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Monophosphoryl lipid A and Poly I:C combination adjuvant promoted ovalbumin-specific cell mediated immunity in mice model

GRADUATE SCHOOL
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Abstract

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Induction of antigen-specific cell-mediated immunity (CMI) as well as humoral immunity is critical for successful vaccination against various type of pathogens. Toll-like receptor (TLR) agonists have been developed as adjuvants to promote vaccine efficacy and induce appropriate immune responses. Monophosphoryl lipid A (MPL); a TLR4 agonist, and polyinosinic acid: polycytidylic acid (Poly I:C); a TLR3 agonist, are known as a strong immuno-stimulator which induce Th1 response. Many studies proved and compared the

efficacy of each adjuvant, but no study has investigated the combination of them. Using ovalbumin protein antigen, MPL+Poly I:C combination induced more effective CMI response than single adjuvants. Production of inflammatory cytokines and population of innate immune cells and CD4/CD8 memory T cell at the immunized site had been significantly enhanced by MPL+Poly I:C combination. Moreover, MPL+Poly I:C combination enhanced ovalbumin-specific serum IgG, IgG1 and IgG2c production and proliferative function of CD4 and CD8 T cell after *in vitro* ovalbumin peptide stimulation. Taken together, these data suggest that combination of MPL and Poly I:C has a potency as a CMI-inducing vaccine adjuvants with highly increased effects.

Keywords: Cell-mediated immunity; Memory T cell; Dendritic cell; Monophosphoryl lipid A; Poly I:C

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Ⅰ. Introduction

Many studies have shown the importance of antigen-specific cell-mediated immunity (CMI) and humoral immunity for the successful vaccination against various types of pathogens [1,2]. Many vaccine adjuvants have been developed and included in vaccine regimens to enhance the efficacy of the vaccine and induce the appropriate immune responses [3]. Adjuvant is an immune-stimulator, which induces innate immunity by activating the pathogen recognition receptors (PRRs), such as the Toll-like receptors (TLRs). Activation of PRRs leads to the production of pro-inflammatory cytokines, recruitment and maturation of antigen-presenting cells (APCs), modulation of the adaptive immunity by influencing the T and B cell responses, and induction of memory cells [4,5]. These memory cells consist of two main subsets: $CD62L^{high}/CCR7^{high}$ central memory T cells (Tcm), and $CD62L^{low}/CCR7^{low}$ effector memory T cells (Tem). Tcm cells are prevalent in lymph nodes and become highly proliferative after an antigen re-encounter, while Tem cells are predominantly found in the circulation and can be easily recruited to the sites of inflammation [6]. The memory T cells remaining after infections provide strong and long-term protection against re-infection [7,8].

Aluminum salts (alum) were the first adjuvants licensed for human vaccines and have been used for decades. They can be used to achieve high antibody responses along with T helper (Th)-2 immune responses; however, they have limited capacity for the stimulation of cell-mediated immunity and induction of Th1 responses, showing poor protection against intracellular pathogens [9]. Thus, induction of CMI, especially with Th1 responses, has become a major challenge in the development of vaccines [10]. Few adjuvants, including alum, MF59, AS03 (squalene based oil-in-water emulsions), and AS04 (alum +

MPL) have got licensed for being used in human vaccine [11]. MPL is a detoxified lipopolysaccharide (LPS), which has been proved to be an effective adjuvant in several studies[12]. Adjuvants systems (AS) means various combinations of adjuvants, to enhance the immune responses via the activation of several PRRs [13]. AS01 (MPL + QS-21 + Liposome) [14], AS02 (MPL + QS-21) [15] are under clinical trial and studies examining the effect of various adjuvants combinations are going on [16,17]. Recently, the combination of MPL and CpG, a TLR9 agonist, immunized with influenza split vaccine elicited strong antigen-specific Th1 immune responses and induced protective efficacy against homosubtypic and heterosubtypic influenza infections [18,19].

MPL is a TLR4 agonist [20], and Poly I:C is a synthetic double-stranded (ds) RNA, a mimic of viral dsRNA. It activates several PRRs including TLR3, retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). Activation of these receptors is important to induce anti-viral immune responses [21]. By activating various signaling pathways simultaneously, Poly I:C elicits strong Th1 and $CD8^+$ T cell immunity [22,23]. Given these properties, we hypothesized that the combination of MPL and Poly I:C might have synergic effect in improving the CMI response with strong memory T cell response

In this study, we investigated the effect of combination of MPL and Poly I:C on immune responses to ovalbumin (OVA) protein antigen, at a relatively low dose (1 μg and 10 μg, respectively) for safety and economic advantages [12,24].

Ⅱ. Materials and methods

Animals and reagents

C57BL/6 and BALB/c mice were purchased from Orient Bio and maintained at the Jeju National University Animal Facility. All mouse experiments were performed according to the guidelines of the Jeju National University approved Institutional Animal Care and Use Committees (IACUC) protocol (protocol number 2020-0022). MPL and Poly I:C were purchased from InvivoGen. OVA protein was purchased from Sigma-Aldrich and OVA peptides, $OVA_{257-274}$ and $OVA_{323-339}$, were purchased from GenScript. All reagents were prepared according to the manufacturer's instructions.

Immunization

To investigate the efficacy of the combination of adjuvants to induce the cellular and humoral immune responses and antigen-specific memory responses, immunizations were performed using the strategy shown in Fig. 1A (15 mice per group). Briefly, the C57BL/6 mice were immunized intranasally with adjuvant combinations twice (prime and boost) at 2-week intervals. The composition of each group was as follows: OVA-only(10 μg/mouse of OVA protein), MPL-adjuvanted (10 μg/mouse of OVA protein and 1 μg/mouse of MPL), Poly I:C-adjuvanted (10 μg/mouse of OVA protein; 10 μg/mouse of Poly I:C), and MPL+Poly I:C-adjuvanted (10 μg/mouse of OVA protein; 1 μg/mouse of MPL; 10 μg/mouse of Poly I:C). And the total volume of immunization was 50 μL/mouse. The control group received 50 μL of phosphate buffered saline (PBS) intranasally. The mice were sacrificed at three time-points to collect samples: One day after prime immunization (Prime 1D), one day after boost immunization (Boost 1D), and 2 weeks after boost immunization (Boost 2w).

Sera were taken at 2 weeks post each immunization. The bronchoalveolar lavage (BAL) fluid (BALF) and lung samples were collected at every time-point. Spleen and bone marrow samples were collected at Boost 2w.

Sample preparation

Sera were taken by centrifugation of blood collected from the caudal vena cava. BALF was collected by inserting 18-gauge catheter into the trachea and washing the airway twice with 650 μL of PBS. After centrifugation, the supernatants were stored at –80℃ for cytokine enzyme-linked immunosorbent assay (ELISA) and the cell pellets were resuspended with 1 mL of PBS containing 2% fetal bovine serum (FBS) (FACS buffer) for flow cytometry. To acquire the lung and spleen cells, the tissues were mechanically disrupted, filtered by using 100 μm cell strainer, and centrifuged. The supernatants were stored for cytokine ELISA at –80℃. The red blood cells (RBCs) were removed and then the cell pellets were resuspended with PBS and filtered by 40 μm cell strainer for further analysis. Bone marrow cells were collected from the femur and tibia of the mice as previously describe [25].

Serum antigen (Ag)-specific antibody ELISA

To measure Ag-specific antibody levels in the serum, OVA protein (400 ng/well) was coated onto the ELISA plate before adding the serum. Serially diluted sera were then added to OVA-coated plates after blocking. Then, anti-mouse immunoglobulin (Ig) G, G1, and G2c horseradish peroxidase (HRP-labeled) secondary antibodies were used to detect the Ag-specific IgG in the serum. Tetramethylbenzidine substrate solution was used as the substrate, and the reaction was stopped by sulfuric acid. The optical density was measured at 450 nm wavelength.

Cytokine ELISA

Cytokines in the BALF and lungs extracts from the immunized mice at different time-points were measured using tumor necrosis factor (TNF)-α, interleukin (IL)-6 Mouse Uncoated ELISA Kit (Invitrogen), IL-12 p40, and interferon (IFN)- γ DuoSet ELISA kit (R&D system).

Memory B cell response

To measure the antigen-specific antibody production by and memory B cell response, the cell culture plates were coated with the OVA protein (400 ng/well) overnight. The plates were washed and blocked with 10% complete media (200 μL/well) for 1 h at room temperature, before adding the cells. Spleen and bone marrow cell harvested from the Boost 2w mice were seeded at the density of 2×10^6 cells/mL onto the plates and incubated at 37°C for 7 d and 1 d, respectively. Anti-mouse IgG-labeled secondary antibodies were used to detect Ag-specific antibodies produced by the cells.

Flow cytometry

In Prime 1D and Boost 1D, the lung and BAL cells were harvested and stained with fluorophore-labeled antibodies specific for anti-mouse CD45 (clone 30-F11), CD11b (Clone M1/70), CD11c (N418), F4/80 (BM8), Ly6c (Clone AL-21), MCH class II (I-A/I-E), and Live/dead(L/D) to investigate the inflammatory cell recruitment. In Boost 2w, to evaluate the memory T cell response, the lung, BAL, spleen cells were stained with CD45 (clone 30-F11), CD3 (clone 17A2), CD4 (clone RM4.5), CD8a (53-6.7), CD44 (clone IM7), CD62L (Clone MEL-14), and L/D. To evaluate the antigen-specific memory T cell proliferation, the lung and spleen cells were stained with carboxyfluorescein succinimidyl ester (CFSE) and then seeded at a density of 5×10^5 cells/mL and 2×10^6 cells/mL in 96-well plates with OVA peptide stimulation. Two types of mixed OVA peptides ($OVA_{257-264}$ and $OVA_{323-339}$) were

used. After 5-day culture, the cells were collected and stained with CD3 (clone 17A2), CD4 (clone RM4.5), and CD8a (53-6.7).

For cell phenotype staining, the harvested cells were washed with the FACS buffer and blocked Fc receptors using anti-CD16/32 (clone 2.4G2) antibody. The antibody cocktail was added to the cells and incubated for 30 mins at room temperature in the dark. The data were acquired using the BD FACS DIVA program and analyzed using the FlowJo software.

The phenotypes of the acquired cells were gated by the phenotypic markers [6,26,27]: alveolar macrophages: $CD45^+$ $CD11b^-$ $CD11c^+$ $F4/80^+$; neutrophils: $CD45^+$ $CD11b^+$ $Ly6c^{lo}$ $F4/80^-$; monocyte-derived macrophages: $CD45^+$ $CD11b^+$ $Ly6c^{high}$ $F4/80^+$; total DCs: $CD45^+$ $F4/80^-$ $CD11c^+$ $MHCII^{high}$; CD4 naïve T cell: $CD45^+$ $CD3^+$ $CD4^+$ $CD44^-$ $CD62L^+$; CD4 central memory T cell (Tcm): $CD45^+$ $CD3^+$ $CD4^+$ $CD44^+$ $CD62L^+$; CD4 effector memory T cell (Tem): $CD45^+$ $CD3^+$ $CD4^+$ $CD44^+$ $CD62L^-$; CD8 naïve T cell: $CD45^+$ $CD3^+$ $CD8^+$ $CD44^-$ $CD62L^+$; CD8 Tcm: $CD45^+$ $CD3^+$ $CD8^+$ $CD44^+$ $CD62L^+$; CD8 Tem: $CD45^+$ $CD3^+$ $CD8^+$ $CD44^+$ $CD62L^-$.

Bone marrow-derived dendritic cells (DCs) culture and allogeneic mixed lymphocyte reaction (MLR)

The bone marrow cells isolated from BALB/c mice were cultured in the Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS and 1x antibiotic-antimycotic (complete media) supplemented with 20ng/ml mouse granulocyte-macrophage colony stimulating factor (mGM-CSF) to enrich the bone marrow-derived DCs as described previously [19]. The floating cells were removed and the old media was replaced with the fresh complete media with mGM-CSF every 2 d.

To perform MLR assay, immature DCs were collected and seeded at a density of 5×10□ cells/mL in 6-well plates, while the immature macrophages were seeded at a density of

1×10^6 cells/mL. The cells were cultured for 2 d with MPL (0.05 μg/mL), Poly I:C (2 μg/mL), or MPL+Poly I:C (MPL, 0.05 μg/mL; Poly I:C, 2 μg/mL).

Allogenic naïve lymphocytes were harvested from the spleen cells of C57BL/6 mice as described previously [28] and stained with 2 μM CFSE at 37°C for 10 min. After washing, the pellet was resuspended in 10% complete media with 1 mM sodium pyruvate, 1× non-essential amino acids, and 55 μM 2-mercaptoethanol. The CFSE-labeled lymphocytes and prepared DCs were then seeded at a density of 2×10□ cells/well and 1×10^4 cells/well of U-bottom 96-well plates in 200 μL of culture media, so that the ratio of DCs to lymphocytes was 1:20. The macrophages were seeded at a density of 1×10^5 cells/well, so that the ratio of macrophages to lymphocytes was 1:2. After 5 d co-culture, the cells were harvested, stained with CD3 (clone 17A2), CD4, and CD8, and analyzed by flow cytometry to determine the T cell proliferation.

For *ex vivo* MLR, the spleen cells were harvested from the immunized mice at 2-week post-boost immunization and co-cultured with OVA pre-treated DCs or macrophages for 5 d. Then, the supernatants were collected and the levels of IFN-γ cytokine were measured by ELISA.

Statistical analysis

All results are presented as the mean ± standard error of mean (SEM) and statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $P < 0.05$ was considered to be statistically significant. All data were analyzed using the Graphpad Prism software 9.1 (GraphPad Software Inc.).

Ⅲ. Results

Combination of MPL and Poly I:C enhanced the OVA-specific IgG antibody responses

To determine the adjuvant effects of the combination of MPL and Poly I:C *in vivo*, we immunized C57BL/6 mice with OVA with or without MPL, Poly I:C, or MPL+Poly I:C intranasally twice (prime and boost) at 2-week intervals (Fig. 1A). After 2 weeks of each immunization, sera were collected and OVA-specific antibodies were measured by ELISA (Fig. 1B-G). The adjuvanted groups significantly enhanced OVA-specific antibody production, whereas OVA-only immunization did not induce any OVA-specific antibody responses, even after the boost immunization. The MPL+Poly I:C-adjuvanted group showed approximately 10-times higher levels of OVA-specific IgG and IgG1 isotype antibodies in sera at 2-week post immunization compared to those in single-adjuvanted groups. After prime immunization, OVA-specific IgG2c was induced by Poly I:C adjuvant, but after boost immunization, both Poly I:C-adjuvanted and MPL+Poly I:C-adjuvanted groups showed similar OVA-specific IgG2c levels in sera.

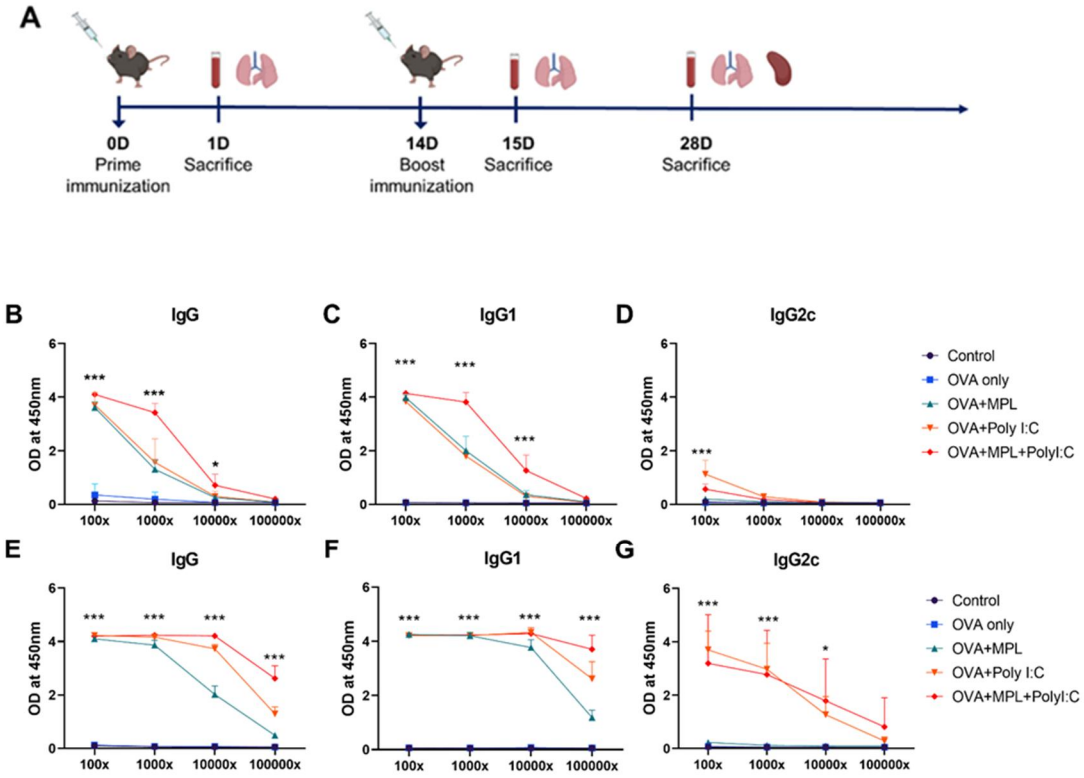

Figure 1. Immunization schedule and OVA-specific antibody levels in sera after immunizations. (A) A scheme of immunization and sample collection schedule. (B-G) OVA-specific antibody levels in the immune sera were measured by ELISA. The sera were taken at 2-weeks after each prime (B-D) and boost (E-G) immunization. All result were shown in mean ± SEM. For statistical analysis, Two-way ANOVA and Tukey's post-multiple comparison tests were performed. *$p<0.05$; and ***$p<0.001$ compared to OVA group.

Combination of MPL and Poly I:C promoted the induction of initial inflammatory cytokines after immunization

Adjuvants have been used to induce inflammatory responses at the site of immunization to enhance adaptive immune responses. To evaluate the initial inflammatory responses after OVA immunization with or without adjuvants, we measured cytokine levels in the lung extracts collected 1-day post-immunization. After the prime immunization (Fig. 2A), OVA-only immunization did not induce any significant cytokine production in the lungs. MPL-adjuvanted immunization induced a moderate level of IL-12p40, but not TNF-α, IL-6, and IFN-γ. The Poly I:C-adjuvanted group induced IL-6 and IL-12p40 production. The combination of MPL and Poly I:C induced significantly higher levels of TNF-α, IL-6, IL-12p40, and IFN-γ production in the lungs compared to the OVA only or single adjuvanted groups. The patterns of cytokine production were maintained after the boost immunization (Fig. 2B), so that the MPL+Poly I:C adjuvanted group showed significantly higher TNF-α, IL-6, IL-12p40, and IFN-γ production in the lung. These data suggested that the MPL+Poly I:C combination elicited strong initial inflammatory immune responses at the site of immunization.

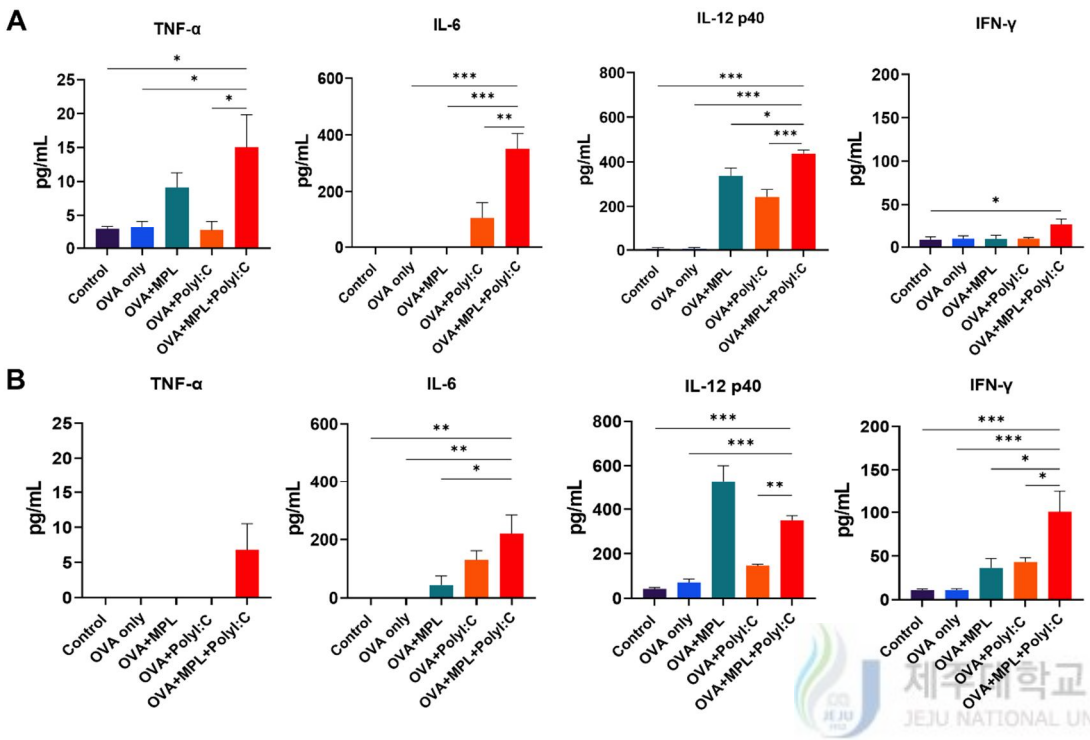

Figure 2. Cytokine production in lung after prime and boost immunizations of mice. Lung extracts were harvested from the mice one day after prime immunization **(A)** and boost immunization **(B).** Levels of cytokine production from each sample were measured by ELISA. All results were shown in mean ± SEM. For statistical analysis, One-way ANOVA and Tukey's post-multiple comparison tests were performed. *$p<0.05$; **$p<0.01$; and ***$p<0.001$ between the indicated groups

OVA immunization with the MPL and Poly I:C combination adjuvant recruited inflammatory cells to the site of immunization

To evaluate the cell recruiting effects of the MPL+Poly I:C combination at the site of immunization, we harvested lung cells from the immunized mice at day 1 post prime and boost immunizations. Cell phenotypes were determined using multi-color flow cytometry (Fig. 3). OVA-only immunization did not induce cell recruitment in the lungs. After prime immunization (Fig. 3A), Poly I:C adjuvanted immunization induced the recruitment of monocytes, neutrophils, and total DC populations in the lungs. The frequencies of monocytes and total DCs were significantly increased by the combination of MPL and Poly I:C. In addition, the activation of alveolar macrophages (AM), which was measured by the expression levels of MHC class II molecules, was significantly enhanced by Poly I:C and MPL+Poly I:C-adjuvanted groups. These data suggested that Poly I:C effectively enhanced innate cell recruitment at the site of immunization, and the recruitment of antigen-presenting cells, such as monocytes and DCs, was synergistically increased by MPL supplementation with Poly I:C after the prime immunization (Fig. 3A). After the boost immunization, the MPL-adjuvanted group also exhibited similar enhanced innate cell recruitment to those of the Poly I:C-adjuvanted group, and more innate cell recruitment was observed in the

MPL+Poly I:C-adjuvanted group (Fig. 3B). Compared to the prime immunization, 1.6 to 3 times more innate immune cells were recruited to the lungs after the boost immunization.

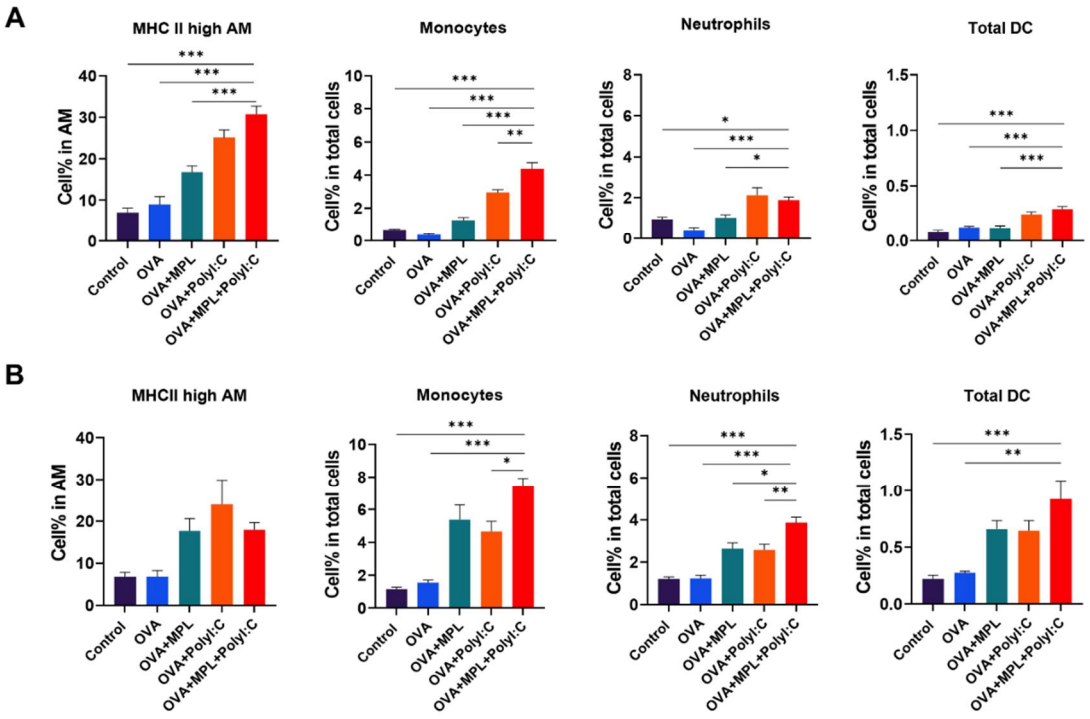

Figure 3. APC recruitment in lungs after immunization. Lung cells were harvested from the mice one day after prime immunization **(A)** and boost immunization **(B).** Cell phenotypes were analyzed by flow cytometry. All results were shown in mean ± SEM. For statistical analysis, One-way ANOVA and Tukey's post-multiple comparison tests were performed. *p<0.05; **p<0.01; and ***p<0.001 between the indicated groups.

Effects of the adjuvants on the memory T cell population and proliferation of antigen-specific T cells

To investigate memory T cell responses in the lung and spleen, cells were harvested from the mice at 2-week post-boost immunization. There was no significant difference between groups in memory T cell populations in spleen, but still increasing trends of memory T cell populations were observed in adjuvanted groups compared with those of the control or OVA-only groups (Fig. 4A). The percentages of Tcm and Tem populations in both CD4 and CD8 T cells in the lung were significantly increased by the combination of MPL and Poly I:C, while single-adjuvanted groups did not induce a significant increase compared with the OVA-only group (Fig. 4B).

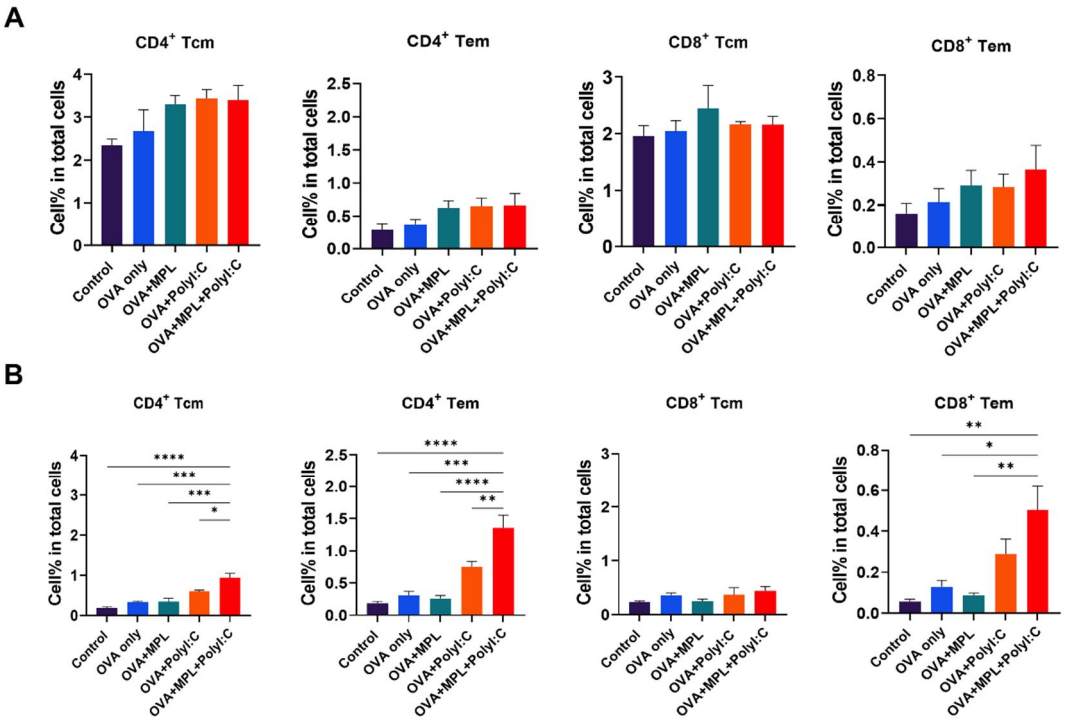

Figure 4. Memory T cell frequencies after immunization. The Tcm and Tem frequencies of CD4 and CD8 T cells in spleen **(A)** and lung **(B)** after immunization were determined by flow cytometry. Lung and spleen cells were harvested from the mice 2 week after boost immunization. All results were shown in mean ± SEM. For statistical analysis, One-way ANOVA and Tukey's post-multiple comparison tests were performed. *$p<0.05$; **$p<0.01$; and ***$p<0.001$ between the indicated groups.

To investigate the OVA-specific memory T cell responses, T cell proliferation and cytokine production were measured after OVA restimulation. The antigen-specific T cell proliferation of lung and spleen cells was stimulated by OVA peptide treatment (Fig. 5A,B). The antigen-specific CD4 T cell proliferation in the lung was induced in the MPL+Poly I:C-adjuvanted group. The antigen-specific CD8 T cell proliferation in the lung was similarly increased by Poly I:C and the combination of MPL and Poly I:C (Fig. 5A). The MPL+Poly I:C-adjuvanted group showed enhanced Ag-specific CD4 and CD8 T cell proliferation after in vitro OVA peptide stimulation (Fig. 5B). To further investigate the function of Ag-specific T cells after immunization, spleen cells were harvested from immunized mice and co-cultured with OVA-loaded macrophages or DCs. IFN-γ cytokine production from co-cultured cells was then evaluated. OVA-specific IFN-γ production was improved by Poly I:C and the combination of MPL and Poly I:C immunized spleen cells after co-culture with OVA-loaded macrophages and DCs (Fig. 5C). These data implied that Poly I:C could efficiently induce memory T cell responses, and the responses can be greatly enhanced by combination with MPL, showing numerical and functional improvement of the memory T cells, especially in the immunized site.

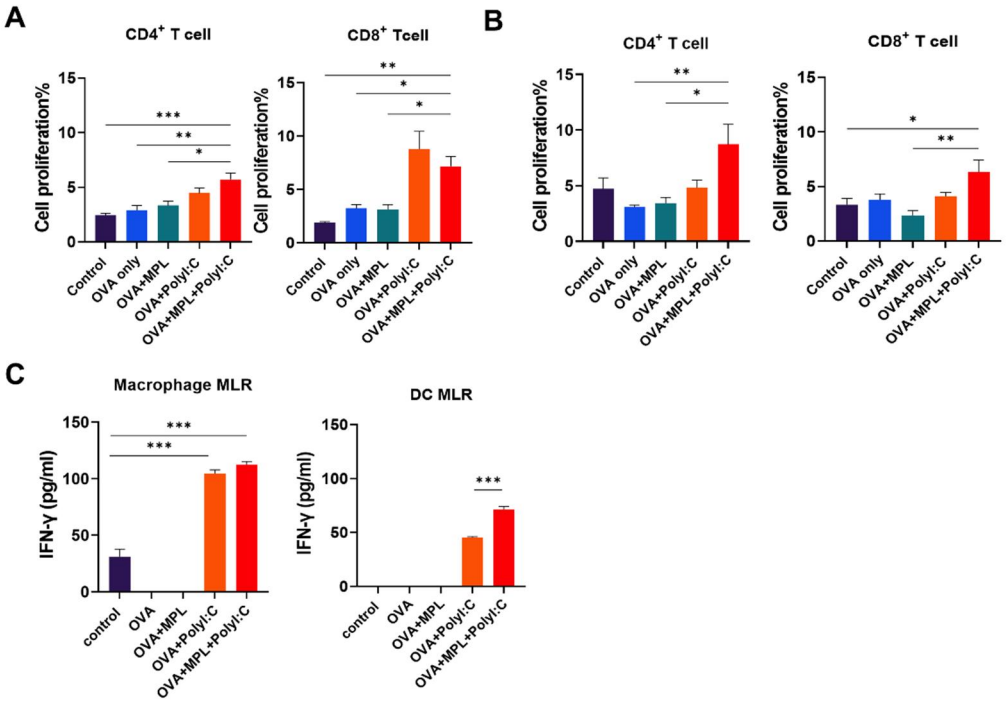

Figure 5. Antigen-specific memory T cell proliferation and cytokine production. Lung **(A)** and spleen **(B)** cells were harvested from the immunized mice 2 week after boost immunization. CFSE-labeled cells were cultured with OVA peptide for 5 days and T cell proliferation was determined by flow cytometry. (**C**) The spleen cells from the immunized mice were harvested at 2 weeks post boost immunization and co-cultured with OVA pre-loaded macrophages or DCs for 5 days. IFN-γ secretion was measured by ELISA. All results were shown in mean ± SEM. For statistical analysis, One-way ANOVA and Tukey's post-multiple comparison tests were performed. *p<0.05; **p<0.01; and ***p<0.001 between the indicated groups.

Combination of MPL and Poly I:C enhanced the antigen-specific memory B cell responses

After immunization, B cells are stimulated and differentiated into antibody-producing plasma cells and memory B cells. The plasma cells migrate to the bone marrow and keep producing antibodies, whereas the memory B cells stay in lymphoid tissues and are activated by antigen stimulation [29]. We harvested bone marrow cells and spleen cells from the immunized mice after 2 weeks of boost immunization to evaluate *the ex vivo* capacity of plasma cells and memory B cells to produce OVA-specific antibodies (Fig. 6). Poly I:C and MPL+Poly I:C adjuvants significantly promoted both antibody-producing cells in the bone marrow and memory B cells in the spleen; in particular, the combination adjuvant showed synergistic effects on inducing antibody-producing cell activity. These data suggest that the MPL+Poly I:C combination elicited strong antibody-producing cells and memory B cell responses, as well as memory T cells.

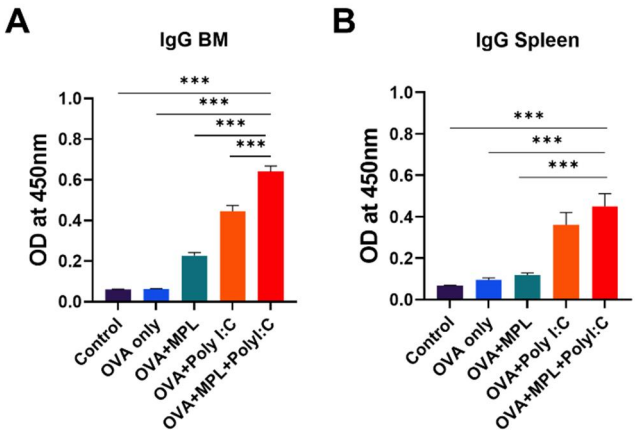

Figure 6. OVA-specific IgG production of bone-marrow cells and spleen cells from the immunized mice. BM cells **(A)** and spleen cells **(B)** were harvested from the immunized mice 2 weeks after boost immunization and cultured with OVA protein for 1 days or 7 days, respectively. Then OVA-specific IgG production was measured by ELISA. All results were shown in mean ± SEM. For statistical analysis, One-way ANOVA and Tukey's post-multiple comparison tests were performed. ***$p<0.001$ between the indicated groups.

Adjuvants promote the APC-T cell reaction

The effects of adjuvants on APCs to stimulate T cells were determined by MLR assay. DC and macrophage stimulation by Poly I:C and MPL+Poly I:C adjuvants significantly promoted CD4 and CD8 T cell proliferation (Fig. 7A and B).

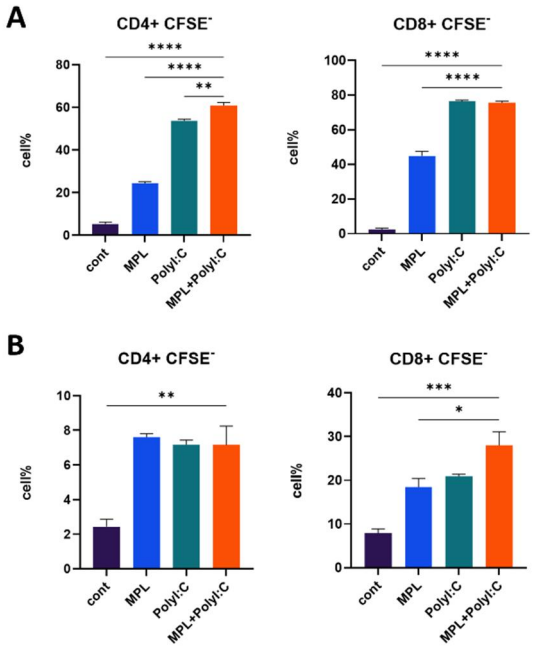

Figure 7. T cell proliferation after co-culture with adjuvant pre-treated DCs and macrophages. DCs and macrophages enriched from bone marrow cells were pre-activated by MPL, Poly I:C, or MPL + Poly I:C for 2 days. Allogeneic lymphocytes were harvested from spleen. CFSE-labeled lymphocytes and pre-activated DCs **(A)** or macrophages **(B)** were co-cultured for 5 days. T cell proliferation was determined by flow cytometry. All results were shown in mean ± SEM. For statistical analysis, One-way ANOVA and Tukey's post-multiple comparison tests were performed. *p<0.0332; **p<0.0021; ***p<0.0002 ****p<0.0001 between the indicated groups.

Ⅳ. Discussion

Vaccination is the best way to protect life against invading pathogens. Most vaccine studies have focused on B cell responses, including neutralizing antibodies, but protection against pathogens such as viruses requires cell-mediated immune responses. Therefore, to develop an effective vaccine adjuvant, antigen-specific T cell responses need to be evaluated in addition to B cell responses and safety [30].

In addition to antigen-specific memory T cell responses, helper T cells affect antibody production via T-B cognition and cytokine production. Th1 immune responses mainly produce IFN-γ and induce IgG2c antibody production, while Th2 secretes IL-4 and elicits IgG1 antibody production [31]. In this study, we examined the effects of MPL and Poly I:C combination on the induction of antigen-specific T cell responses as vaccine adjuvant candidates. MPL stimulates the TLR4 signaling pathway, which elicits initial inflammatory responses via the TRIF-TRAM pathway [32,33]. It is known to be a safe immuno-stimulator to induce Th1 immune responses [34], but MPL-adjuvanted OVA immunization induced poor OVA-specific IgG2c antibody production in this study, suggesting Th2-skewed responses by MPL. However, the TLR3, RIG-1, and MDA5 signaling pathway, stimulated by Poly I:C, activates Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Interferon regulatory factor 3 (IRF-3); virus-mediated signaling, which directly enters the nuclear membrane and elicits fast antiviral responses [35]. In this study, Poly I:C induced more effective CMI responses with higher IgG2c antibody

responses compared to those of the MPL-adjuvanted group, showing consistent results with other previous studies comparing adjuvant effects [24,36]. The combination of MPL and Poly I:C adjuvants enhanced the initial inflammatory responses at the immunized site, showing higher levels of inflammatory cytokines and recruiting more APCs to the lungs. Consequently, it induced stronger T cell and antibody responses at 2-weeks after boost immunization. Improved IgG, IgG1, and IgG2c production in sera and OVA-specific IgG production in spleen cells and bone marrow cells demonstrated that B cells and T cells were functionally improved by MPL+Poly I:C combination. We found that Poly I:C and MPL+Poly I:C adjuvants increased the CD4 T cell population and decreased the CD8 T cell population (data not shown). However, the population of memory T cell subsets has increased in both CD4 and CD8 T cells, maintaining the ratio of Tcm and Tem. In addition to the numerical increases, the proliferation capacity of memory T cells after *in vitro* OVA treatment was also improved by MPL+Poly I:C adjuvanted immunization.

The Ag-presenting process of APCs, such as DCs or macrophages, is crucial to induce Ag-specific T cell immunity [37]. MPL and Poly I:C stimulated DC activation and upregulated the co-stimulatory molecule expression on DCs, which was critical to initiate T cell response [12,38]. Additionally, Poly I:C contributed to the antiviral response of macrophages by promoting the differentiation of type 1 macrophages and pro-inflammatory phenotype [39]. Our *in vitro* MLR data supported the potency of MPL and Poly I:C adjuvants in the activation of DCs and macrophages. MPL+Poly I:C-treated DCs induced high proliferation of CD4 T cells, and MPL+Poly I:C-treated macrophages induced high proliferation of CD8 T cells. The MPL+Poly I:C

combination induced Ag-specific T cell immunity through various stimulation pathways on APCs and T cells, leading to successful induction of memory T cell responses.

In summary, we demonstrated the distinct effects of MPL and Poly I:C combination on the induction of antigen-specific T cell responses, which might be an effective vaccine adjuvant for vaccines against intracellular pathogens, like viruses. The MPL+Poly I:C combination has shown the great capacity of inducing strong and extensive cellular and humoral immunity. Future studies can further explore the vaccine adjuvant efficacies with viral antigens and determine the efficiency of their protection against various viral infections.

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