/Cal) H1N1 virus was used as the split vaccine strain and for homologous challenge. Reverse genetics (rg) reassortant H5N1 (rgH5N1) virus, containing HA and NA derived from A/Vietnam/1203/2004 and six internal genes from A/Puerto Rico/8/1934, as described previously (Song et al., 2011), was used as a heterologous challenge virus. A/Cal H1N1 and rgH5N1 viruses were propagated using embryonated chicken eggs. Inactivated viruses used as coating antigens for Enzyme-linked immunosorbent assay (ELISA) were prepared as described previously (Ko et al., 2018). Briefly, A/Cal H1N1 or rgH5N1 influenza viruses were inactivated with 1% formalin and concentrated by ultracentrifugation (SW32 Ti rotor, 123,760×g, 1 hour). The inactivated virus pellet was resuspended in phosphate-buffered saline (PBS) and was used as the ELISA coating antigen. For influenza split vaccine (sCal) preparation, inactivated A/Cal H1N1 virus was treated with 1% Triton X-100 to disrupt virion particles. The disrupted virus particles were dialyzed against PBS 3 times overnight. The total protein concentration of the inactivated viruses was determined by the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

2.2. Immunization and virus challenge of mice

To determine the protective efficacy of combination adjuvants against homologous virus (A/Cal H1N1), groups of 6-week-old C57BL/6 mice (n = 7) were intramuscularly immunized with sCal (3 μg/mouse) alone or sCal (3 μg/ mouse) plus CWS (25 μg/ mouse), QS-21 (10 μg) + MPL (1 μg)/mouse, or CpG (4 μg) + MPL (1 μg)/mouse. Young adult (6 weeks old) BALB/c mice (n = 4) were prime immunized with sCal (0.6 μg/mouse) alone vaccination for comparison with C57BL/6 mice. Blood samples were collected at 2 weeks after immunization, and sera were separated. Three weeks after prime immunization, the mice were challenged with a lethal dose of homologous A/Cal H1N1 virus ($2\times LD_{50}$, equivalent to $Log10^{3.7}\times EID_{50}$). For cross-protection studies, C57BL/6 mice (n = 6) were intramuscularly immunized (prime and boost) with sCal alone or sCal plus CWS, QS-21+MPL, or CpG+MPL at a 3-week interval. Three weeks after boost, the mice were challenged with a lethal dose of heterosubtypic rgH5N1 virus ($2\times LD_{50}$, equivalent to $Log10^{3.4}\times EID_{50}$) in the BSL-2+ designated animal facility at Georgia State University. Considering the availability and further aging disadvantages in response to vaccination, BALB/c strain was used for the aged mouse model studies. BALB/c mice (n = 12), at 17 to 20 months of age, were intramuscularly immunized (prime and boost) with sCal (1 μg/ mouse) alone or sCal plus CWS, QS-21+MPL, or CpG+MPL at a 3-week interval. Four weeks after boost, the immunized mice were challenged with a lethal dose of homologous A/Cal H1N1 virus ($2\times LD_{50}$). After challenge, body weight changes and survival rates were monitored for 14 days, and lung viral titers and detailed immunological profiles were determined in bronchoalveolar lavage (BAL), mediastinal lymph nodes (MLN), lung and spleen tissues collected at day 5 post infection.

2.3. Antibody Enzyme-linked immunosorbent assay (ELISA) and *in vitro* IgG antibody detection

To measure antigen-specific antibody levels, inactivated A/Cal H1N1 or rgH5N1 viruses (200 ng/well) were coated onto ELISA plates and then incubated with diluted immune sera as detailed previously (Ko et al., 2018; Ko et al., 2016). IgG isotypes were measured using anti-mouse immunoglobulin IgG, IgG1 and IgG2a (or IgG2c), horse-radish peroxidase (HRP)-conjugated secondary antibodies (Southern Biotechnology, Birmingham, AL), and tetramethylbenzidine (TMB) substrate (Invitrogen, Waltham, MA). Antibody levels are presented as optical density absorbance values at 450 nm (BioTek ELISA plate reader) or as concentrations calculated using standard IgG (Southern Biotech). Additionally, consensus group I hemagglutinin (HA) stalk protein (50 ng/well), prepared as described (Chae et al., 2019), and N1 neuraminidase (NA) protein (20 ng/well, BEI Resources, NR-19234) derived from A/California/04/2009 (H1N1) virus were used to determine HA stalk and NA-specific IgG antibodies.

Bronchoalveolar lavage fluid (BALF) was obtained by infusing 1.5 mL of phosphate-buffered saline (PBS) into the lungs. Lung extracts were prepared in 1.5 mL of Roswell Park Memorial Institute (RPMI) 1640 without fetal bovine serum (FBS) by mechanical grinding of lung tissues harvested at day 5 after challenge. BALF and lung extracts were used to determine vaccine antigen (A/Cal)-specific antibody levels.

Secreted IgG antibodies specific for A/Cal H1N1 were determined from mediastinal lymph nodes (MLN, 5×10^5 cells/well) and spleen tissues (5×10^5 cells/well) from C57BL/6 and BALB/c mice. The cells from MLN and spleen were isolated at day 5 post infection and cultured for 1 day and 5 days in 96-well cell culture plates (Corning, Kennebunk, ME) pre-coated with inactivated A/Cal H1N1 virus (200 ng/well). The combined levels of IgG antibodies secreted into the culture supernatants and those captured on the plate were analyzed by ELISA.

2.4. Hemagglutination inhibition (HAI) assay

HAI titers in immune sera treated with receptor destroying enzymes (RDE, Sigma-Aldrich) and inactivated (at 56 °C for 30 min) were determined by using 4 HA units of A/Cal H1N1 or rgH5N1 virus and 0.5% chicken red blood cells (RBC, Lampire Biological Laboratories, Pipersville, VA) as previously described (Ko et al., 2018; Ko et al., 2016).

2.5. Lung viral titration

Lung extracts prepared in 1.5 mL of RPMI 1640 without FBS by mechanical grinding of lung tissues harvested at day 5 after challenge were used to determine viral titers in embryonated chicken eggs (Hy-Line North America, LLC, Mansfield, GA), as described (Kim et al., 2019). Virus titers as 50% egg infection dose (EID_{50})/mL were evaluated according to the Reed and Muench method (Reed and Muench, 1938).

2.6. Enzyme-linked Immunospot (ELISpot) assay

Interferon (IFN)-γ secreting cell spots were determined by culturing splenocytes (5×10^5 or 10^6 cells/well) and lung cells (5×10^5 or 3×10^5 cells/well) for 72 hours on multi-screen 96-well plates (MilliporeSigma, St. Louis, MO) coated with cytokine-specific capture antibodies as described (Song et al., 2011). Inactivated influenza A/Cal H1N1 was included as an antigenic stimulator (4 μg/mL) and IFN-γ secreting T cells were counted using an ELISpot reader (Biosys, Miami, FL).

2.7. Flow cytometry analysis

Lung cells were harvested from the layer of percoll gradients between 44% and 67% and stimulated with 4 μg/mL inactivated A/Cal H1N1 virus in the presence of Brefeldin A (20 μg/mL) for 5 hours at 37 °C as described (Lee et al., 2019; Lee et al., 2018). *In vitro* cultured cells (lung and BAL cells) were stained with anti-CD3-PacificBlue (Clone 17A2, Biolegend, San Diego, CA), anti-CD4-PE/Cy5 (Clone RM405, BD Biosciences, San Jose, CA) antibodies, and then fixed and permeabilized using BD Cytofix/CytopermTM Plus Kit (BD Biosciences). After staining the cells with anti-IFN-γ-APC/Cy7 (Clone XMG1.2, BD), anti-TNF-α-PE/Cy7 (Clone MP6-XT22, Biolegend), and anti-Granzyme B-FITC (Clone NGZB, eBioscience) antibodies, live lymphocytes were first gated by forward versus side scatter strategic gating, followed by the gating of $CD3^+$ T cells and then CD4 T cells secreting cytokines. The number of effector T cells in BAL and lung were expressed by reflecting the frequency gated out of the total cells from each mouse. Cells positive for intracellular cytokines were revealed through acquisition on a Becton-Dickinson LSR-II/

Fortessa flow cytometer (BD, San Diego, CA) and analyzed by Flowjo software (Tree Star Inc., Ashland, OR).

2.8. Analysis of acute innate immune responses to intraperitoneal adjuvant treatment

Naïve C57BL/6 mice were intraperitoneally injected with 200 μL of PBS, QS-21 (10 μg) + MPL (1 μg), or CpG (4 μg) + MPL (1 μg). Sera were collected from the mice at 2 hours and 20 hours after adjuvant injection to analyze systemic cytokine and chemokine levels using Ready-SET-Go kits with IL-6 or MCP-1 specific antibodies (eBioscience, San Diego, CA). Immune cells in peritoneal exudates from the mice were collected in 2 mL of PBS at 20 hours after adjuvant treatment and cellular phenotypes were determined by cell-specific phenotypic markers as described (Ko et al., 2018).

2.9. Statistical analysis

All results are presented as mean ± standard errors of the mean (SEM). The statistical significance for all the experiments was calculated by one-way or two-way analysis of variance (ANOVA). P-values ≤ 0.05 were considered significant. Data analysis was performed using Prism software (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Adjuvants enhance the protective efficacy of split vaccination despite differential IgG responses in C57BL/6 mice

C57BL/6 strain is a relatively low responder mouse model in humoral immune responses to experimental vaccination (Chen et al., 1999; Ko et al., 2018; Misplon et al., 2010). To compare the efficacy of different adjuvants, groups of C57BL/6 mice were intramuscularly primed with split sCal (H1N1) vaccine (3 μg) plus CWS, QS-21+MPL, or CpG+MPL (Fig. 1A). To directly compare the levels of IgG responses between young adult BALB/c and C57BL/6 mice, a group of BALB/c mice was intramuscularly primed with split sCal (H1N1) vaccine (Figs. 1B, C). The BALB/c mice with low dose sCal (0.6 μg) vaccination induced higher levels of IgG and IgG1 antibodies specific for the virus antigen, compared to those in C57BL/6 mice immunized with sCal (3 μg), as shown by ELISA OD values (Figs. 1D–F). Therefore, we reasoned that C57BL/6 mouse strain would be an appropriate model to compare the potency of different vaccine adjuvants. CWS was not combined with MPL because CWS exhibits adjuvant effects via TLR2 and TLR4 signaling pathways (Tsuji et al., 2000; Uehori et al., 2003; Yuk et al., 2010). The QS-21+MPL adjuvanted sCal group showed the highest levels of IgG1 and IgG2c isotype antibodies for A/Cal H1N1 after prime vaccination in C57BL/6 mice, followed by the CpG+MPL group, whereas the CWS group induced the lowest levels of IgG2c (Figs. 1D–F). The inclusion of adjuvants significantly enhanced IgG responses specific for HA stalk domain and HAI activities against homologous A/Cal H1N1 virus at similar levels (Figs. 1G, H). All adjuvanted vaccine groups were protected against lethal challenge with homologous A/Cal H1N1 virus, displaying 100% survival rates without severe weight loss, in contrast to the sCal alone and naïve groups that showed severe weight loss and no survival rates (Fig. 1I). At day 5 post challenge, approximately 100-fold lower levels of lung viral titers were detected in all adjuvanted vaccine groups than those in sCal alone and naïve infected groups (Fig. 1J).

3.2. Adjuvanted vaccination enhances IgG levels at respiratory sites, IgG secreting plasma and cytokine-producing T cells at early time post challenge in C57BL/6 mice

Adjuvant effects on inducing vaccine antigen-specific B and T cell responses were compared at day 5 post challenge with A/Cal H1N1 virus (Fig. 2). Higher levels of A/Cal-specific IgG antibodies were observed in BALF and lung extracts from the adjuvanted vaccine groups (Figs. 2A, B). Also, after *in vitro* culturing MLN and spleen cells for day 1 or 5, A/Cal-specific IgG antibodies from the adjuvanted vaccine groups were produced at higher levels than those from sCal and naïve mice at day 5 post infection (Figs. 2C, D). IFN-γ producing spots from lung and spleen samples and IFN-γ^+ $CD4^+$ T cells from BALF were induced at higher levels in the adjuvanted sCal vaccinated C57BL/6 mice than those in sCal vaccinated and naïve mice at early time post challenge (Figs. 2E–G).

3.3. Prime boost adjuvanted vaccination induces humoral and cellular immune responses contributing to heterosubtypic cross-protection in C57BL/6 mice

A regimen of prime boost vaccination and heterosubtypic rgH5N1 virus challenge is presented (Fig. 3A). The highest levels of IgG and IgG2c isotype antibodies specific for A/Cal H1N1 virus were induced in the QS-21+MPL adjuvanted sCal group after booster dose, followed by the CpG+MPL and CWS groups (Figs. 3B–D). Substantial levels of IgG and IgG1 (Figs. 3B, C) but not IgG2c isotype antibodies (Fig. 3D) were induced after boosting with sCal vaccine alone. IgG antibodies binding to heterosubtypic rgH5N1 virus (Fig. 3E), HA stalk domain (Fig. 3F), and N1 NA (Fig. 3G) were induced at the highest levels in the QS-21+MPL adjuvanted sCal group after boost, followed by the CpG+MPL and CWS groups. Consistent with the IgG levels, the highest titers of HAI were induced in the QS-21+MPL adjuvanted sCal group, followed by the CWS and CpG+MPL groups at 2 weeks and 3 months after boost vaccination (Figs. 3H, I).

We determined whether prime boost adjuvanted vaccination would induce cross-protection in C57BL/6 mice after lethal challenge with rgH5N1 virus. The CpG+MPL and QS-21+MPL adjuvanted sCal groups displayed the least weight loss and rapidly recovered by day 8 post challenge (<5 %), whereas the average peak weight loss in the CWS group was approximately 11% (Fig. 3J). The sCal only and naïve groups showed severe weight loss, and all mice died of infection in contrast to 100% protection in the adjuvanted sCal vaccine groups (Figs. 3J, K). The CWS adjuvant group showed the lowest levels of HA stalk and N1 NA antibodies (Figs. 3F, G) after vaccination and the highest T cell responses at recall compared to the other groups (Figs. 2E–G). To determine the roles of T cells in conferring cross-protection, both CD4 and CD8 T cells were depleted before and after challenge, and body weight changes and survival rates were monitored (Supplementary Fig. S1). The CWS adjuvanted sCal group with CD4/8 depletion was not protected (Supplementary Fig. S1A), suggesting dependency on T cell immunity. Meanwhile, the QS-21+MPL and CpG+MPL groups (Supplementary Figs. S1B, C) with CD4/8 depletion displayed severe weight loss in a wide range of 12 to 18% against lethal challenge with rgH5N1 virus, suggesting that both humoral and cellular immunity contribute to cross-protection.

3.4. Adjuvanted vaccination enhances IgG antibodies, HAI titers, and protection in aged BALB/c mice

A regimen of prime boost vaccination and homologous A/Cal H1N1 virus challenge in aged BALB/c mice is presented (Fig. 4A). QS-21+MPL adjuvanted sCal prime vaccination of aged BALB/c mice induced a measurable level of IgG2a isotype, in contrast to CWS adjuvanted sCal prime dose promoting IgG1 antibody production, but the overall IgG and isotype levels were significantly lower than those in young adult C57BL/6 mice (Supplementary Figs. S2A–C). Adjuvanted sCal boost vaccination substantially enhanced the induction of IgG, IgG1, and IgG2a isotype antibodies, with the QS-21+MPL group displaying the highest IgG2a levels in aged BALB/c mice (Figs. 4B–D). IgG antibodies binding to the HA stalk domain (Fig. 4E) and N1 NA (Fig. 4F) were induced at the highest levels in the QS-21+MPL adjuvanted sCal group after boost, followed by the CWS and CpG+MPL groups. The HAI functional antibodies against homologous A/Cal H1N1 virus were induced at higher levels in the CWS and QS-21+MPL groups than those in the CpG+MPL group, while the sCal vaccine alone group could not induce detectable levels of HAI titers in aged BALB/c mice (Fig. 4G).

Both CpG+MPL and QS-21+MPL adjuvanted sCal groups were 100% protected against lethal challenge with A/Cal H1N1 virus, with the CpG+MPL group displaying a trend of lower weight loss (~4%) compared to the QS-21+MPL vaccine group (~6%) and quicker recovery, but there were no statistical differences (Fig. 4H). The CWS group also provided 100% protection against A/Cal H1N1 virus challenge, despite a moderate weight loss (~9 %) without significant differences among the adjuvanted groups. In contrast, heat-killed lactic acid bacterium (LAB) shown to be an effective adjuvant for influenza vaccination in C57BL/6 mice (Jung et al., 2020) did not improve the vaccine efficacy, resulting in severe weight loss in aged BALB/c mice (Supplementary Fig. S3). The sCal alone and naïve aged BALB/c mice experienced severe weight loss and did not survive A/Cal virus challenge (Fig. 4H). Approximately 100-fold lower lung viral titers were detected in the adjuvanted sCal groups at day 5 after challenge, compared to those in naïve infected aged BALB/c mice (Fig. 4I).

3.5. Aged BALB/c mice with adjuvanted vaccination induce IgG levels at respiratory sites, IgG secreting plasma and cytokine-producing T cells at early time post challenge

Correlating with protection, higher levels of A/Cal-specific IgG antibodies were induced in BALF and lung extracts at day 5 post challenge from the adjuvanted vaccine groups, compared to the significantly low levels in the sCal vaccine only and naive aged BALB/c mice (Fig. 5A). Also, one- or five-day *in vitro* culture of MLN cells and spleen cells produced higher levels of A/Cal virus-specific IgG antibodies from the adjuvanted vaccine groups than those in sCal alone and naïve aged BALB/c mice (Figs. 5B, C). These IgG antibodies produced *in vitro* recognized HA proteins (Supplementary Figs. S2D, E) in a similar pattern as the binding to A/Cal H1N1 virus. Intracellular cytokine staining results showed that higher levels of $CD4^+$ T cells secreting IFN-γ (in all three adjuvants), TNF-α (highest in CWS), or granzyme B (highest in QS-21+MPL) were detected in lung tissue samples from the adjuvanted sCal groups at day 5 post infection than those in sCal alone and naïve groups (Figs. 5D–F). In the cytokine ELISpot data (Figs. 5G, H), the QS-21+MPL

adjuvanted sCal group showed the highest levels of IFN-γ^+ spots in lung and spleen tissues, followed by the CWS group. The CpG+MPL group also showed substantial levels of IFN-γ^+ spots in lung and spleen tissues, but at a slightly lower level than the QS-21+MPL and CWS adjuvant groups.

3.6. Acute innate immune responses by QS-21+MPL combination adjuvant and unique features of enhancing adaptive immune responses to vaccination

To better understand the QS-21+MPL combination adjuvant effects, we analyzed acute innate immune responses after intraperitoneal injection of QS-21+MPL in C57BL/6 mice (Fig. 6). QS-21+MPL acutely induced inflammatory cytokine IL-6 and chemokine MCP-1 at higher levels in sera within 2 h than CpG+MPL, and then returned to near-basal levels, whereas both combination adjuvants acutely recruited monocytes, neutrophils, and dendritic cells at the site of injection (Fig. 6). Also, the addition of MPL to QS-21 (QS-21+MPL) resulted in over 4-fold higher increases in IgG2b and IgG2c isotype antibody levels than QS-21 adjuvant alone suggesting immunological advantages of the combination adjuvant (Supplementary Fig. S4). Interestingly, QS-21 equivalent Quil-A adjuvanted influenza vaccination could induce virus-specific IgG antibody responses in CD4-deficient mice at a significant level but not CpG adjuvant (Supplementary Fig. S5), suggesting less dependence of Quil-A on CD4 T cells in exhibiting adjuvant effects.

4. Discussion

QS-21 and MPL are the active components of a liposome-based AS01 adjuvant included in the highly effective Shingrix vaccine product for the elderly population and immunocompromised patients (Cunningham et al., 2016; Lal et al., 2015; Vink et al., 2020), progressively replacing an old live attenuated vaccine, Zostavax. Our previous studies reported potent adjuvant effects of CWS (Kim et al., 2021) and CpG+MPL combination adjuvant (Ko et al., 2018) on influenza vaccination. Here, we compared these potent adjuvant effects on enhancing the immunogenicity of influenza vaccination and efficacy of protection against homologous and heterosubtypic viruses in adult C57BL/6 and aged BALB/c mouse models. All three different adjuvants were able to increase IgG antibodies specific for virus and HA stalk domain, and HAI titers as well as confer homologous protection after prime dose in an adult C57BL/6 model, heterosubtypic protection after adjuvanted boost vaccination in adult C57BL/6 mice, and homologous protection after prime-boost vaccination in an aged BALB/c mouse model with differential efficacies. Both humoral and cellular immunity in mucosal and systemic sites induced by adjuvanted vaccination might have contributed to cross-protection.

A head-to-head comparison of the effects of potent adjuvants on vaccine immunogenicity and efficacy is rare, although there is a significant focus on developing and licensing combination adjuvants such as AS04 (Alum+MPL), AS03 (α-tocopherol in oil-in-water emulsion), and AS01. Beyond the use of AS01 in Shingrix, malaria and tuberculosis vaccines containing AS01 were reported to be protective in clinical trials (Rts, 2014; Van Der Meeren et al., 2018), adding to the value of AS01. Consistent with the results in this study, a synergy of QS-21 and MPL actions was shown to recruit neutrophils and monocytes

(Dendouga et al., 2012) and induce IFN-γ by stimulating macrophages and inflammatory cytokines (Coccia et al., 2017). Recently, CpG adjuvant was approved for use in licensed hepatitis B virus vaccine (O'Hagan et al., 2020). Our previous studies demonstrated that MF59 oil-in-water emulsion, AS04, and MPL, but not alum adjuvant, could enhance vaccine immunogenicity and protection in CD4-deficient mice as in wildtype mice (Ko et al., 2016). BCG-CWS was shown to exhibit adjuvant effects on vaccination mainly via TLR2 and partly TLR4 signaling pathways (Tsuji et al., 2000) and even in CD4-deficient mice (data not shown). These three potent adjuvants appear to share a common TLR4 signaling pathway and stimulate innate immune responses leading to enhanced immunogenicity and protective efficacy of sCal vaccination.

Although all adjuvanted single-dose vaccinations induced comparably enhanced protection against homologous virus in C57BL/6 mice as evidenced by lung viral clearance and lower weight loss compared to the naïve infected mice, the effects of CWS, QS-21+MPL, and CpG+MPL adjuvants were differential in inducing Th1 type IgG2c (or IgG2a) isotype antibodies specific for the virus. QS-21+MPL adjuvant in influenza vaccination was found to be more effective in promoting T helper type 1 (Th1) IgG2c (in C57BL/6 mice), IgG2a antibodies (in BALB/c mice), and protection in both mouse strains, whereas CpG+MPL was a potent adjuvant in enhancing the protection by influenza vaccination in both adult C57BL/6 and aged BALB/c mouse models. Th1 IFN-γ cytokines promote B cells to produce IgG2c isotype in C57BL/6 mice (IgG2a in BALB/c mice), whereas Th2 cytokine responses promote IgG1 isotype antibodies (Stevens et al., 1988). Murine IgG2a isotype is known to be the most effective in inducing antibody-dependent cell-mediated cytotoxicity and protection via Fc receptor-mediated mechanisms (Kipps et al., 1985; Oishi et al., 1992; Wiedinger et al., 2020), suggesting an immunological advantage in promoting Th1 type IgG antibody responses by QS-21+MPL adjuvanted vaccination.

Prime boost vaccination of C57BL/6 mice with sCal alone was shown to induce HAI antibodies lowering lung viral titers by 100 folds against homologous A/Cal H1N1 virus after challenge, which was dependent on CD4 T cell help (Ko et al., 2017). MF59 and AS04 were demonstrated to overcome CD4 T cell deficiency defect in exhibiting adjuvant effects on enhancing homologous protection of sCal vaccination (Ko et al., 2016; Ko et al., 2017). Here, we extended potent adjuvant effects on heterosubtypic protection after boost dose in an adult C57BL/6 mouse model and homologous protection in an aged BALB/c mouse model. CWS was also effective in inducing mucosal IgG and IFN-γ^+ CD4 T cell responses at early time post challenge, as observed in the QS-21+MPL and CpG+MPL groups. The least weight loss against rgH5N1 virus was observed in the CpG+MPL group, followed by the QS-21+MPL group, although QS-21+MPL adjuvanted sCal (H1N1) vaccination was most effective in inducing humoral IgG2c and HAI titers against A/Cal H1N1 virus. Particularly, the adjuvant effects on enhancing IgG antibodies to stalk and NA were prominent. Under a condition without cross-reactive HAI antibodies against rgH5N1, this study suggested that adjuvants enhanced T cell immunity, IgG antibodies for stalk domain, and binding IgG antibodies to rgH5N1 and N1 NA, contributing to cross-protection. Notably, CpG+MPL adjuvant effect on enhancing cross-protection was comparable to QS-21+MPL, preventing weight loss more effectively than the CWS adjuvanted vaccination, although there were no significant differences.

Older aged mice were found to be very poor in inducing protective immune responses to influenza vaccination. Our previous study reported that CWS has potent adjuvant effects on increasing the immunogenicity and protective efficacy of influenza vaccination in young infant, adult, and older aged BALB/c mice (Kim et al., 2021). MF59-adjuvanted sCal vaccination shown to be protective even in CD4-knockout mice (Ko et al., 2016) could not prevent severe weight loss against homologous challenge in aged BALB/c mice despite enhanced IgG antibody responses (data not shown). Heat-killed LAB, which also exhibited adjuvant effects on improving influenza vaccine efficacy even in CD4 knockout mice (Jung et al., 2020) displayed weak adjuvant effects on influenza vaccination, resulting in severe weight loss and partial survival after lethal challenge with homologous virus in aged BALB/c mice. QS-21+MPL adjuvant in sCal vaccination was more effective than CWS and CpG+MPL in enhancing IgG2a antibodies in addition to comparable HAI titers and IFN-γ^+ CD4 T cell responses in aged BALB/c mice. The CpG+MPL adjuvanted sCal vaccination of aged BALB/c mice was most effective in preventing weight loss after challenge, although there were no statistically significant differences among the adjuvants compared in this study.

5. Conclusion

This study has significance in providing a comparative assessment of the effects of three different potent adjuvants or combination adjuvants on enhancing the humoral and cellular immunogenicity and protection against homologous and heterosubtypic viruses in both adult C57BL/6 and aged BALB/c mouse models. Combination adjuvant, QS-21+MPL, was found to be the most effective in enhancing the immunogenicity of the sCal vaccine, whereas CpG+MPL was highly effective in improving the vaccine efficacy of cross-protection in adult mice and homologous protection in aged mice despite no statistically significant differences between these two potent combination adjuvants. Considering the differential effects of different adjuvants on inducing stalk antibodies, it will be informative to assess the efficacy of cross-protection in naïve mice by passive transfer of immune antisera from the mice with different adjuvanted vaccinations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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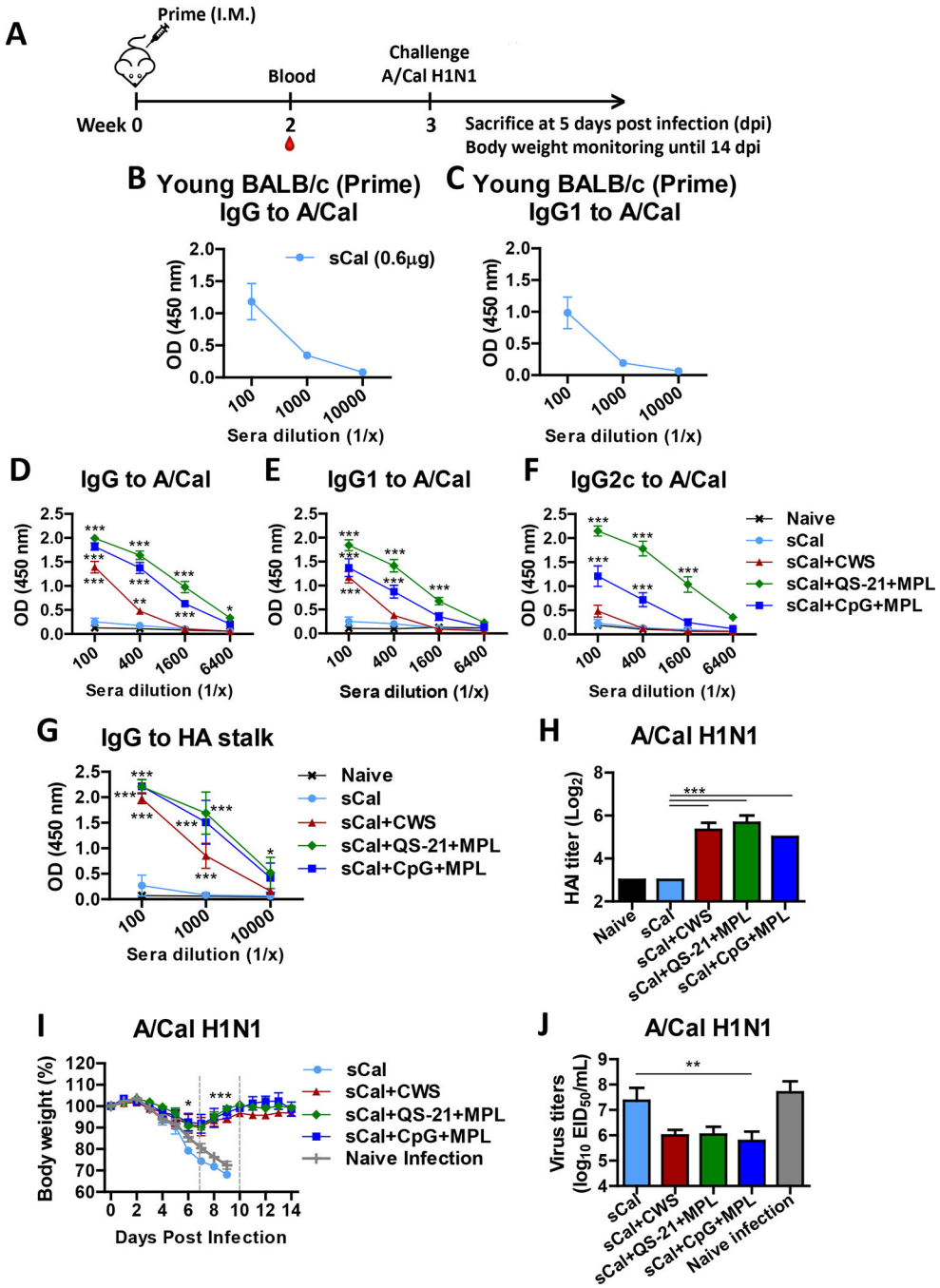

Figure 1. A single dose of adjuvanted influenza vaccination confers protection against lethal challenge with homologous A/Cal H1N1 virus in C57BL/6 mice.

(A) Immunization scheme (n = 7). C57BL/6 mice were intramuscularly immunized with sCal (3 μg) split vaccine alone or sCal plus CWS (25 μg), QS-21 (10 μg) + MPL (1 μg) or CpG (4 μg) + MPL (1 μg). A timeline for collection of blood samples and challenge is indicated. (**B** and **C**) Antibodies to A/Cal H1N1 virus were determined in prime sera from young adult BALB/c mice (n = 4) intramuscularly immunized with low dose sCal (0.6 μg). **(D-F)** A/Cal H1N1 virus-specific IgG and isotype antibody levels were determined in

prime sera. **(G)** Antibody responses specific for consensus group I full-length HA stalk in prime sera. **(H)** HAI titers against A/Cal H1N1 virus in prime sera. Three weeks post immunization, the mice were challenged with a lethal dose of A/Cal H1N1 virus ($2\times LD_{50}$). **(I)** Body weight changes were monitored for 14 days. **(J)** Lung viral titers as 50% egg infectious titers (EID_{50}) at day 5 post infection using embryonated chicken eggs. Statistical significance was calculated by using one- or two-way ANOVA and Dunnett's or Bonferroni's post-multiple comparison tests. Error bars indicate the mean ± standard errors of the mean (SEM). *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$ compared to sCal group.

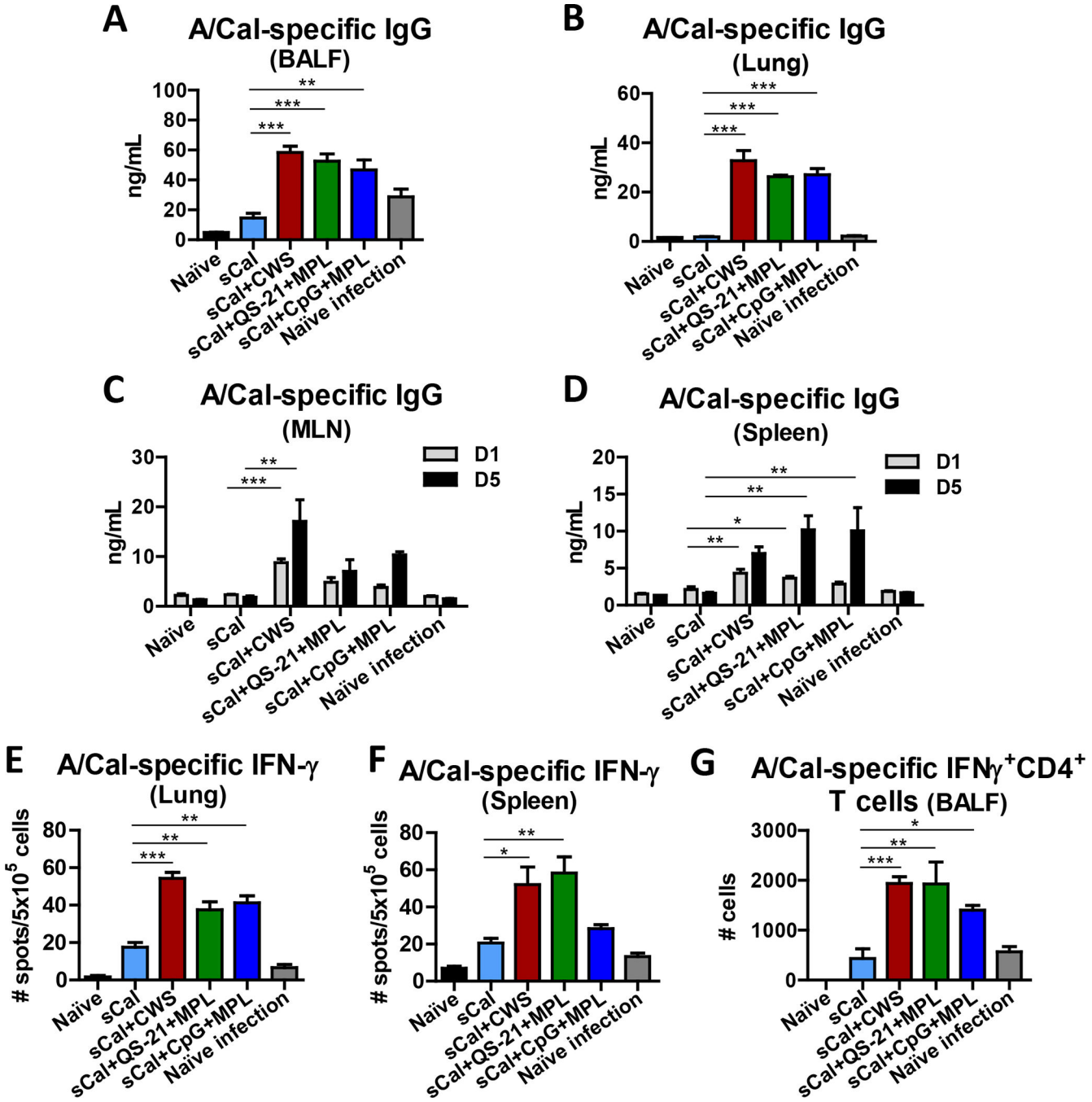

Figure 2. Adjuvanted vaccination enhances antibody-secreting cells and effector T cells upon influenza virus infection in young C57BL/6 mice.

(A and **B)** A/Cal virus-specific IgG levels in BALF and lung samples collected at day 5 post challenge (n = 4). **(C** and **D)** A/Cal virus-specific IgG production from *in vitro* cultures of MLN cells and splenocytes harvested on day 5 post infection, for 1 day and for 5 days in the presence of inactivated A/Cal H1N1 virus (200 ng/well). **(E** and **F)** Cytokine ELISpot of lung cells and splenocytes at day 5 post infection after *in vitro* stimulation with inactivated A/Cal (4 μg/mL per well). **(G)** Flow cytometry data of IFN-γ-secreting $CD4^+$ T cells in

BALF airway samples. Statistical significance was calculated by using one-way ANOVA and Dunnett's post-multiple comparison tests. Error bars indicate the mean ± standard errors of the mean (SEM). *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$ compared to sCal group.

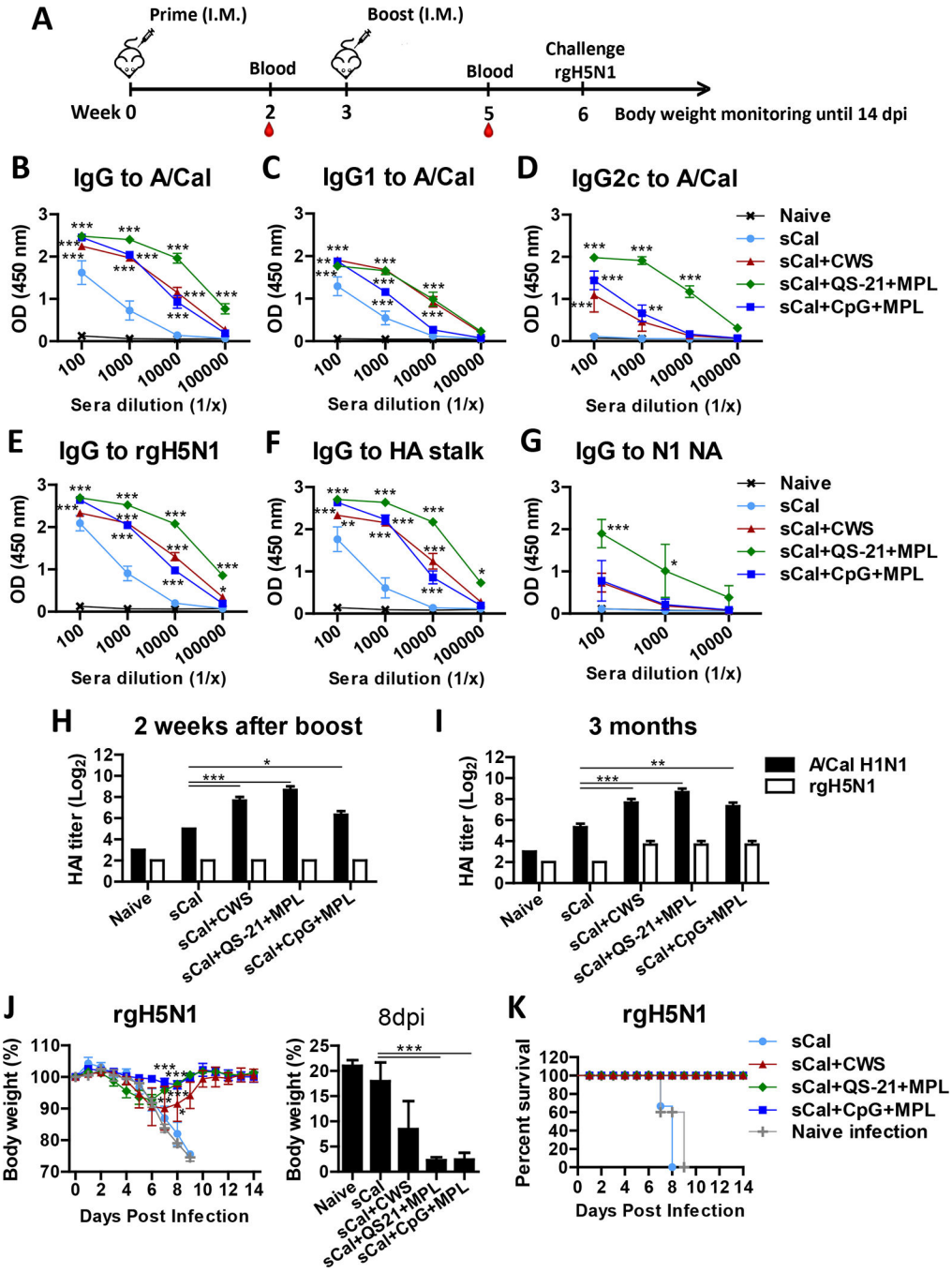

Figure 3. Adjuvanted prime boost vaccination confers protection against lethal infection with heterosubtypic rgH5N1 virus in young C57BL/6 mice.

(A) Immunization scheme (n = 6). C57BL/6 mice were intramuscularly prime and boost immunized at a 3-week interval with sCal alone or sCal plus CWS, QS-21+MPL, or CpG+ MPL. **(B-D)** A/Cal H1N1 virus-specific IgG and isotype antibody levels in boost sera. **(E)** rgA/Viet H5N1 virus-specific IgG antibody levels in boost sera. **(F)** Consensus group I full-length HA stalk-specific IgG antibody levels in boost sera. **(G)** N1 NA-specific IgG antibody levels in boost sera. **(H** and **I)** HAI titers against A/Cal H1N1 virus at two weeks

(H) and three months (I) after boost. **(J)** Body weight changes and **(K)** Survival rates after lethal infection with rgH5N1 virus ($2\times LD_{50}$). Statistical significance was calculated by using one- or two-way ANOVA and Dunnett's or Bonferroni's post-multiple comparison tests. Error bars indicate the mean ± standard errors of the mean (SEM). *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$ compared to sCal group.

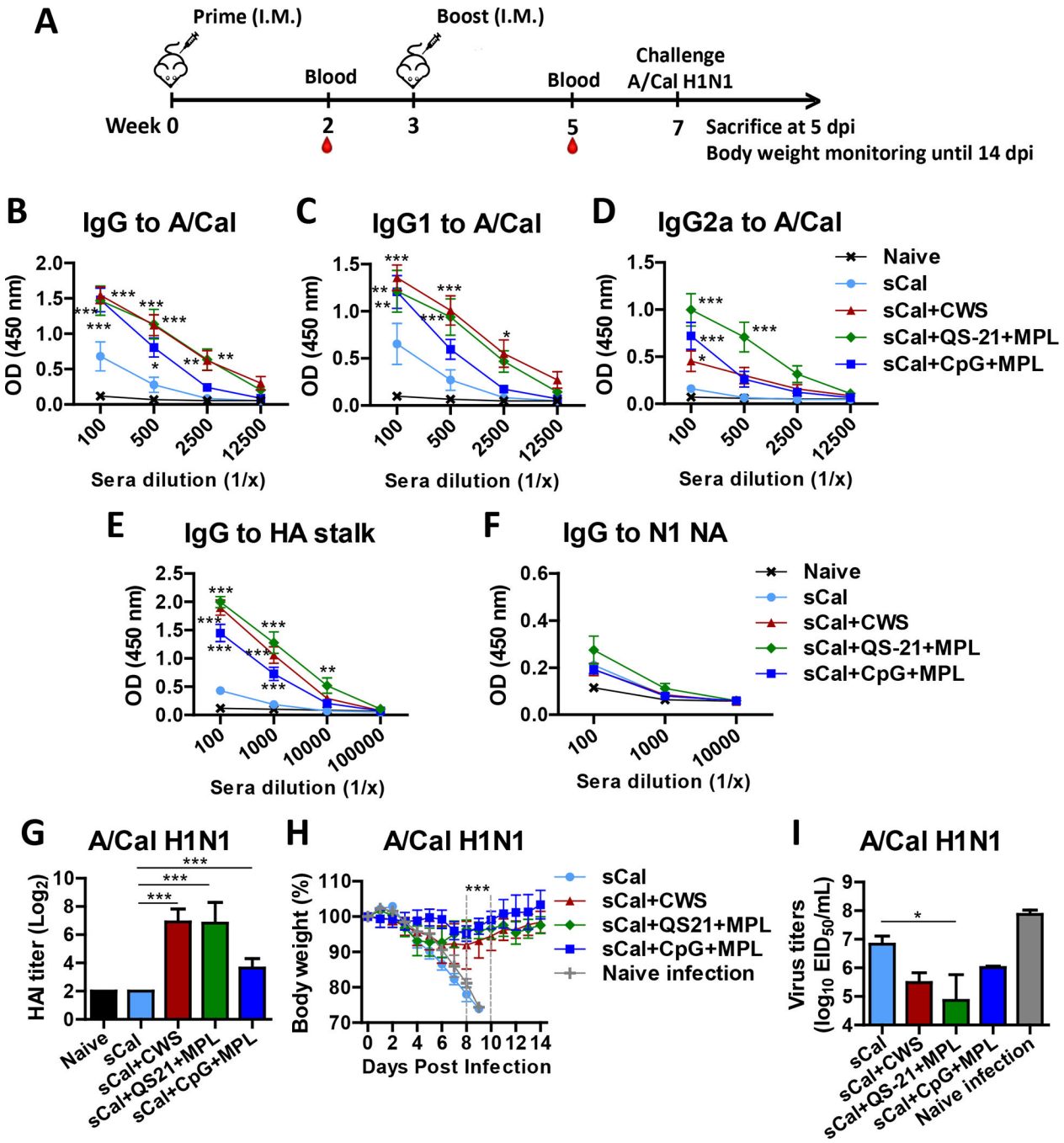

Figure 4. Adjuvanted boost vaccination confers protection against lethal challenge with homologous A/Cal H1N1 virus in aged BALB/c mice.

(A) Immunization scheme. Aged BALB/c mice (n = 12) were intramuscularly prime and boost immunized with sCal (1 μg) alone or sCal plus CWS, QS-21+MPL, or CpG+MPL. **(B-D)** A/Cal H1N1 virus-specific IgG and isotype antibody levels in boost sera. (**E** and **F**) IgG antibody specific for HA stalk and N1 NA proteins in boost sera. **(G)** HAI titers against A/Cal H1N1 virus in boost sera. **(H)** Body weight changes after challenge with a lethal dose of A/Cal H1N1 virus (2×LD_{50}). **(I)** Lung viral titers (EID_{50}) at day 5 post infection.

Statistical significance was calculated by using one- or two-way ANOVA and Dunnett's or Bonferroni's post-multiple comparison tests. Error bars indicate the mean ± standard errors of the mean (SEM). *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$ compared to sCal group.

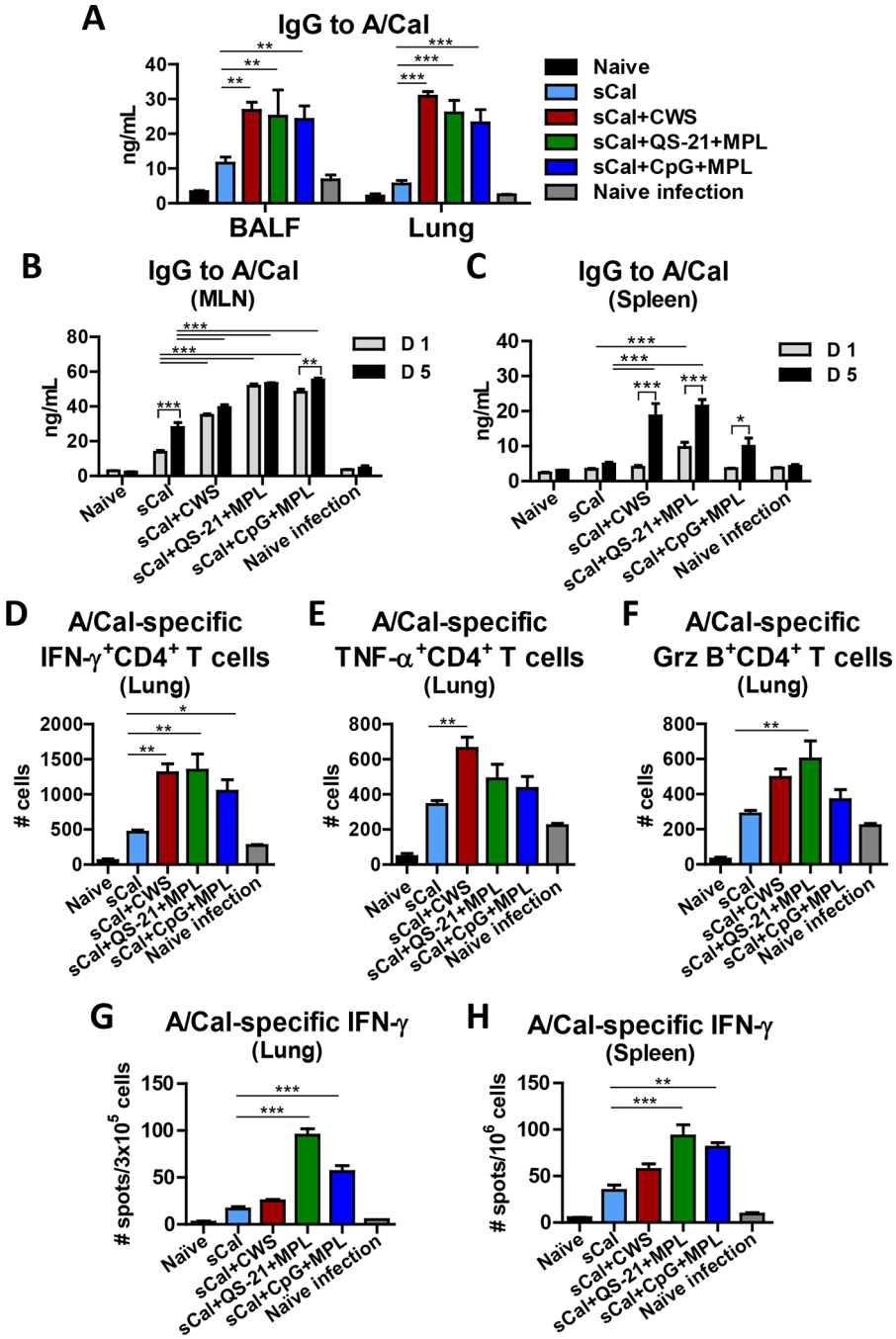

Figure 5. Adjuvanted boost vaccination enhances antibody-secreting cells and effector T cells upon influenza virus infection in aged BALB/c mice.

(A) A/Cal virus-specific IgG levels in BALF and lung samples harvested on day 5 post infection (n = 4). **(B and C)** A/Cal virus-specific IgG production from MLN cells and splenocytes harvested on day 5 post infection after *in vitro* culture for 1 day or 5 days with inactivated A/Cal H1N1 virus antigen. **(D-F)** Flow cytometry data of IFN-γ^+ CD4$^+$ T cells, TNF-α^+ CD4$^+$ T cells, and Granzyme B$^+$ CD4$^+$ T cells in lung cells after intracellular cytokine staining. (**G** and **H**) Cytokine ELISpot of lung cells and splenocytes at day 5

post infection after *in vitro* stimulation with inactivated A/Cal H1N1 virus (4 μg/mL per well). Statistical significance was calculated by using one-way ANOVA and Dunnett's post-multiple comparison tests. Error bars indicate the mean ± standard errors of the mean (SEM). *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$ compared to sCal group.

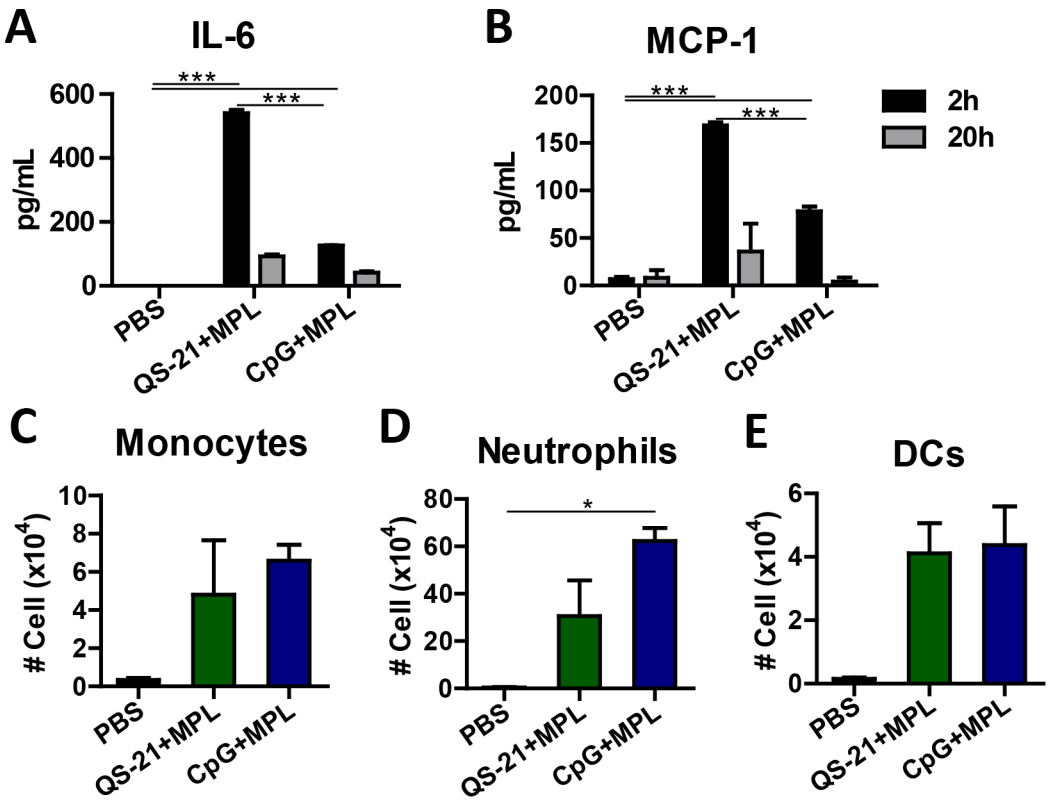

Figure 6. Acute induction of innate immune responses to intraperitoneal injection with adjuvants.

Naïve C57BL/6 mice (n = 3) were intraperitoneally injected with PBS, QS-21+MPL, or CpG+MPL. **(A** and **B)** Cytokine (IL-6) and chemokine (MCP-1) levels. **(C-E)** Cellular phenotypes in peritoneal exudates at 20 h after injection. Cell numbers of each cell population per mouse were calculated by multiplying cell percentages with total cell numbers. **(C)** Monocytes ($CD11b^{+}CD11c^{-}F4/80^{+}Ly6c^{hi}$), **(D)** Neutrophils ($CD11b^{+}CD11c^{-}F4/80^{-}Ly6c^{+}$), **(E)** Dendritic cells (DCs, $CD11b^{-}CD45^{+}F4/80^{-}CD11c^{+}$). Statistical significance was calculated by using one-way ANOVA and Dunnett's post-multiple comparison tests. Error bars indicate the mean ± standard errors of the mean (SEM). *; $p < 0.05$, ***; $p < 0.001$.