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FOR THE DEGREE OF MASTER OF SCIENCE

Exploring the adjuvant potential of *Peyssonnelia caulifera* extract on the efficacy of influenza vaccine in a mice model

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Abstract

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Adjuvants sourced from natural origins have attracted significant attention for their immune-boosting properties within vaccine development. This study focused on assessing the effectiveness of *Peyssonnelia caulifera (PC)*, a species of marine algae, in serving as a natural adjuvant candidate for the inactivated split A/Puerto Rico/8/1934 H1N1 influenza vaccine (sPR8). PC effectively stimulates antigen-presenting cells (APCs), leading to increased pro-inflammatory cytokine production and T cell activation *in vitro*. Furthermore, vaccines adjuvanted with PC elicited higher levels of antigen-specific IgG1 and IgG2a antibodies than traditional

alum-adjuvanted vaccines, indicating a robust humoral response. Notably, the PC-adjuvanted vaccine was superior in inducing a potent T helper type 1 (Th1) cellular immunity, as reflected in the elevated IgG2a antibody levels. In addition, vaccination with PC significantly enhanced the generation of memory B and T cell populations, providing a foundation for sustained immune protection. *In vivo* protection assays revealed a decrease in viral load and inflammatory responses in PC-adjuvanted vaccinated mice upon viral challenge, highlighting the adjuvant's effectiveness in triggering a strong immune response. These findings offer insights into the immune-modulating effects of natural adjuvants and support the enhancement of vaccination strategies against infectious diseases.

Keywords: *Peyssonnelia caulifera* (PC), natural adjuvant, influenza vaccine, Th1 immunity.

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I. INTRODUCTION

Annually, seasonal influenza infections are responsible for the deaths of an estimated 300,000 individuals worldwide [1]. Influenza outbreaks primarily rely on vaccination as the key method to decrease illness and death rates, while also resulting in significant reductions in health care expenses [2-4]. Among the various formulations of influenza vaccines, the split virus vaccine is widely used. While the split vaccine increases safety, it reduces immunogenicity, especially in individuals, primarily children, who have no prior immunity to influenza [5, 6] . Therefore, it is necessary to enhance the immune response through novel delivery systems and/or the use of adjuvants [7].

Employing adjuvants in vaccines significantly enhances immunogenicity, offering multiple immunological benefits. These include antigen dose minimization, improved vaccine efficacy in elderly populations, unexposed individuals, and those with compromised immune systems, as well as a broader influenza-specific immune response [8-10]. While current commercial adjuvants like AS03, Alum, MF59 and others licensed for pandemic influenza vaccines are effective, they also come with some limitations [11, 12] . For example, Alum is known to be particularly effective in triggering an antibody response, known as the Th2 (T helper type 2) response [13].This response leads to the production of antibodies, which are vital in recognizing and neutralizing pathogens such as viruses and bacteria. However, Alum is less effective in stimulating the cell-mediated immune response, known as the Th1 (T helper type 1) response. This pathway involves activating cells that directly attack infected cells in

the body, an essential defense against intracellular pathogens like viruses. Hence, identifying and creating safer adjuvants for influenza vaccines is a critical step in vaccine research. Safer adjuvants could enhance vaccine efficacy and reduce the health risks associated with the influenza virus. Moreover, developing such adjuvants is pivotal for improved protection against influenza and remains a primary focus of public health initiatives.

Marine algae, widely used in the food, cosmeceutical, and pharmaceutical industries, yield extracts composed of critical bioactive molecules [14-17]. Furthermore, these molecules show therapeutic promise in treating a variety of various pathologies, including tumors, metabolic disorders such as obesity, and infectious diseases [18-20]. Owing to their rich of metabolic products, algae synthesize diverse array of bioactive compounds, such as polysaccharides, lipid as well as proteins, vitamin, which are known for their significant immunostimulatory properties [21-24]. Consequently, the immunomodulatory activities of marine algae have captured scientific interest, establishing them as potential sources of novel therapeutic agents, especially in terms of the development of vaccine adjuvants.

Recent many researches underscored the potential of specific algae species to elicit potent immune reactions when used as adjuvants in vaccines [25-27]. The potential biological functions of the vast array of marine algae species remain largely unexplored. Each type of algae could have unique bioactive compounds beneficial for health and medicine, yet our current understanding is limited. With continued research, these diverse algae extracts may offer groundbreaking applications in biotechnology and therapeutics.

In light of this, this research focused on evaluating the potential of *Peyssonnelia caulifera* (PC), a type of marine algae, to serve as an adjuvant in influenza vaccine formulations. Initially, I conducted a preliminary exploration of the immunostimulatory effects of PC extracts by investigating PC's impact on antigen-presenting cells, including dendritic cells (DCs) and macrophages, through checking the activation of specific markers and cytokine secretion. Subsequently, I proceeded to assess the efficacy of PC as an adjuvant in a seasonal influenza vaccine, using mice model. This phase involved observing and analyzing the immune responses in mice after they were given the vaccine enhanced with PC adjuvant via intramuscular injection.

II. MATERIALS AND METHODS

1. Animals

Female BALB/c mice, aged 6 to 8 weeks, were obtained from OrientBio Co. (Gyeonggi, Korea). They were housed at the laboratory animal center of Jeju National University. All experiments involving these mice were carried out following the protocols sanctioned by the Institutional Animal Care and Use Committee (IACUC) of Jeju National University, as per the approval code 2021-0051.

2. Chemicals and reagents

The split A/PR8 vaccine, derived from the Influenza A virus strain A/Puerto Rico/8/1934 H1N1, underwent a preparation process beginning with an overnight inactivation using 1% neutral formalin. This step was followed by ultracentrifugation at 30,000 round per minute (rpm) for an hour to concentrate the mixture. The inactivated virus was then collected as a pellet and resuspended in phosphate-buffered saline (PBS). To disrupt the viral particles, 1% Triton X-100 was added, and its subsequent removal was accomplished by dialysis against PBS three times over night. The protein content in the final split virus preparation (sPR8) was measured using the EZ-BCA protein assay kit (DoGenBio, Seoul, Korea). The prepared vaccine was then stored at -80°C for preservation.

Peyssonnelia caulifera (PC) extract was sourced from the Marine Biobank of Korea (Chungcheongnam-do, Korea). Ingredients such as Monophosphoryl lipid A (MPL) and aluminum were obtained from InvivoGen, and all were prepared following the specific guidelines provided by their manufacturers.

3. DC and macrophage generation and treatment *in vitro*

The process for generating bone marrow-derived dendritic cells (BMDCs) and macrophages (BMDMs) followed established methods from previous studies [28, 29]. This involved extracting bone marrow cells from the femurs and tibias of BALB/c mice. To grow BMDCs and BMDMs, the cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, which included 10% fetal bovine serum and 1x antibiotic-antimycotic (Gibco) and was further enhanced with 20 ng/mL each of mouse granulocyte-macrophage colony-stimulating factor (mGM-CSF) for BMDCs and mouse macrophage colony-stimulating factor (mM-CSF) for BMDMs. The culture medium was refreshed every two days for a total of six days, adding either mGM-CSF or mM-CSF. After this period, the immature DCs and 0.2×10^6 cells/well were harvested.

For cytokine production analysis, immature DCs were seeded at 0.2×10^6 cells/well and macrophages at 1×10^5 cells/well in 96-well plates. These cells were treated with either 0.1 μg/mL MPL or 20 μg/mL PC extract and incubated at 37 °C. After 48 hours, cytokine levels of interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-12p40 in the culture supernatants were quantified using ELISA kits.

To analyze the effect of PC on the activation markers of DCs and macrophages, immature DCs at 2.5×10^6 cells/well and macrophages at 1.25×10^6 cells/well were placed in 6-well plates, with each well containing 2.5 mL of culture. The cells were treated with either 0.1 μg/mL MPL or 20 μg/mL PC. Following a two-day incubation, the cells were harvested and stained with fluorescently-labelled antibodies for phenotype analysis and to evaluate the expression of activation markers.

4. Assessment of allogeneic mixed lymphocyte reaction (MLR)

In this part of study, I investigated the impact of PC on the activation of dendritic cells and macrophages to T cell proliferation. Both BMDCs and BMDMs were seeded and subsequently exposed to PC for a duration of two days, mirroring the conditions utilized in the studies assessing the expression of activation markers. I obtained naïve lymphocytes (LPC) from C57BL/6 mouse spleen cells, following the method outlined in [28] . These lymphocytes were marked with carboxyfluorescein succinimidyl ester (CFSE) and then placed in co-culture with PC-treated DCs (0.4×10^5 cells/well) and macrophages (0.2×10^5 cells/well). The co-culture was set up in 96-well U-bottom plates, with each well holding 200 μL of the medium, at a density 4×10^5 CFSE-lymphocytes/well. The proliferation of T-cells over a five-day incubation was quantified using flow cytometry, and ELISA was employed to check cytokine secretion in the culture supernatants.

5. *In vivo* intraperitoneal injection

To assess the ability of PC to recruit cells into the peritoneal cavity of mice, we carried out an intraperitoneal injection experiment. Groups of six BALB/c mice were given intraperitoneal injections of 200 μL PBS, which contained either MPL (1 μg per mouse) or PC (100 μg per mouse). The peritoneal cells and exudates were collected from three mice at each of the two post-injection time points, 6 and 24 hours, using peritoneal lavage with 2 mL of PBS. After lavage, the fluids were centrifuged at 1600 rpm for 5 minutes at 4°C to isolate the peritoneal cells, which were then prepared for

flow cytometry. Additionally, we measured the levels of cytokines and chemokines in the peritoneal exudates using ELISA.

6. Animal immunization and viral infection

In this experiment, I divided mice into groups of eight and administered intramuscular injections. The injections were of either the sPR8 vaccine alone at a dose of 0.5 μg per mouse or the vaccine combined with adjuvants - specifically, Alum at 100 μg per mouse or PC at 200 μg per mouse, all in a 100 μL PBS solution. We also had a control group that received only PBS injections. The vaccination protocol involved an initial prime injection followed by a boost after two weeks. Blood samples were taken from the mice two weeks following each immunization.

Sixteen weeks after the booster, the mice were challenged intranasally with a lethal dose (1×LD50) of the A/PR8 virus. Subsequently, half the mice in each group were monitored daily for 14 days for any changes in weight and survival rates. The other half were euthanized on the seventh day after infection, and their lung and spleen tissues were collected for additional analysis.

7. Memory B cell response assay

To evaluate the activity of memory B cells and antibody-producing cells in vaccinated mice, we initially prepared cell culture plates by coating them with 400 ng per well of inactivated PR8 (iPR8) and left them overnight. These plates were then blocked with a complete medium, which consisted of RPMI media with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco). We used 200 μL/well of this medium for blocking, which was carried out for 1 hour at room temperature. Spleen and bone marrow cells, harvested from the mice seven days

post-infection, were seeded at a density of 4×10^5 cells / well. The spleen cells were incubated for 7 days, while the bone marrow cells were incubated for 1 day. The detection of antigen-specific antibodies produced by these cells was accomplished using anti-mouse immunoglobulin IgG, IgG1, and IgG2a antibodies, all conjugated with horseradish peroxidase (HRP).

8. Enzyme-linked immunosorbent assay (ELISA) for antibody, cytokine and chemokine levels

To evaluate the levels of cytokines and chemokines in both the culture supernatant and peritoneal lavage fluid, as well as in lung tissue extracts, I utilized specific ELISA kits. These kits, designed to measure interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1β, IL-12p40 (from Invitrogen, Waltham, MA, USA), and interferon gamma (IFN-γ), chemokine (C-C motif) ligand 2 (CCL2), and chemokine (C-C motif) ligand 5 (CCL5) (from R&D Systems, Minneapolis, MN, USA), were applied as per the manufacturer's instructions.

Two weeks following both the prime and boost vaccinations, immune sera were collected for analysis. The levels of antigen-specific antibodies in these samples were determined using an ELISA assay. For this purpose, ELISA plates were coated with the inactivated A/PR8 virus at 400 ng/well, and then blocked using a 1% bovine serum albumin (BSA) and 0.05% Tween 20 solution in PBS. The immune sera samples were serially diluted and added to the coated plates. Following this, the plates were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, targeting antigen-specific IgG, IgG1, and IgG2a antibodies. The detection reaction was

carried out using tetramethylbenzidine (TMB) as a substrate, and the optical density (OD) readings were taken at 450 nm using an ELISA plate reader.

9. Lung viral titration

At sixteen weeks after the boost vaccination, the mice were exposed to a lethal viral infection. Seven days post infection, lung tissues were collected from the infected mice for virological analysis. The lung samples were processed through homogenization and centrifugation to procure the lung extracts, which were then used to determine viral titers within the lung tissue. The lung extracts underwent serial dilutions before being inoculated into embryonated chicken eggs that were 10 days old. The allantoic fluid from the eggs was collected for the assessment of viral hemagglutination activity after three days of incubation. Reed and Muench method was utilized to calculate the infectious virus concentration in the lung tissue, quantified as the egg infectious dose 50 (EID50) [29].

10. Flow cytometry

The activation levels of DCs and macrophages were evaluated using flow cytometry. To prevent non-specific antibody binding, Fc receptors on these cells were blocked prior to antibody staining. DCs were labeled with CD11c-PE/Cy7 (N418, eBioscience™, Massachusetts, USA), MHC class II (MHCII)-PE (M5/114.15.2, eBioscience™), CD40-BV605 (3/23, BD OptiBuild™, New Jersey, USA), and CD86-FITC (GL1, BD Pharmingen™, New Jersey, USA) to assess activation marker expression. Macrophages were similarly stained using CD11b-APC (M1/70, BD Pharmingen™), MHC II (M5/114.15.2, eBioscience™), CD40-BV605 (3/23; BD Biosciences, Franklin Lakes, NJ, USA), and CD86-FITC (GL1; BD Pharmingen™).

For T-cell proliferation analysis post co-culture with antigen-presenting cells, lymphocytes were stained with CD3-BV421 (17A2; BD Horizon™, Piscataway, NJ, USA), CD4-PE/Cy7 (RM4.5, BD Pharmingen™), and CD8-APC (53-6.7, BD Pharmingen™).

In exploring cell phenotypes migrating to the peritoneal cavity post-PC treatment, harvested cells were first treated with anti-CD16/32 to block Fc receptors, then stained with a cocktail of fluorescent antibodies, including CD45-PerCP (30-F11, BD Pharmingen™), F4/80-FITC (BM8, eBioscience™), MHC II (M5/114.15.2, eBioscience™), CD11c-PE/Cy7 (N418, eBioscience™), CD11b-APC (M1/70, BD Pharmingen™), CD206 (C068C2), and Ly6c-AF700 (AL-21, BD Pharmingen™). Flow cytometry was performed on a BD LSRII Fortessa with FACS Diva software, and data were analyzed using FlowJo (Version 10.1, Tree Star Inc., Ashland, OR, USA). Immune cell gating strategies in the peritoneal cavity were depicted in Figure I.

Sixteen weeks post-boost immunization, the mice underwent a lethal viral challenge. On day seven post-infection, lung samples were collected to analyze inflammatory cell infiltration. Lung cells, prepared as single-cell suspensions, were stained with antibodies targeting CD45 (30-F11), F4/80 (BM8), MHC II (M5/114.15.2), CD11c (N418), CD11b (M1/70), CD170 (Siglec-F) (S17007L), and Ly6c (AL-21). Initially, lung cells were washed and treated with anti-CD16/32 (Biosciences) to block Fc receptors, followed by incubation with the antibody cocktail for 30 minutes at 4°C. Post-washing, cells were acquired using Beckman CytoFLEX,

USA, and analyzed using FlowJo software. The gating strategies for identifying immune cells and assessing inflammatory cell infiltration in the lung are shown in Figure II.

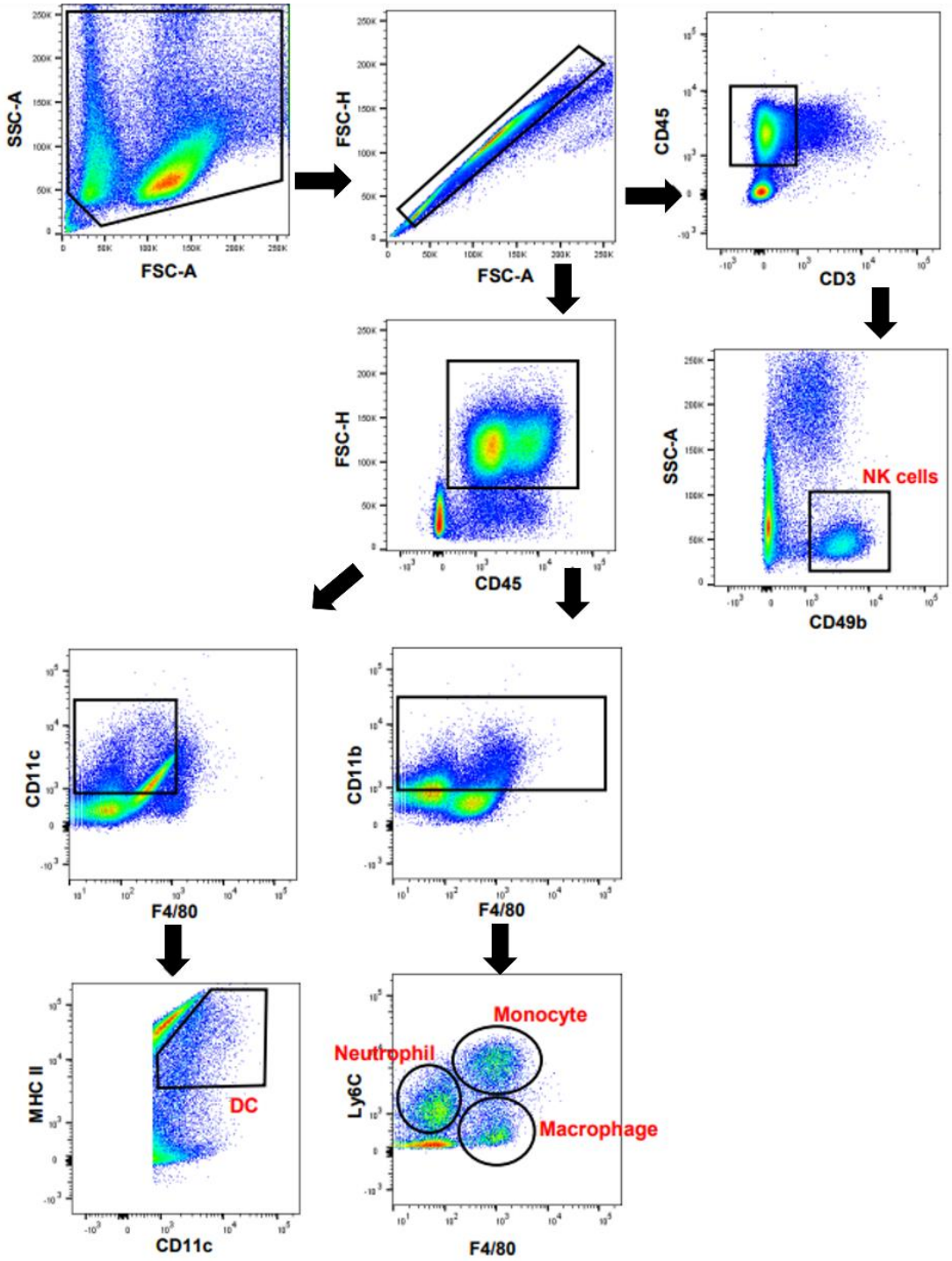

Figure I. Gating strategies for immune cells in peritoneal cavity. The harvested peritoneal cells after 24 hours of the MAE injection were stained with fluorescencelabeled antibodies and analyzed using flow cytometry. Macrophages; $CD45^{+}CD11b^{+}F4/80^{+}$, Monocytes; $CD45^{+}CD11b^{+}Ly6c^{high}$, Neutrophils; $CD45^{+}CD11b^{+}Ly6c^{int}$, DCs; $CD45^{+}CD11c^{+}F4/80^{-}MHCII^{+}$, NK cells; $CD45^{+}CD3^{-}CD49b^{+}$.

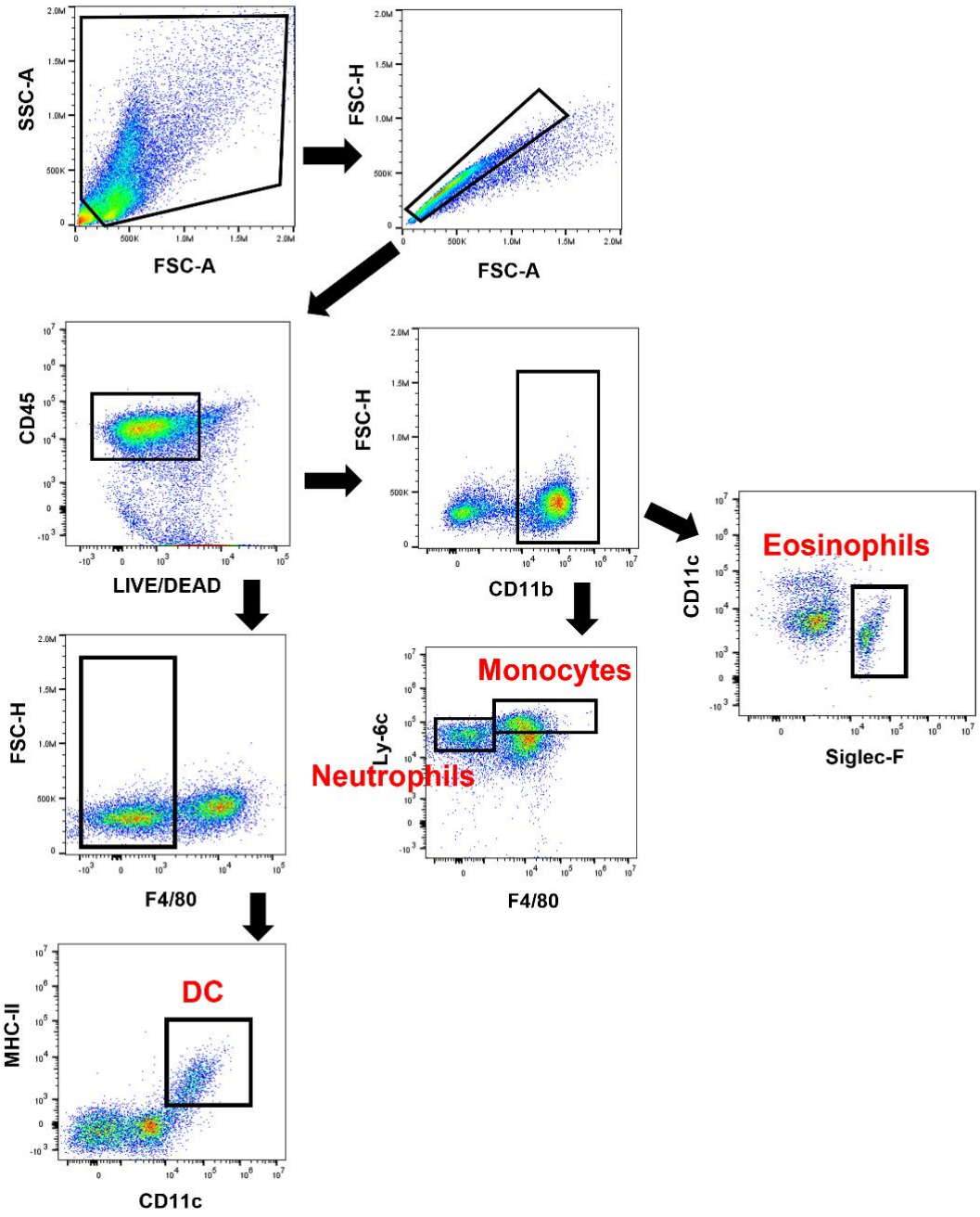

Figure II. Gating strategies were employed to identify inflammatory cell infiltrates in the lungs. Cells from the lungs were harvested on day 7 post-infection with a lethal dose of the virus. These cells were then stained with fluorescence-labeled antibodies and analyzed via flow cytometry. Monocytes; $CD45^{+}CD11b^{+}Ly6c^{high}$, Neutrophils; $CD45^{+}CD11b^{+}Ly6c^{int}$, Eosinophils; $CD45^{+}CD11b^{+}SiglecF^{+}$, DCs; $CD45^{+}CD11c^{+}F4/80^{-}MHCII^{+}$.

11. Statistical analysis

Statistical analyses were performed using One-way ANOVA, Two-way ANOVA, and Tukey's multiple comparison test. The results are presented as mean ± Standard Error of Mean (SEM). Graphpad Prism software version 9.2.0 (GraphPad Software Inc., San Diego, CA, USA) was utilized for all data analyses. p value of less than 0.05 was considered statistically significant.

III. RESULTS

1. PC promoted cytokine secretion and activation of DCs and macrophages *in vitro*

My initial goal was to explore how the PC adjuvant affects the activation of the innate immune response. I investigated cytokine release and activation of BMDCs and BMDMs *in vitro* (Figure 1). Immature BMDCs and BMDMs were treated with 20 μg/ml of PC and 0.1 μg/ml of MPL. Two days post-incubation, I used ELISA to quantify cytokine levels from the cell culture supernatants, while flow cytometry was employed to detect the activation marker expression on DCs and macrophages.

The data indicated that when stimulated with PC, there was a significant rise in the levels of TNF-α, IL-6, and IL-12p40 in DCs, a result that was similar to what was observed with the MPL control (Figure 1A). Moreover, PC treatment increased the expression of CD40 on DCs, while CD86 remained unchanged.

As presented in Figure 1B, BMDMs showed a significant increase in the production of cytokines (TNF-α, IL-6, and IL-12p40) and Nitric Oxide (NO) after PC treatment. Furthermore, PC led to an induction of both CD40 and CD86 expressions, although the changes were not significant. Summarizing, PC was effective in inducing the secretion of pro-inflammatory cytokines, elevating activation markers in APCs, and strongly stimulating both DCs and macrophages.

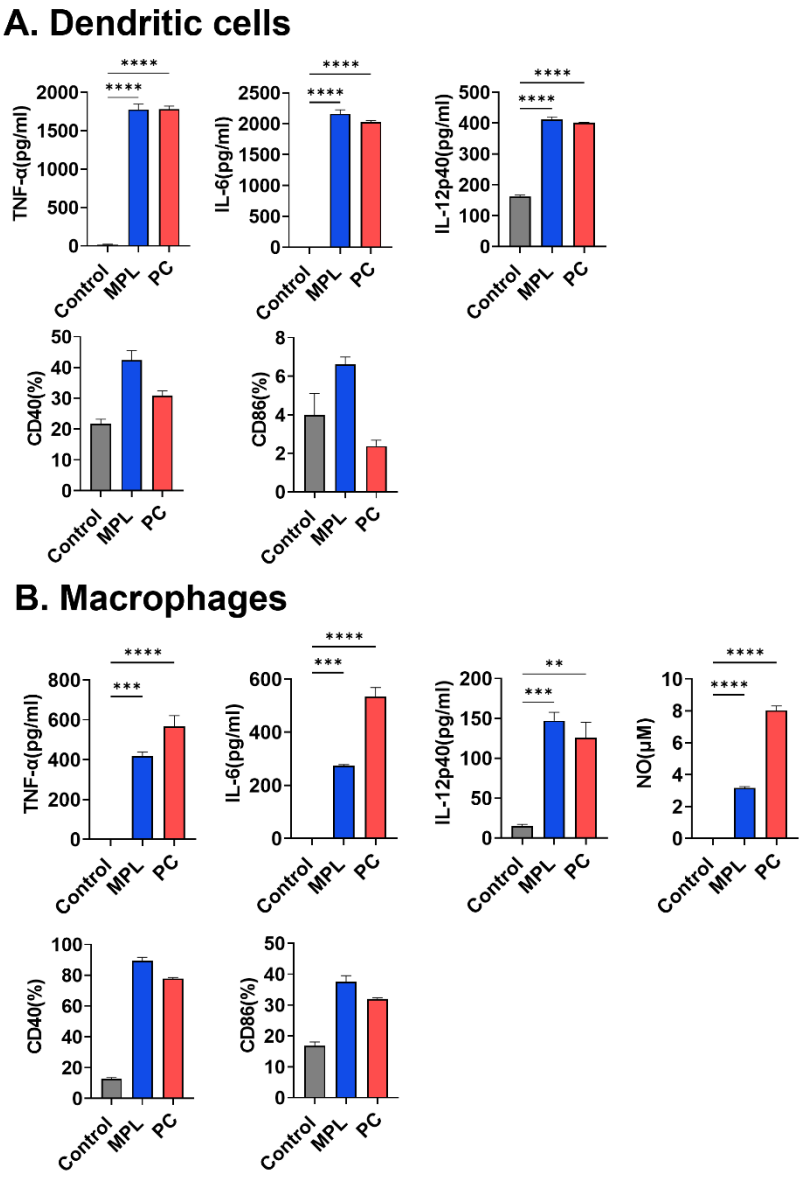

Figure 1. Cytokine secretion and activation marker expression in BMDCs and BMDMs by PC treatment. BMDCs and BMDMs were exposed to PC at a concentration of 20 μg/mL and MPL at 0.1 μg/mL *in vitro*. Following a culture period of two days, the production of Nitric Oxide (NO), interleukin (IL)-6, tumor necrosis factor (TNF)-alpha, and IL-12p40 were quantified using an ELISA kit. The expression of activation markers MHC class II, CD40, and CD86 on dendritic cells (DCs) and macrophages were measured through flow cytometry and data were processed using FlowJo software. The populations of DCs and macrophages were identified based on CD11c and CD11b surface markers, respectively. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison test. *; $p<0.0332$, **; $p<0.0021$, ***; $p<0.0002$, ****; $p<0.0001$, compared to control group.

2. PC pretreatment stimulated T cell proliferation and IFN-γ production output in co-cultured lymphocytes

To assess the active engagement of dendritic cells (DCs) and macrophages treated with PC, I conducted an allogeneic mixed lymphocyte reaction assay. In this setup, allogeneic lymphocytes were co-cultured with DCs or macrophages pre-treated with PC for five days. During this period, I measured the proliferation of T cells and their production of INF-γ. The results showed that treating DCs with either MPL or PC led to an augmented proliferation of $CD4^+$ and $CD8^+$ T cell subsets, compared to the control group, with PC showing a more pronounced effect (Figure 2A). Additionally, IFN-γ secretion was significantly higher following PC treatment compared to both the control and MPL treatments. Similarly, macrophages co-cultured with T cells after MPL and PC treatments also displayed an increased proliferation of T cells, including both $CD4^+$ and $CD8^+$ subsets, and heightened IFN-γ production, with PC having a stronger impact than MPL (Figure 2B). Consequently, PC demonstrated a strong activation capacity for both DCs and macrophages, effectively engaging these cells functionally.

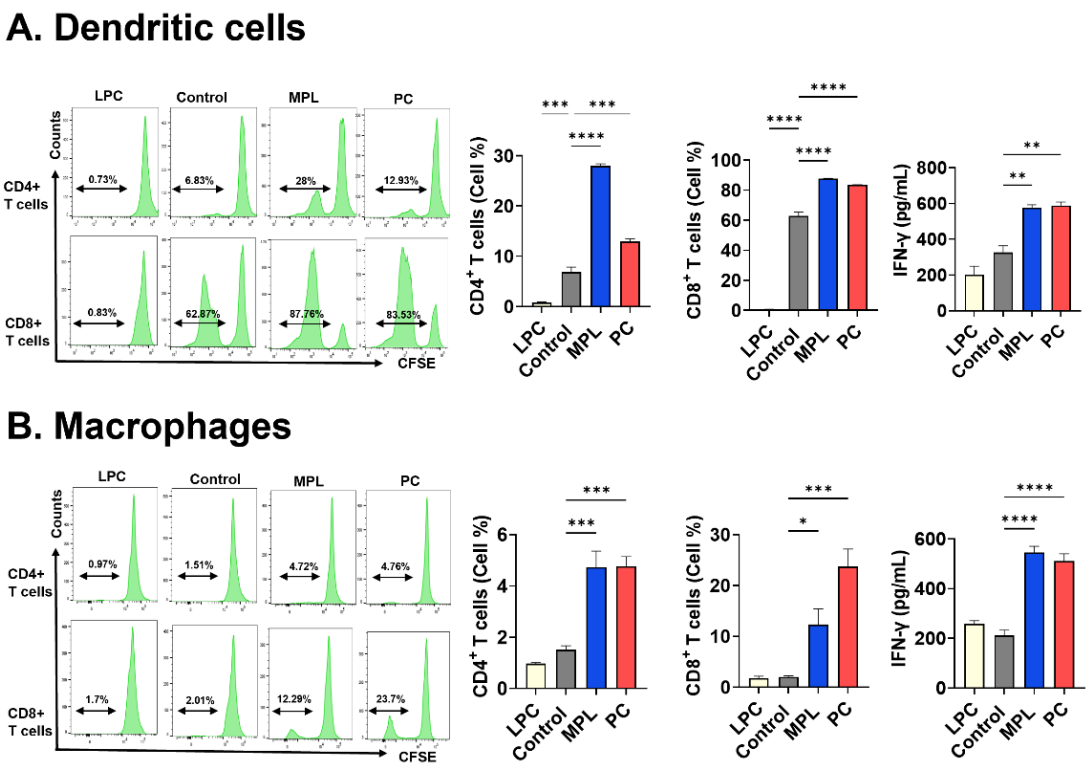

Figure 2. Proliferation of T cells and IFN-gamma secretion after co-culture with PC-treated BMDCs and BMDMs. BMDCs and BMDMs from BALB/c mice were prepared with a pre-treatment of either MPL at 0.1 μg/ml or PC at 20 μg/ml. Post a culture period of two days, these primed cells, specifically the dendritic cells (DCs) (A) and the macrophages(B), were co-cultured with CFSE-stained naive T cells derived from the spleens of C57BL/6 mice for five days. Subsequently, the percentage of proliferated T cells (CFSE-negative), was determined for both $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ T cell subsets using flow cytometry and the data were processed using FlowJo. Additionally, the culture's supernatant was tested to quantify IFN-gamma secretion using ELISA. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison test. *; $p<0.0332$, **; $p<0.0021$, ***; $p<0.0002$, ****; $p<0.0001$, compared to control group.

3. PC injection promoted cytokine, chemokine production and innate immune-cell recruitment in peritoneal cavity

To investigate the *in vivo* immunostimulatory effects of PC, I administered an intraperitoneal injection of PC (100 μg/mouse) and subsequently measured cytokine and chemokine levels in the peritoneal cavity at 6 hours post-injection. Additionally, 24 hours after PC injection, I analyzed the infiltration of innate immune cells in the peritoneal cavity using flow cytometry. MPL (1 μg/mouse) served as a positive control (Figure 3). I noted an increase in the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β in the peritoneal cavities of mice treated with PC, although the increase was not statistically significant. Chemokines, associated with the recruitment of inflammatory cells, were notably elevated following PC treatment (Figure 3A).

In the control group, macrophages constituted the majority of cells in the peritoneal cavity. Post PC treatment, the proportion of macrophages decreased as other immune cells were recruited to the site. PC injection led to the recruitment and proliferation of DCs, monocytes, neutrophils, and natural killer (NK) cells in the peritoneal cavity. Notably, the levels of DCs and monocytes were significantly higher with PC treatment compared to MPL. These findings indicate that PC is effective in stimulating immune responses and activating cells *in vivo.*

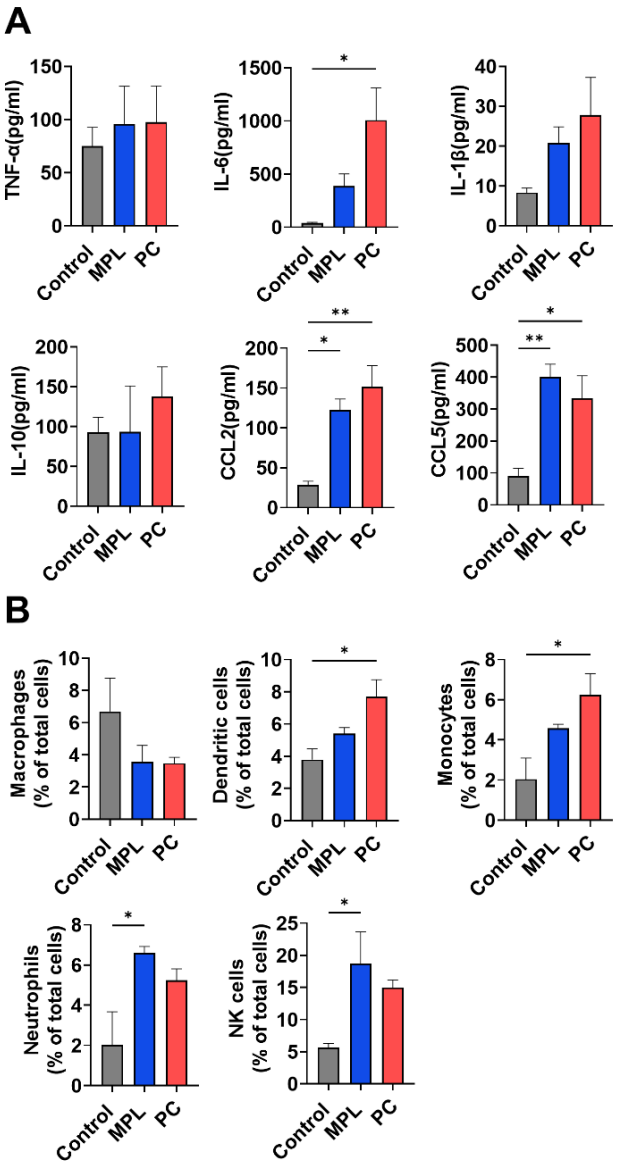

Figure 3. Peritoneal immune response post PC injection. BALB/c mice (n = 6) were received an intraperitoneal injection of PC (100 μg/mouse) or MPL (1 μg/ mouse).6-hour post-injection, the concentrations of cytokines and chemokines were measured in the peritoneal exudates and lavage from the peritoneal cavity via using ELISA (A). After a period of 24 hours following the injection, the peritoneal cells were isolated through the centrifugation of the peritoneal lavage. Subsequently, the proportions of various immune cells present in the peritoneal cavity including dendritic cells, macrophages, monocytes, neutrophils, and NK cells were determined using flow cytometry and the data were further analyzed with FlowJo software (B). Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison test. *; $p<0.0332$, **; $p<0.0021$, ***; $p<0.0002$, compared to control group.

4. PC induced antigen-specific IgG antibody responses

To assess the *in vivo* adjuvant potential of PC, i administered with prime and boost of the sPR8 vaccine to mice with PC as an adjuvant, using Alum as a positive control for adjuvant efficacy. Fourteen days after each vaccination, I obtained immune sera from the mice and conducted ELISA assays to evaluate antigen-specific IgG responses and the presence of various antibody subtypes in all the serum samples.

Two weeks after the prime vaccination, the group receiving the Alum adjuvant exhibited the highest levels of IgG and IgG1 antibodies. The addition of PC also significantly increased IgG production compared to the group administered with sPR8 alone, with a notable rise in IgG2a levels compared to those in the Alum-adjuvanted group, as detailed in Figure 4A.

Following the booster vaccination, the presence of PC was associated with significantly higher IgG2a antibody levels, surpassing both the no-adjuvant group and the Alum-adjuvanted group. Although the highest IgG and IgG1 responses were observed with Alum, as shown in Figure 4B, the data suggest that PC has a unique capacity to enhance overall antibody production, particularly by increasing IgG2a levels. This enhancement in IgG2a levels is indicative of a bias towards a Th1-type immune response, underscoring the distinct immunomodulatory properties of the PC adjuvant.

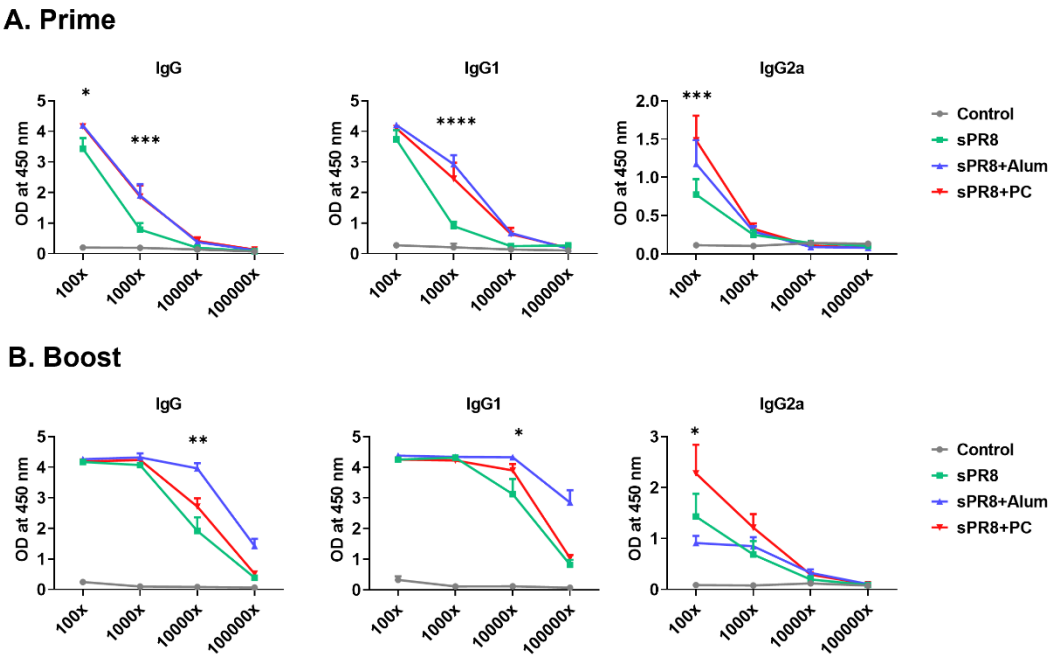

Figure 4. Antigen-specific IgG isotypes in immunized mice serum. Mice were vaccinated with the sPR8 vaccine alone (0.5 μg per mouse) or in combination with adjuvants, including Alum (100 μg per mouse) and PC at 200 μg per mouse). Blood samples were collected from these mice two weeks after prime and boost immunizations. The immune serum from these samples was then methodically diluted to evaluate the levels of antigen-specific IgG, IgG1, and IgG2a antibodies using an ELISA assay. Data are presented as mean ± SEM. Statistical significance was determined by One-way and Two-way ANOVA followed by Tukey's multiple comparison test. *; $p<0.0332$, **; $p<0.0021$, ***; $p<0.0002$, ****; $p<0.0001$, compared to sPR8 group.

5. PC improved the protective efficacy of influenza vaccine after lethal virus infection

To assess the protective efficacy of the PC-adjuvanted sPR8 vaccine, naive and immunized mice were challenged with a lethal dose of the A/PR8 influenza virus, with observations made over a 14-day period for weight fluctuation and survival outcomes. Post-challenge, the vaccinated group exhibited robust protection, as evidenced by negligible weight loss and sustained survival. In contrast, naive mice experienced a precipitous weight reduction, with a maximal decline of 24.17% on day seven post-infection, and a 50% mortality rate was recorded at the same time point. Among the vaccinated groups, the PC-adjuvanted group demonstrated enhanced resistance to the PR8 virus, indicated by a marginal weight decrease of 2.14% and a 100% survival rate, outperforming both the Alum-adjuvanted group, which showed a 4% reduction in body weight yet maintained a 100% survival rate, and the group administered with sPR8 alone, which presented a 17.36% weight reduction but also maintained a full survival rate.

Additionally, histological analyses were performed on lung tissues seven days after A/PR8 exposure. The naive group displayed pronounced inflammation, evident tissue damage, and significant inflammatory cell infiltration. On the other hand, the PC-immunized group presented with diminished inflammatory cell presence and reduced inflammation, as depicted in Figure 5C. Corroborating these observations, the vaccine-immunized groups showed a significant decrease in lung viral loads (Figure 5D).

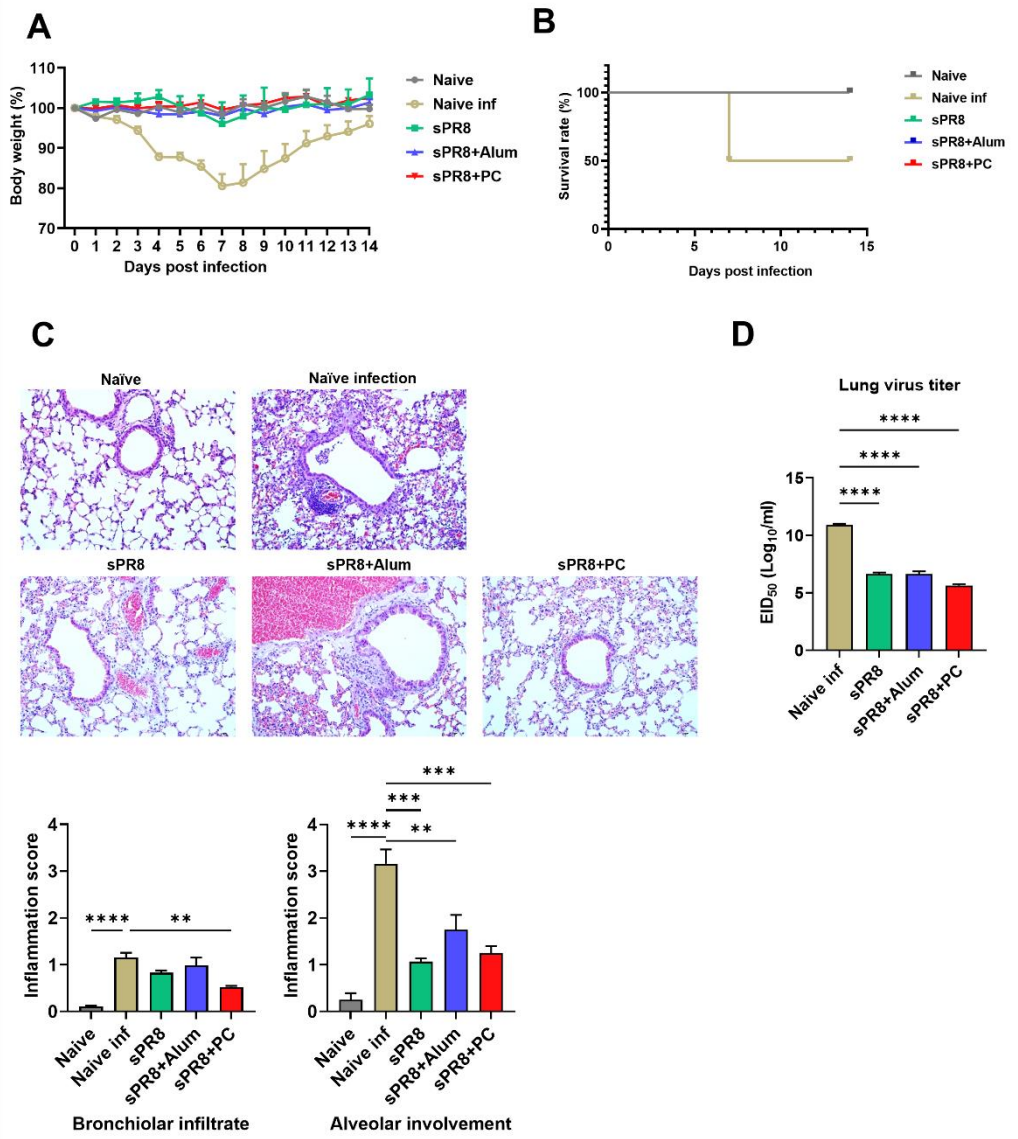

Figure 5. Protective effectiveness of sPR8 vaccine with adjuvants against lethal influenza infection. Sixteen weeks following boost immunization, naïve and vaccinated mice were exposed to a lethal dose of the A/PR8 influenza virus, (1×LD50). The changes in body weight (A), the survival rates (B) of the mice over a 14-day period post infection. Furthermore, seven days after the infection, lung tissues were collected for histological and virological analyses. The lung sections were fixed, processed, and stained with hematoxylin and eosin to evaluate the histopathological impact of the virus(C). Additionally, the viral load within the lungs (D) was quantified by inoculating embryonated eggs and determining the egg infectious dose 50 (EID50). Data are presented as mean ± SEM. Statistical significance was determined by One-way ANOVA followed by Tukey's multiple comparison test. **; $p<0.0021$, ***; $p<0.0002$, ****; $p<0.0001$, 0.0001 compared to between the indicated groups.

6. PC prevented lung inflammation after a lethal A/PR8 influenza virus infection

Sixteen weeks after receiving the boost immunization, mice were exposed to a lethal dose of the A/PR8 virus. Seven days subsequent to this infection, lung tissues were harvested for analysis. Levels of inflammatory markers such as TNF-α, IL-1β, IL-6, IL-12p40, IFN-γ, and CCL2 were quantified in the lung tissue by ELISA, and the presence of infiltrating inflammatory cells was assessed using flow cytometry.

Following this infection, the naïve group showed a significantly higher presence of monocytes, neutrophils, and dendritic cells (DCs), as illustrated in Figure 6A. By contrast, the group that received the PC adjuvant significantly displayed reduction in the percentages of monocytes and DCs, aligning with the levels found in the Alum-adjuvanted group. Despite this, the PC-adjuvanted mice had higher neutrophil counts compared to their Alum-adjuvanted counterparts. The Alum-adjuvanted group also stood out due to a significantly increased number of eosinophils in their lungs, whereas the PC-adjuvanted and sPR8 groups both showed a moderate presence of eosinophils. Remarkably, in the naïve infection group, eosinophil recruitment was not significantly observed.

Further investigation as illustrated in Figure 6B reveals that naïve mice exhibited elevated levels of IL-1β, IL-6, IL-12p40, TNF-α and CCL2 after viral infection. In contrast, mice that received the Alum and PC adjuvants showed a significant reduction in the levels of these cytokines.

In summary, the gathered data suggests that the PC adjuvant significantly reduced the inflammatory response in the lungs prompted by the influenza virus, indicating its potential utility in modulating immune reactions during viral challenges.

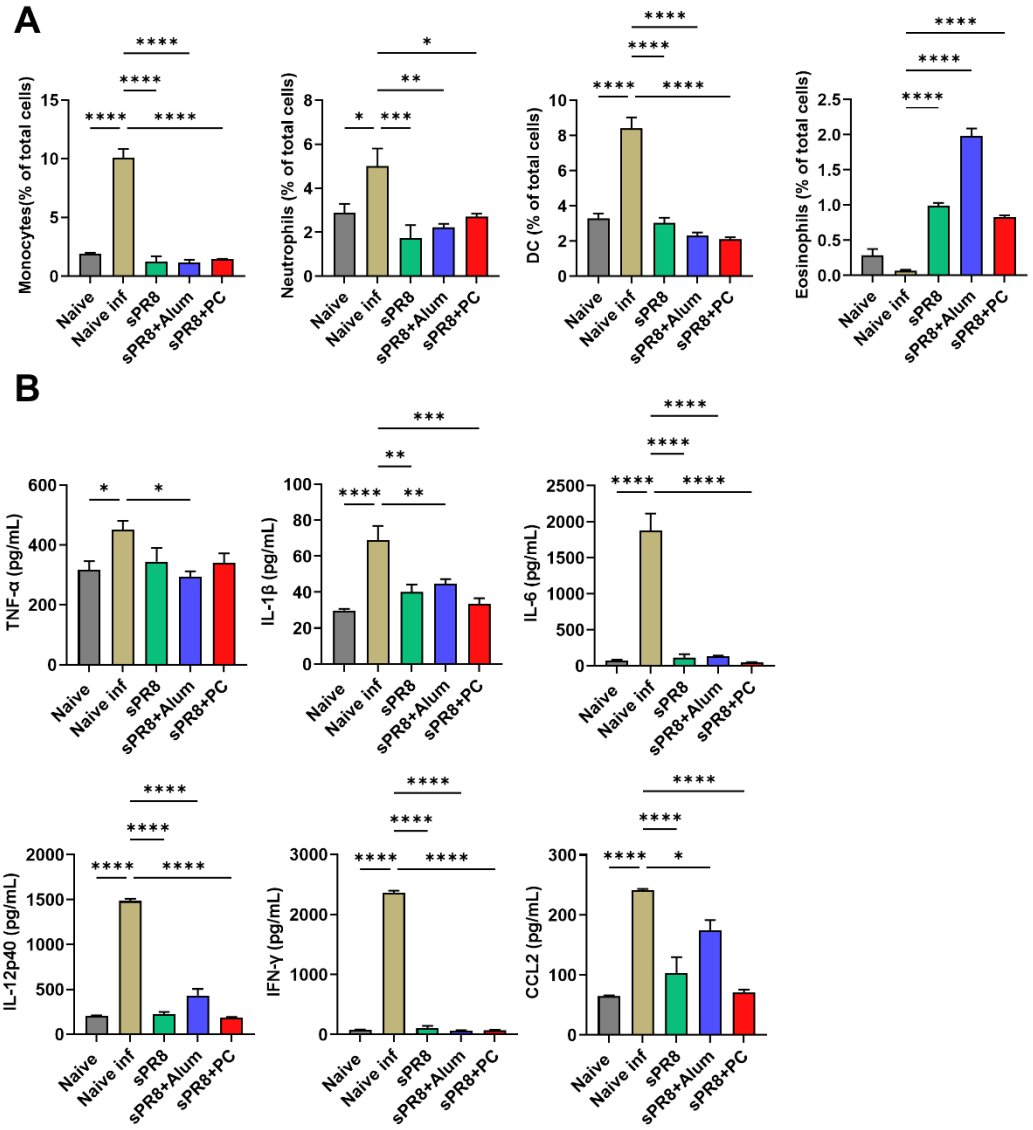

Figure 6. Lung cellular infiltration and cytokine response following PR8 lethal viral infection. Immunized mice (n=4) that were infected with the A/PR8 virus at a lethal dose (1×LD50) after receiving a boost immunization 16 weeks prior. Seven days post-infection, lung was collected to identify cellular phenotypes via flow cytometry, and cytokine level in the lung samples were quantified using ELISA. Data are presented as mean ± SEM. Statistical significance was determined by One-way ANOVA followed by Tukey's multiple comparison test. *; $p<0.0332$, **; $p<0.0021$, ***; $p<0.0002$, ****; $p<0.0001$, 0.0001 compared to between the indicated groups.

7. PC enhanced Memory T and B cell responses

Memory T cells and B cells are essential components of the adaptive immune system, providing long-term immunity and swift response upon re-exposure to pathogens such as the influenza virus [30] [31]. To determine the effect of PC on memory T cell responses, the proliferation of T cells after antigen stimulation was checked. I employed FACS to assess the presence of antigen-specific memory $CD4^+$ and $CD8^+$ T cells in lung and spleen tissues seven days following a lethal challenge with the influenza virus. Vaccination with the PC adjuvant resulted in significant proliferation of antigen-specific $CD4^+$ cells in the lungs after in vitro re-stimulation. Additionally, the level of $CD8^+$ T cells also increased, although this increase did not show a significant difference (Figure 7A). In the spleen, antigen-specific $CD4^+$ and $CD8^+$ memory T cell populations were most abundant in the group receiving the PC-adjuvanted vaccine. The sPR8-vaccinated group elicited a moderate increase in $CD4^+$ T cell numbers, in contrast to the Alum-adjuvanted group, which failed to induce significant proliferation of T cells in both the lungs and spleen.

Consistent with the enhanced memory T cell response in the lungs and spleen, PC-adjuvanted group also showed the most substantial induction of antigen-specific antibody-secreting cells in both the bone marrow and spleen when compared to other groups. The Alum-adjuvanted group demonstrated a moderate ability to stimulate IgG antibody production from bone marrow-derived cells, while the sPR8 group elicited higher antibody levels in splenic cells than the Alum group. This data underscores the potency of the PC adjuvant in enhancing responses from not only memory T cells but also memory B cells and cells responsible for antibody production.

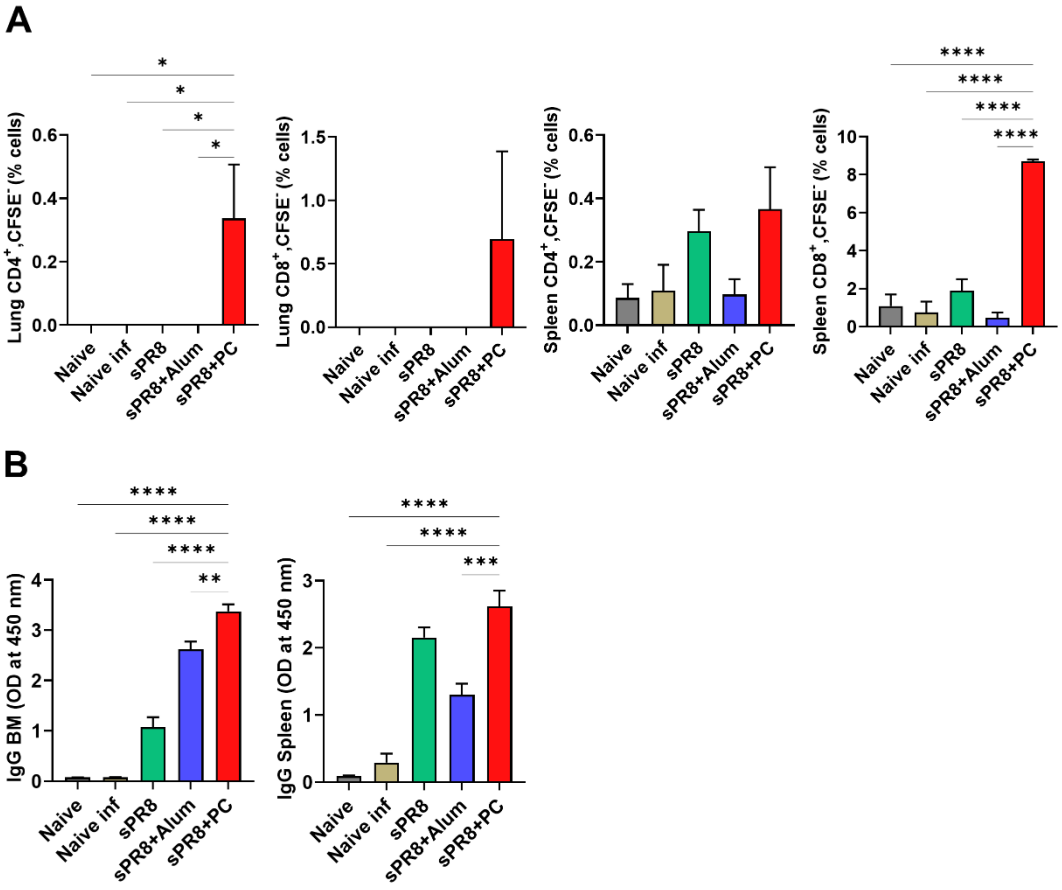

Figure 7. Antigen-specific memory T cell and B cell responses after lethal virus challenge. At sixteen weeks after receiving a boost immunization, mice were exposed to a lethal virus. Seven days after infection, cells from the lung, spleen, and bone marrow were collected for analysis. Cells from the lungs and spleen were marked with CFSE and then cultured for five days with an sPR8 antigen stimulation (10 μg/ml) to assess T cell proliferation, which was determined using flow cytometry (A). Additionally, bone marrow and spleen cells were seed onto the cell culture plates which coated with 400 ng/well iPR8 overnight before, then incubated for one day and seven days respectively, after which the production of antigen-specific IgG antibodies was measured via ELISA, after which the production of antigen-specific IgG antibodies was measured via ELISA (B). Data are presented as mean ± SEM. Statistical significance was determined by One-way ANOVA followed by Tukey's multiple comparison test. *; $p<0.0332$, **; $p<0.0021$, ***; $p<0.0002$, ****; $p<0.0001$, 0.0001 compared to between the indicated groups.

IV. DISCUSSION

The limited immunogenicity of split influenza vaccines requires the incorporation of adjuvants to enhance their effectiveness [32]. As the influenza virus undergoes constant mutation, the employment of adjuvants could be a key strategy to improve vaccine efficacy. In this study, I explored the efficacy of PC, a compound extracted from marine algae, as a potential adjuvant for split influenza vaccines. I initial examination focused on PC's ability to stimulate antigen-presenting cells and to promote the recruitment of immune cells. Subsequently, I assessed the immunological impact of PC when used in conjunction with prime and boost vaccinations in mice, administered intramuscularly. This included analyzing the influence of PC on the generation of antigen-specific IgG1 and IgG2a serum antibodies, the response of memory T and B cells, and the overall protection afforded against viral challenges.

The innate immune system plays a crucial role as the initial defense mechanism against infections, such as the influenza virus [33]. In this context, two types of antigen-presenting cells (APCs), macrophages and dendritic cells (DCs), are extensively studied to evaluate the biological and immunological effects of novel compounds, including drugs and extracts. This research is vital for understanding their functions and potential in immunotherapy [34]. In my *in vitro* studies, I observed that PC enhanced the production of pro-inflammatory cytokines and the expression of co-stimulatory molecules in APCs compared to the control (Figure 1). Notably, PC induced cytokine secretion in DCs at levels comparable to MPL and even higher in macrophages than MPL. APCs are also instrumental in driving adaptive immune

responses by activating and fostering the proliferation of T-cells [35]. As shown in Figure 2, DCs and macrophages stimulated by PC significantly enhanced the growth of both $CD4^+$ and $CD8^+$ T cells and increased IFN-γ production. In my *in vivo* experiments, I found that an injection of PC activated immune responses in the peritoneal cavity. Mice injected with PC exhibited increased levels of pro-inflammatory cytokines in their peritoneal cavities. Furthermore, this treatment encouraged the recruitment of immune cells such as DCs, monocytes, neutrophils, and natural killer (NK) cells to the peritoneal cavity (Figure 3).

Adjuvants, acting as immune potentiators, specifically target these APCs to initiate the secretion of proinflammatory mediators. This process creates an optimal environment for enhancing the immune response [36]. In my initial studies, PC exhibited an immunostimulatory effect on both dendritic cells (DCs) and macrophages. This was evidenced by the upregulation of activation markers, the enhanced production of pro-inflammatory cytokines in antigen-presenting cells (APCs), and improved T cell proliferation capacities *in vitro*. Furthermore, *in vivo* experiments showed an increased peritoneal immune response and recruitment of immune cells following PC injection. Therefore, these findings suggest that PC has the potential to act as an effective candidate adjuvant to enhance vaccine efficacy.

Antibodies are central to vaccine research as they help assess a vaccine's potential effectiveness as well as indicating immune response levels and guide the design of vaccines to elicit strong protection [37]. Consequently, by investigating antibody responses, researchers can fine-tune vaccines to ensure they activate the necessary

immunity to prevent disease. In this context, this study revealed that incorporating PC as an adjuvant significantly enhanced antigen-specific IgG, IgG1, and IgG2a levels after both prime and boost vaccinations (Figure 4). Notably, the group receiving the PC adjuvant exhibited higher IgG2a levels in comparison to the group with alum. Moreover, in line with previous studies on alum, my research confirmed its limited capacity to induce IgG2a production [38-40]. While alum is known to promote IgG1, indicative of a Th2-type humoral immune response, it does not similarly enhance IgG2a, which is associated with Th1-type cellular immunity [41]. These findings underscore the potential of PC as an effective adjuvant for stimulating comprehensive immune protection, encompassing both cellular and humoral responses.

Memory T and B cells play crucial roles in providing long-term immunity through vaccines and in mounting immune against pathogens like the influenza virus [42]. Memory B cells, once formed in response to a vaccine, can swiftly produce antibodies during a subsequent encounter with the pathogen. Memory T cells, including both $CD4^+$ T helper and $CD8^+$ T cytotoxic types, ready to mount a faster and stronger response if the antigen reappears. This cellular memory forms the basis for vaccine-induced immunity, ensuring that when the body is exposed to the actual pathogen in the future, it can neutralize it quickly and effectively, often preventing illness altogether. In this study, mice vaccinated with a PC-adjuvanted exhibited a significant proliferation of antigen-specific T cells within the lungs, particularly among $CD4^+$ T cells, upon *in vitro* stimulation (Figure 7A). Further analysis revealed a significant increase in the populations of antigen-specific $CD4^+$ and $CD8^+$ memory T cells in the spleen. Additionally, the PC-adjuvanted vaccine group showed a marked elevation in

the number of antibody-producing cells in both the bone marrow and spleen compared to other groups, as depicted in Figure 7B. These findings highlight the effectiveness of the PC adjuvant in actively stimulating memory T cells, memory B cells, and antibody-producing cells, providing a foundation for strengthened immune protection.

Characteristics such as a proinflammatory cytokine storm, cellular infiltration, and viral load are typically observed following infection [43]. Within the vaccinated group, there was a significant decline in the levels of inflammatory cytokines and the number of infiltrating cells in the lungs on the seventh day following exposure to a lethal virus dose. This was in contrast to the naïve group, which presented with increased levels (Figure 6). The mice that received the PC-adjuvanted vaccine displayed a superior ability to suppress viral replication post-infection. This was evidenced by the maintenance of body weight (Figure 5A), the significantly decreased viral presence in the lungs (Figure 5D), and the considerable reduction in both cytokine production and inflammation in lung tissues (Figure 6). The observed protective effect can be attributed to the heightened presence of antigen-specific IgG antibodies, a response incited by the PC adjuvant. Moreover, the PC adjuvant enhanced memory immune responses, which was evidenced by an increase in antigen-specific output from plasma cells and memory B cells (Figure 7B). Additionally, it is established that vaccine adjuvant could enhance the presence of lung-resident memory T cells, which play a crucial role in preventing respiratory illnesses in mice challenged with the influenza virus [44]. This research demonstrated that incorporating PC into the sPR8 vaccine formulation significantly increased the levels of memory T cells in the lungs,

especially $CD4^+$ T cells (Figure 7A). Collectively, these findings indicated that the PC adjuvant offers a potent and reliable defence against viral infections.

In conclusion, this study indicated that PC had significant potential as an adjuvant for split influenza vaccines. My investigation showed that PC activated antigen-presenting cells, including macrophages and dendritic cells, leading to an increase in pro-inflammatory cytokines and T-cell proliferation. These results demonstrated PC's potent immunostimulatory effects. Moreover, using PC as an adjuvant enhanced the levels of antigen-specific IgG antibodies, especially IgG1 and IgG2a, essential for long-term immunity and immediate defense against pathogens. Notably, the PC-adjuvanted vaccine induced a robust population of memory T and B cells. Enhanced immune responses were correlated with protective effects, evidenced by reduced inflammatory cytokines, decreased cellular infiltration, and lower viral loads in the lungs post-vaccination in the addition of PC. Overall, these findings indicated that incorporating PC as an adjuvant in split influenza vaccines could significantly enhance their effectiveness, providing more robust protection against the influenza virus.

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