A Thesis
For the Degree of Master of Interdisciplinary Graduate Program in Advanced Convergence Technology and Science

A combination adjuvant of monophosphoryl lipid A and Poly I:C enhanced vaccine efficacy by functional natural killer cell activation and DC maturation

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

Department of Interdisciplinary Graduate Program in Advanced Convergence Technology and Science

Chau Thuy Tien Le

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Chau Thuy Tien Le

(Supervised by Professor Eun-Ju Ko)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

2022. 2

This thesis has been examined and approved.

Thesis director, Inhae Kang, Professor, Department of Interdisciplinary Graduate Program in Advanced Convergence Technology and Science, Jeju National University

Eun-Ju Ko, Professor, Department of Interdisciplinary Graduate Program in Advanced Convergence Technology and Science, Jeju National University

Minu Ha, Professor, Department of Interdisciplinary Graduate Program in Advanced Convergence Technology and Science, Jeju National University

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Jeju National University

Abstract

The traditional vaccines were appeared to be concerned either safety or efficacy. Natural killer cells were demonstrated as an effective targeting immune cell used in immunotherapy and cancer therapy for several decades with their well-known ability to control tumor cell spread and microbial infections. Beside to their lysis capacity of target cells, NK cells were also defined as regulatory cells with immunoregulatory cytokine producing functions. Notably, most of markers expressing on NK cell can be triggered by Toll-like receptor-signaling pathways, resulting in utilization of Toll-like receptor agonists as a potential approach to stimulate NK activation. Herein, we studied the effects of monophosphoryl lipid A (MPL) and polyriboinosinic polyribocytidylic acid (Poly I:C) on NK cell cytotoxic

and regulatory functions after intranasally immunization with Ovalbumin. The findings provided that individual Poly I:C was effective in recruitment and stimulation of NK cell expressing CD69 and CD107a activation markers at prime dose, and exhibiting highly cytolytic activity as well, but not that of boost dose and regulatory functions. The combination of MPL and Poly I:C, whereas, displayed a significantly efficacy not only in inducing NK activation release of cytokines, up-regulation of lyse ability and also in enhancement of collaboration with Dendritic cells (DCs) as compared to individual adjuvants or naïve mice. Taken together, these data suggest that combination adjuvant of MPL and Poly I:C might be a promised adjuvant in vaccination with a capacity to strongly enhance immune responses particularly NK cells, leading to improve vaccine efficacy.

Key words: Nature killer cells, Dendritic cells, MPL, Poly I:C, Toll-like receptors

CONTENT

Introduction

The innate immune system serves as a first guard recognize and against non-self antigens invading mammalian body [1]. Therein, NK cells are lymphocytes in the same family as T and B cells but belong to the innate immunity which are among the first immune effector cells to migrate to inflammation site and induce apoptosis of target cells [2]. Not only are NK cells best known for killing virally infected cells via death receptor-mediated pathways and for capacity to secrete dominantly interferon-gamma (IFN-γ) and Granzyme B, but activated NK cells are also capable of inducing Dendritic cell (DCs) maturation which further drives the development of T helper type 1 lymphocytes (Th1) via priming DCs for production of interleukin (IL)-12p40 [3, 4]. Indeed, the interaction between DCs and NK cells were reported as a crucial mechanism of innate immune system and of modulation of initially adaptive immune responses. Mature DCs (mDCs) might augment NK-cell cytotoxicity, cytokine production and NK proliferation by IL-12 and IL-18 [5-7]. NK cells furthermore can undergo differentiation into memory-like NK cells once exposed to these cytokines [8, 9]. Conversely, NK cells were profound strongly in mediating DC maturation and promoting optimal cytokine production such as IL-12 and IL-18 which play roles in polarization of T cell, production of IFN-γ and proliferation of NK cells [7, 10]. Additionally, NK cells have been demonstrated the importance of decrease in the susceptibility to virus infection in adolescent human [11] and mortality during influenza infection in mice [12]. These findings, collectively, suggested that NK cells may play the important roles in the development of vaccine efficacy.

Vaccines are the most effective strategy preventing infectious diseases, which are classified into live-attenuated organism, inactivated organism and subunit

components. Live vaccines induce strong immune response, but cause significant disease manifestation. By contrast, non-lived vaccines pose no risk to immunocompromised individual but show weaker immune responses [13]. For this reason, adjuvanted vaccines presented sufficient immune-stimulatory activity and heighten innate immune responses to pathogens, subsequently is guaranteed from side effects. Alum-based compounds, oil-in-water emulsion and liposome-base adjuvants have been licensed in the past few decades. AS04, an adjuvant composed of aluminum salt and monophosphoryl lipid A (MPL), a toll-like receptor 4 agonist, has been licensed for vaccine against human papilloma virus [13, 14].

The innate immune cells express pattern-recognition receptors that can bind to a wide variety of infectious agents and stimulate rapid mechanism of detecting viral infection. Herein, Toll-like receptors are key receptors of the innate immune system, which can recognize both of exogenous and endogenous pathogens, resulting in rapidly triggering innate immune responses and modulating adaptive immune responses via a variety of cell signaling pathways such as Myeloid differentiation factor 88 (MyD88) and Toll-IL-1R domain-containing adaptor-inducing IFN-β factor (TRIF) dependent pathway leading to an increase in the host's capacity of eliminating pathogens [15, 16]. Several recent studies suggested TLR agonists were potent vaccine adjuvants induced TLR signaling pathway which can directly activate antigen-specific immune responses.

Synthetic double-stranded RNA polyriboinosinic polyribocytidylic acid (Poly I:C) which can induce the activation of NF-κB, Mitogen-activated protein kinase and the production of cytokine through a signalling pathway dependent or independent on MyD88, and also promote DC maturation [17]. In a murine model, intranasal co-administration of Poly I:C with inactivated hemagglutinin vaccine induced a

high anti-HA immunoglobulin A response in the nasal wash and IgG antibody response in the serum, which provided a high protective effect against influenza virus infection [18, 19]. Monophosphoryl lipid A, besides, which is a TLR4 agonist component of lipopolysaccharide, was known as a novel licensed adjuvant targeting TLR4 with a purpose of inducing TLR4 signaling pathway and has demonstrated safety and effective concerns on co-administration with respiratory syncytial virus vaccine at high dose [20]. Additionally, another studies investigated that combination of CpG and MPL adjuvant either in respiratory syncytial virus F protein or in influenza vaccine showed a contribution to stimulating strong immune responses and preventing either inflammatory RSV disease or IAV infection after a single dose vaccination, furthermore providing a cross-protection against to heterosubtypic influenza virus infection [21-23].

In this study, we investigated the adjuvant effects of MPL, poly I:C, and combined MPL and poly I:C added to the ovalbumin (OVA) protein antigen on immune responses, particularly the NK cell responses in murine models. Herein, we found that a combination adjuvant of MPL and poly I:C promoted the production of immune-regulatory cytokines, including IL-12p70, IL-18, IFN-γ, and enhanced NK cytotoxic activities, rather than single adjuvants. Additionally, we used an *in vitro* DC-NK cell co-culture system and found that the DC and NK cells treated with the combination adjuvant were capable of directly activating each other. Therefore, this study demonstrates the potential of using MPL and poly I:C as a novel vaccine combination adjuvant that functions via the activation of NK cells.

Material and Methods

Mice and reagents

Female C57BL/6 (n= 6 each group) mice (OrientBio Co.) were used in this study. The mice were 6–8 weeks old at the time of priming immunization. OVA was purchased from Sigma-Aldrich, and poly I:C was purchased from InvivoGen. All reagents were prepared according to the manufacturer's instructions..

Immunization of mice with OVA + Adjuvants

Before the intranasal administration of OVA and adjuvants, the female C57BL/6 (B6) mice were anesthetized with isoflurane using an oxygen controlling machine and then intranasally administered: 50 μL containing 10 μg of OVA, 50 μL containing 10 μg of OVA + 1 μg of MPL, 50 μL containing 10 μg of OVA + 10 μg of poly I:C, or 50 μL containing 10 μg of OVA + 1 μg of MPL + 10 μg of poly I:C at day 0 (prime) and day 14 (boost). The control group consisted of mice that did not receive any such immunization (naïve mice).

Sample harvest and preparation

The mice in each group were euthanized at the indicated dates post-immunization (prime D1, boost D1). The bronchoalveolar lavage fluid (BALF) was collected from the trachea using an 18-gauge Excel Safelet Catheter and phosphate-buffered saline (PBS). The BALF was then centrifuged at 1,600 rpm at 4°C for 5 min. The supernatant was stored at -20°C for cytokine enzyme-linked immunosorbent assay (cytokine ELISA), and the cell pellet was resuspended in 2% fetal bovine serum (FBS) containing PBS (fluorescence-activated cell sorting (FACS) buffer) for the analysis of the cell phenotype. The lungs and spleens were harvested aseptically and homogenized in the Roswell Park Memorial Institute (RPMI) medium 1640

(Fisher Scientific, Corning, NY, USA) using a 70 μm cell strainer. The homogenates were then centrifuged at 1,600 rpm at 4°C for 5 min, and the supernatant was stored at –20°C for cytokine ELISA. Next, the red blood cells (RBCs) were removed from the cell pellets and the single cells were used for FACS and other assays.

Flow cytometry

The prepared lung and BAL cells were stained with different surface markers for NK cells including Live/Dead-Amcyan (LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, ThermoFisher Scientific), CD3-BV421 (clone 17A2, BD HorizonTM), CD45-PerCP (clone 30-F11, BD PharmingenTM), CD49b-PE (Natural killer Cells, clone HMα2, BD PharmingenTM); CD107a-APC (degranulation marker for NK cells, clone 1D4B, BD PharmingenTM); CD69-AF700 (activation marker for NK cells, clone H1.2F3, BD PharmingenTM); CD11b-APC/Cy7 (clone M1/70, BD PharmingenTM), CD27-PE/Cy7 (NK cell subsets, clone LG.3A10, BD PharmingenTM), and intracellular cytokine marker being IFN-γ-APC/Cy7 (clone XMG1.2, BD PharmingenTM). Stained cell samples were performed by Becton-Dickenson LSR flow cytometer and data analysis by Flowjo Software Program (Tree Star Inc.).

Cytokine-specific ELISA

The levels of gamma interferon (IFN-γ, R&D Systems, a Bio-Techne brand), Granzyme B (R&D Systems), interleukin-12p70 (IL-12p70, R&D Systems), interleukin-12p40 (IL-12p40, Invitrogen), interleukin-15 (IL-15, Invitrogen) and interleukin-18 (IL-18, R&D Systems) were measured in cultured supernatants by capture ELISA, following instruction from the manufacturers. Concentrations of

the cited cytokines are expressed by reference to a standard curve constructed by assaying serial dilutions of the respective murine standard cytokine.

Preparation of resting NK and activated NK cells

$CD3^{-}CD49b^{+}$NK cells were isolated from C57BL/6 mice spleen by negative magnetic selection using EsaySepTM mouse NK Cell Isolation Kit and EasySepTM Magnet (STEMCELL Technologies Inc.). To generate activated NK (ANK) cells, purified CD49b+ NK cells (5 x 10^5 cells/mL) were cultured in 6-well plate for 48 h in 10% LCM in the presence either of 100 ng/mL MPL, 1000 ng/mL Poly I:C, 100 ng/mL MPL and 1000 ng/mL Poly I:C combination or only medium. Before using ANK cells for next experiment, ANK cells were harvested, washed to remove all excess reagents and resuspend in 10% LCM.

***In vitro* NK cell cytotoxicity assay**

To evaluate the cytotoxic activities of the ANK cells (effector cells, E), YAC-1 cells were utilized as the target cells (T). ANK cells were first stained with 2000 nM carboxyfluorescein succinimidyl ester (CFSE; Sigma) in PBS at 37 °C for 10 min. Next, the YAC-1 cells (1 × 10^5 cells/mL) were seeded in a 96-well U-bottomed plate and co-cultured with CFSE-stained ANK cells (5 × 10^5 cells/mL). After 4 h of co-culture, the cells were harvested and stained with 7-aminoactinomycin D (eBioscienceTM 7-AAD Viability Staining Solution; Thermo Fisher Scientific). The frequency of target cell death (CFSE-7-AAD+ YAC-1 cells) was later determined by flow cytometry.

Preparation of immature and mature DCs

Bone marrow cells were collected from the femur and tibia and the RBCs were removed. Then, the bone marrow cells were cultured in the presence of 20 ng/mL

of recombinant mouse granulocyte-macrophage colony-stimulating factor (mGM-CSF) at 37 °C with 5% carbon dioxide (CO_2). The culture medium containing mGM-CSF was replaced every 2 d. After 6 d of culturing, the immature DCs were harvested by pipetting and seeded at a concentration of 5×10^5 cells/mL in 6-well plates. Thereafter, 100 ng/mL MPL, 1000 ng/mL poly I:C, or the combination of 100 ng/mL MPL, and 1000 ng/mL poly I:C or only medium were treated and cultured for 2 d to facilitate the maturation of DCs.

***In vitro* NK cell-mediated DC maturation and production of IL-12p70 and IL-18**

iDCs (2 x 10^5 cells/mL) and activated NK cells (ANK, 4 x 10^5 cells/mL) were either culture in media only or co-culture at a ratio of 1 : 2 in the 96-well U-bottomed plate. After 2 days of incubation, cultured cells were harvested. The supernatants were stored for cytokine ELISA and the pellet cells were resuspended in FACS buffer and stained with different surface markers including Live/Dead-Amcyan (ThermoFisher Scientific), CD49b-PE (BD PharmingenTM), CD69-AF700 (BD PharmingenTM); CD11c-PE/Cy7 (Monoclonal Antibody N418, ThermoFisher Scientific), CD40-BV605 (clone 3/23, BD Biosciences), CD86-FITC (clone GL1, BD PharmingenTM). Cell phenotypes were performed by Becton-Dickenson LSR flow cytometer and data analysis by Flowjo Software Program (Tree Star Inc.)

***In vitro* mDCs-mediated NK cell activation and production of IFN-gamma and Granzyme B**

Inactivated NK cells (iNK, 2 x 10^5 cells/mL) and mature DCs (mDCs, 1 x 10^6 cells/mL) were either culture in media only or co-culture at a ratio of 1: 5 in the 96-

well U-bottomed plate for 2 days. Cultured cells were then harvested. The supernatants were stored for cytokine ELISA and the pellet cells were resuspended in FACS buffer which were stained with the different surface markers including Live/Dead-Amcyan (ThermoFisher Scientific), CD49b-PE (BD PharmingenTM), CD69-AF700 (activation marker for NK cells, BD PharmingenTM); CD11c-PE/Cy7 (ThermoFisher Scientific), CD40-BV605 (ThermoFisher Scientific); and intracellular cytokine marker being IFN-γ-APC/Cy7 (BD PharmingenTM). Cell phenotypes and the frequency of gamma interferon were performed by Becton-Dickenson LSR flow cytometer and data analysis by Flowjo Software Program (Tree Star Inc.).

Statistical analysis

All data were statistically analyzed using a GraphPad Prism9® software (GraphPad Software, Inc.).

Results

NK cells are recruited to the site of immunization after prime and boost vaccination.

NK cells were demonstrated to play the vital roles in innate immune responses with a lyse capacity and indirect modulation of adaptive immunity by a release of immune-regulatory cytokines [24]. Here, we observed NK recruitment in BAL and lung one-day after prime and boost intranasal vaccinations (Figure 1). Poly I:C and the combination of MPL+Poly I:C-adjuvanted OVA immunization significantly enhanced NK cell frequencies in both BAL and lung compared to those of naïve mice after primary inoculation. On the other hand, OVA + MPL immunization promoted highly NK cell recruitment to the lung at day 1 post boost immunization, but no significant differences observed between adjuvant treatments at the time (Figure 1D). These data suggested that Poly I:C and a combination adjuvant enhanced NK cell recruitment at the site of immunization.

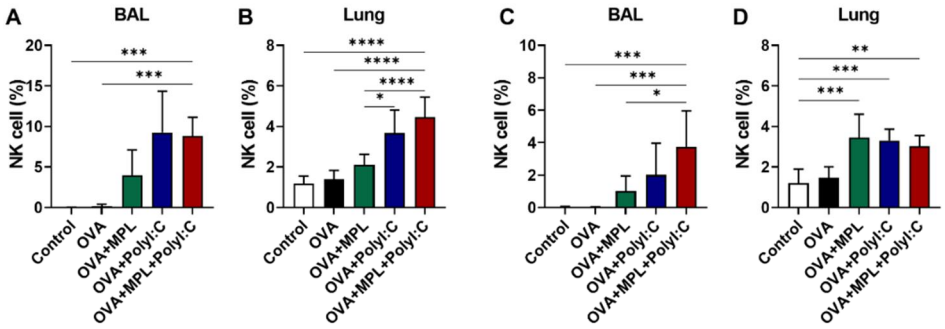


Figure 1. NK cell recruitment after intranasal immunizations of OVA with adjuvants. C57BL/6 mice were intranasally immunized with ovalbumin alone or plus MPL, Poly I:C or MPL + Poly I:C. The immunizations were given two times with 2 weeks interval (prime and boost). At day 1 post prime **(A, B)** and boost **(C, D)** immunizations, NK cell population in BAL and Lung cells were analyzed by flow cytometry. $CD45^{+}CD3^{-}CD49^{+}$ cells were gated as NK cells. All data show the mean ± SD. Statistical analysis between groups were performed by One-way ANOVA and Tukey's multiple comparison test. *; p<0.0332, **; p<0.0021, ***; p<0.0002 and ****; p<0.0001.

Combination of MPL + Poly I:C adjuvanted OVA immunization enhanced NK maturation and activation

Murine NK cells are further differentiated by their expression levels of CD11b and

CD27. The $CD11b^{-}CD27^{-/+}$ populations are considered the immature NK cell phenotypes, and $CD11b^{-}CD27^{+}$ appears as a relatively second stage of NK differentiation with a high potential of proliferation. $CD11b^{+}CD27^{-}$ and $CD11b^{+}CD27^{+}$ NK cells, meanwhile, are the most mature NK cells with a limited capability of proliferation, but a highly capacity for production of cytokines and the strong effector functions, distinctively $CD11b^{+}CD27^{+}$ [25]. The $CD11b^{-}CD27^{-}$ NK cell subset was decreased, but $CD11b^{-}CD27^{+}$, $CD11b^{+}CD27^{+}$, and $CD11b^{+}CD27^{-}$ NK subsets were increased in the adjuvanted groups after both prime and boost immunizations (Figure 2A and D). Therein, the MPL + Poly I:C combination adjuvant greatly induced NK cell maturation compared to OVA only or OVA + single adjuvanted groups. Additionally, we analyzed activation marker expressions on the lung NK cells after prime (Figure 2B and C) and boost (Figure 2I and K) immunizations. CD69 is considered as a general NK cell activation marker and CD107a is a degranulation marker which are increased when NK cells are functionally activated [26]. OVA only or OVA+MPL immunization could not affect the activation marker expression on NK cells. Meanwhile, Poly I:C adjuvanted OVA immunization enhanced both CD69 and CD107a expression after the prime immunization, but not after the boost immunization. NK cells from MPL + Poly I:C combination adjuvanted group exhibited the significantly enhanced CD69 and CD107a expressions at day 1 post immunizations. These data indicated that MPL + Poly I:C combination adjuvant could elicit strongly NK cell maturation and activation at the site of immunization comparing to the other groups.

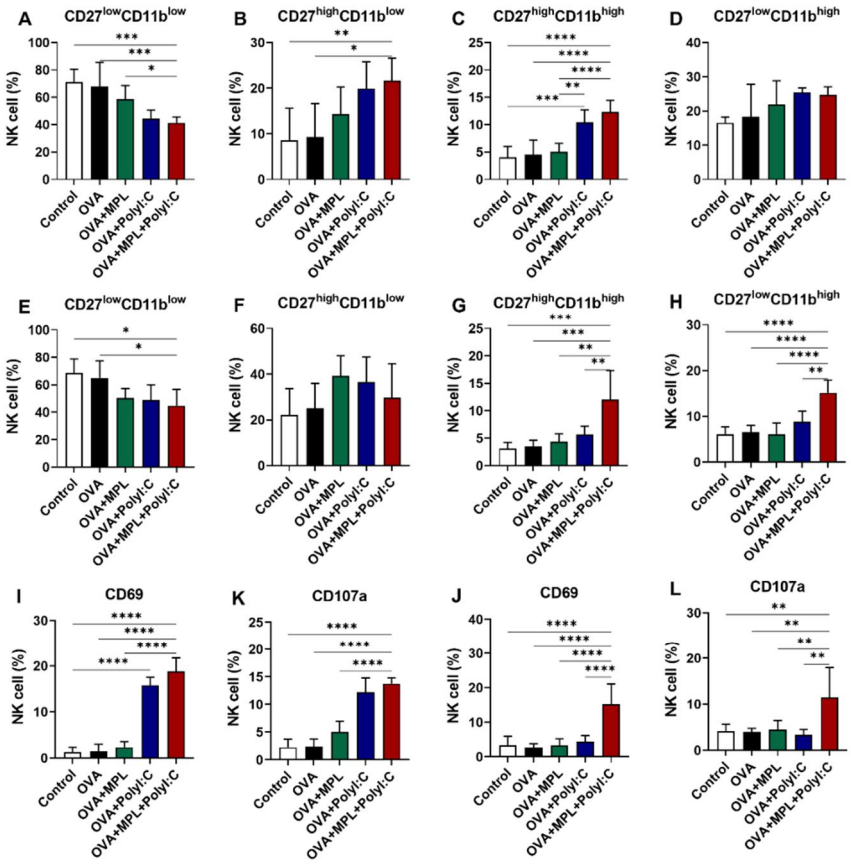


Figure 2. NK cell differentiation and activation marker expressions in lung after intranasal immunizations of OVA with adjuvants. Lung samples were harvested at day 1 post prime and boost vaccinations. **(A-D, I-K)** NK cell subsets and activation marker expressions after the prime immunization. **(E-H, J-L)** NK cell subsets and activation marker expressions after the boost immunization. The data show the mean ± SD. Statistical analysis between groups were performed by One-way ANOVA and Tukey's multiple comparison test. *; p<0.0332, **; p<0.0021, ***; p<0.0002 and ****; p<0.0001.

MPL + Poly I:C adjuvanted OVA immunization promoted cytokine production of NK cells in the lungs

We next examined the cytokine secretion by NK cells and immune cells-resided lung from the vaccinated mice. IFN-γ-producing NK cells in lung were analyzed by intracellular cytokine staining and flow cytometry (Figure 3A and D), and IFN-γ and Granzyme B secretion in lung homogenates were determined by ELISA (Figure 3B, C, E, and F). Similarly to the activation marker expressions on NK cells, Poly I:C adjuvant increased the IFN-γ production of NK cells after prime immunization (Figure 3A), but not boost immunization (Figure 3D). However, MPL + Poly I:C combination adjuvant could enhance IFN-γ production of NK cells

in both prime and boost immunizations. IFN-γ in the lung homogenates were induced by MPL + Poly I:C adjuvanted prime and boost immunizations (Figure 3B and E), but granzyme B production was promoted by MPL, Poly I:C and MPL + Poly I:C adjuvants (Figure 3C and F). Collectively, the combination of MPL + Poly I:C indicated a highly efficiency in cytokine production by immune cells including NK cells as well as enhancement of NK cell activation.

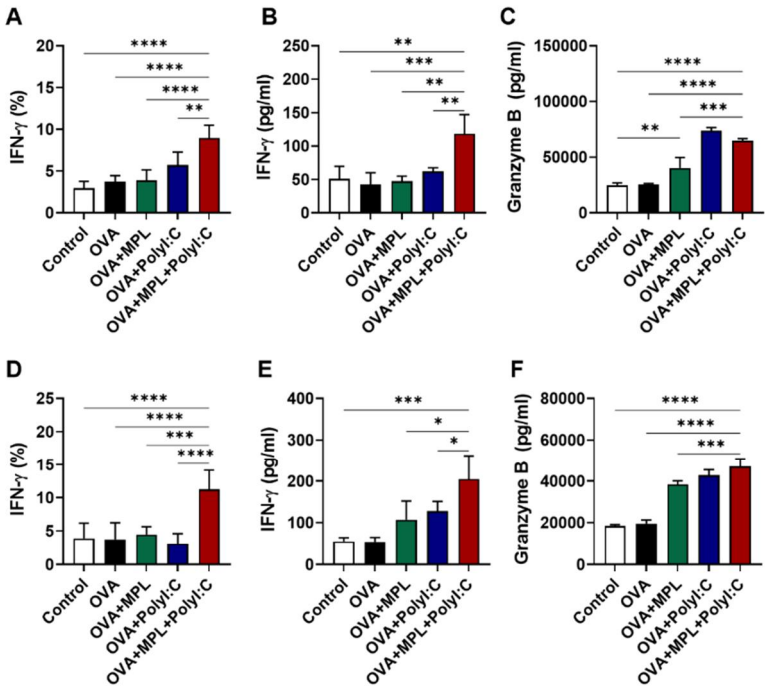


Figure 3. Cytokine production after intranasal immunizations of OVA with adjuvants. Lung samples were collected after 1 day post prime **(A-C)** and boost **(D-F)** immunizations. **(A, D)** The frequencies of IFN-γ-positive NK cells were analyzed by flow cytometry. **(B, C, E, F)** The concentration of IFN-γ and Granzyme B in lung homogenates were measured by ELISA The data show the mean ± SD. Statistical analysis between groups were performed by One-way ANOVA and Tukey's multiple comparison test. **; p<0.0021, ***; p<0.0002 and ****; p<0.0001.

Treatment with MPL + Poly I:C combination strengthened NK cytolytic activity both *in vivo* and *in vitro*

To elucidate the functional responses of adjuvant-pretreated NK cells in immune responses to infected cells, we investigated the effects of adjuvants on NK-

mediated cytotoxicity against YAC-1 cell. Splenic NK cells were either freshly purified from naïve mice and then were stimulated with MPL, Poly I:C, MPL + Poly I:C in vitro for 2 days, or isolated from adjuvant-treated mice *in vivo* at day 1 post second immunization. Activated NK cells were subsequently cultured with YAC-1 cells that are typical target cells for NK cells. As described above, the mortality rate of YAC-1 cells was analyzed by flow cytometry. The Poly I:C adjuvanted OVA and the combination of MPL + Poly I:C adjuvanted OVA group showed highest cytotoxic activity among the vaccination groups both *in vitro* and *in vivo*. NK cell-induced killing of YAC-1 cell, otherwise, was partially stimulated by individual MPL treatment *in vitro* (Figure 4A), but not *in vivo* (Figure 4B).

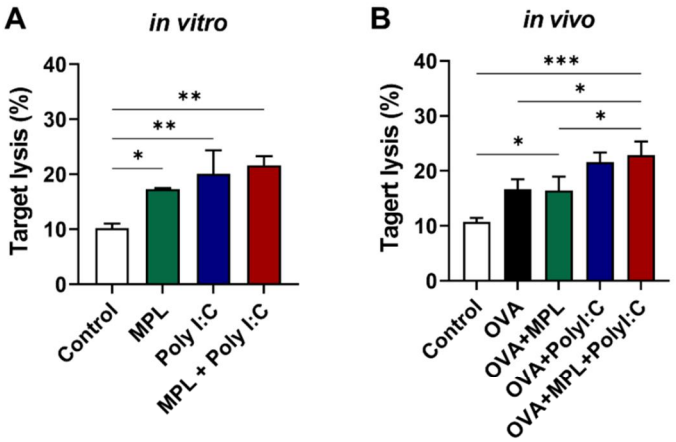


Figure 4. Cytotoxicity mediated by NK cells after *in vitro* adjuvant treatment and *in vivo* immunizations with OVA+adjuvants. (A) splenic NK cells were isolated from naïve mice and then treated with MPL, Poly I:C, and MPL+Poly I:C for 2 days. **(B)** NK cells were isolated from spleen cells of naïve or immunized mice at day 1 post boost immunization. The effector cells (**E**), *in vitro* adjuvant pre-treated-NK cells and the *in vivo* isolated-NK cell from the immunized mice, were cultured with target cells (T), YAC-1 cells, with a ratio of 5 (E) : 1 (T). Cells were collected after 4 hours incubation and the dead cell frequencies of target cells were determined by flow cytometry. The data show the mean ± SD. Statistical analysis between groups were performed by One-way ANOVA and Tukey's multiple comparison test. *; $p<0.0332$, **; $p<0.0021$, and ***; $p<0.0002$.

Correlations between frequencies of NK cells and DCs were found after *in vivo* immunizations

It is well-known that NK cells and DCs can interact with each other to regulate

their own activations by releasing cytokines. Subsequently, DCs initiate antigen-specific adaptive immune responses after antigen uptake and activation. To evaluate the correlation between NK cells and DCs after the adjuvanted immunizations, we analyzed total DC populations in the lung and performed correlation analysis between NK cells and DC populations. DC populations residing lung were significantly increased in Poly I:C and MPL + Poly I:C adjuvanted groups after the prime immunization, (Figure 5A), and in MPL, Poly I:C and MPL + PolyI:C adjuvanted groups after boost immunization (Figure 5C). Strong correlations between DCs and NK cells were found after both prime and boost immunizations (r = 0.6294 and r = 0.7363, respectively) (Figure 5B and D).

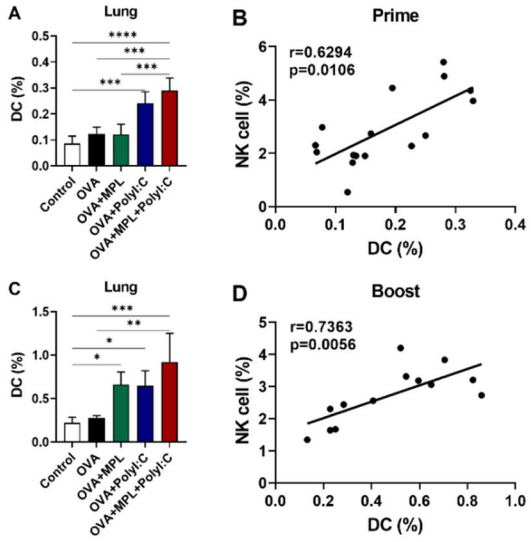


Figure 5. Correlation between DC and NK cell recruitment after intranasal immunizations of OVA with adjuvants. Lung samples of the immunized mice were harvested at day 1 prime (A, B) and boost (C, D) immunizations and then cell phenotypes were identified by flow cytometry. The frequencies of DC were analyzed as $CD45^{+}F4/80^{-}CD11c^{+}MHCII^{high}$ population. The correlation of NK cell (Fig. 1B, D) and DC frequencies were analyzed. The data show the mean ± SD. Statistical analysis between groups were performed by One-way ANOVA and Tukey's multiple comparison test. *; 0.0332, **; 0.0021, ***; 0.0002 and ****; 0.0001. For correlation analysis, Spearman's rank correlation coefficients (r) and p value are calculated and shown.

The combination of MPL + Poly I:C-treated NK cells have a capability to elicit DC maturation and enhance cytokine production *in vitro*

For further details of NK-DC interaction promoted by adjuvants, NK cell freshly purified from C57BL/6 mice were stimulated with either MPL, Poly I:C or MPL + Poly I:C for 2 d, and then were harvested and cultured with bone marrow-derived DCs (BM-DCs) taken from naïve C57BL/6 mice. After 2 d of culture, DC

maturation was performed flow cytometric analysis staining with DC activation markers, subsequently cultured media was used for measuring cytokine-secreted DCs by ELISA. MPL only and MPL + Poly I:C-pretreated NK cell showed a significant capacity to trigger DC maturation expressing activation makers with the extremely high levels of CD40, CD86 and MHC II high when compared to the rest groups (Figure 6A-C). Meanwhile, the productions of IL-12p70, IL-12p40, IL-18 and TNF-alpha by immature DC co-cultured with MPL + Poly I:C pre-treated NK cell exhibited the highest levels, which doubled that of MPL treatment, and the cytokine-produced DC-stimulated inactivated NK cell underwent the trivial levels (Figure 6D). Overall, combination of MPL + Poly I:C pretreated-NK cells were more effective in stimulating DCs activation expressing CD40 and CD86 markers as well as IL-12, IL-18 and TNF-alpha production, compared to individual MPL or Poly I:C.

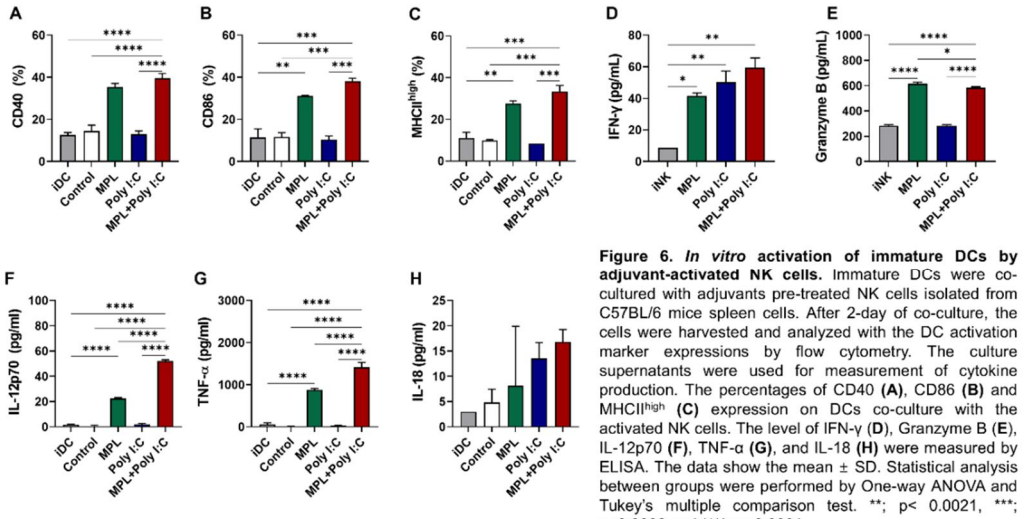


Figure 6. *In vitro* activation of immature DCs by adjuvant-activated NK cells. Immature DCs were co-cultured with adjuvants pre-treated NK cells isolated from C57BL/6 mice spleen cells. After 2-day of co-culture, the cells were harvested and analyzed with the DC activation marker expressions by flow cytometry. The culture supernatants were used for measurement of cytokine production. The percentages of CD40 **(A)**, CD86 **(B)** and MHCII^{high} **(C)** expression on DCs co-culture with the activated NK cells. The level of IFN-γ (**D**), Granzyme B (**E**), IL-12p70 **(F)**, TNF-α **(G)**, and IL-18 **(H)** were measured by ELISA. The data show the mean ± SD. Statistical analysis between groups were performed by One-way ANOVA and Tukey's multiple comparison test. **; p< 0.0021, ***; p<0.0002 and ****; p<0.0001.

Mature DCs by adjuvant treatment could trigger NK activation *in vitro*

To address whether bone marrow-derived DC (BM-DC) treated with either MPL alone, Poly I:C alone or combination of MPL + Poly I:C influence on activation of

NK cell and cytokine productions, we directly cultured adjuvant-pretreated BM-DCs with splenic NK cell isolated from naïve mice and tested NK activation marker by flow cytometry, and estimated IFN- γ and Granzyme B production of NK cell by ELISA. Correspondence to the results of activated NK induced-DC maturation in vitro, either MPL or MPL + Poly I:C pretreated DCs were able to strongly heighten NK activation expressing CD69 marker (Figure 7A), and secreting IFN-gamma (Figure 7B) and Granzyme B (Figure 7C) comparing to control or Poly I:C pretreated-DCs. Interestingly, treatment of Poly I:C alone showed a significantly higher effective to NK activation in vivo than other treatments excepting MPL + Poly I:C, but not in vitro. In contrast, MPL appeared to be more effective in activating NK cell and DC maturation in vitro treatment rather than in vivo. These data suggested that the combination of MPL + Poly I:C was more efficient to strengthen NK-DC interaction not only in vitro but also in vivo rather than the other individual adjuvant treatments.

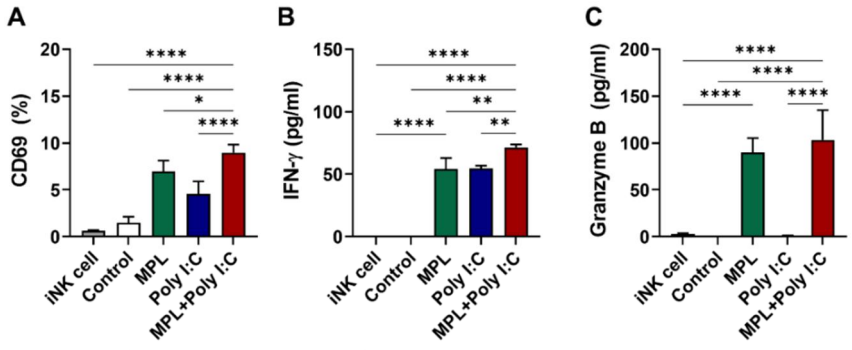


Figure 7. *In vitro* activation of immature NK cell from naïve mice by adjuvant pre-treated matured DCs. BMDCs were pre-activated by MPL, Poly I:C, or MPL + Poly I:C for 2 days, and then co-cultured with NK cell isolated from spleen cells of naïve C57BL/6 mice for 2 days. CD69 NK activation marker expressions were analyzed by flow cytometry **(A)** and IFN-γ **(B)** and Granzyme B **(C)** levels in culture supernatant were determined by ELISA. The data show the mean ± SD. Statistical analysis between groups were performed by One-way ANOVA and Tukey's multiple comparison test. *; 0.0332, **; 0.0021, and ****; 0.0001.

Discussion

Despite of the continuous development of vaccine, erasing viral infectious diseases meet vaccine efficacy problems because of the uninterrupted viral mutation and generation of antigenically differences, and safe concern as well, as some of vaccine such as pneumococcal polysaccharide vaccine (PS23) causes systemic inflammatory reaction after vaccination [27]. Therefore, the improvements of vaccine efficacy and heterosubtypic cross-protection of vaccine without causing chronic inflammation and easing side effects are highly priority.

The successful of eradicating invading pathogens depends on the corporation of the innate and adaptive immunity. NK cells were reported an ability to directly kill infected cells, subsequently collaborate with other innate immune cells, leading to indirectly modulation adaptive immune responses. Toll-like receptors are pattern-recognition receptors responsible for directly or indirectly triggering NK activation. MPL and Poly I:C are TLR4-agoinist and TLR3-agonist, respectively, which were separately demonstrated as the potential adjuvants to enhance the efficacy of vaccines. Therein, TLR4 agonist MPL, which is a licensed adjuvant, has been approved for used in several types of vaccines at high dose range (5-100 μg) [21]. TLR4 agonist Poly I:C was reported as a promised cancer vaccine adjuvant as well as a considered adjuvant for vaccines targeting cellular immune response [19].

In this study, we have formed addressing to hypothesis of whether combination of MPL and Poly I:C might be highly efficient to acceleration of immune responses specifically NK cell responses even at low dose rather than either MPL or Poly I:C adjuvant alone at early time points (24 h post immunization). As a result, the combined adjuvant of MPL+Poly I:C was highly capable of eliciting NK cell secreting cytokines and activating cytotoxic function, additionally strengthening

the interaction of NK cells and DCs leading to stimulation furthering B cell producing antibody and administering T cell polarization via cytokine IL-15 and IL-12p40 productions, comparing to the individual adjuvant or OVA alone.

Poly I:C and the combination of MPL + Poly I:C groups, meanwhile, showed a higher effect to stimulating NK cell recruiting to the site of vaccination than others at early time point of immunizations, MPL indicated a stimulation of NK recruitment to lung only at day 1 post boost immunization. Additionally, the adjuvants combination had also a high capability to trigger NK maturation expressing high level of CD11b. As demonstrated by fore-researches, differentiated NK cell phenotypes indicated different functional potentials, respectively. Double-positive and $CD27^{low}$ NK cell subset appears a greater expression of effector function, responsiveness chemokine, proliferation capacity and cytokine production, whereas $CD27^{high}$ display a high rate of proliferative capacities [25, 28, 29]. Indeed, MPL + Poly I:C exhibited a significant level of CD69 and CD107a NK activation markers at early time points (Figure 2I and K), resemblance with the high frequency of CD11b expression on NK cells (Figure 2C and D). Instead of indicating a relentlessly increase in the expressions of CD69 and CD107a on NK cell resembling to that in prime vaccination, however, NK cell-resided lung from Poly I:C-treated-mice showed an extremely decrease in the level of CD69 and CD107a appearing resemblance to OVA, OVA + MPL treatment or naïve mice at day 1 post boost vaccination (Figure 2J and 2L). The poor productions of CD69 and CD107a in individual adjuvant immunized groups at boost dose were correlated with the low frequency of NK cell differentiating into $CD11b^{high}CD27^{high}$ NK subsets from lung at the same analyzing time (Figure 2G). In addition, we found the correlation of the production of IFN-γ by NK cell and the level of

Granzyme B produced by lung-resided immune cell with the expression of CD27 and CD11b on NK cell. The combined adjuvant of MPL + Poly I:C mice showed the highest frequency of $CD27^{+/-}CD11b^{+}$NK cell at both prime and boost immunizations, while NK cell in Poly I:C mice group indicated a decrease in differentiating into $CD27^{+/-}CD11b^{+}$ at 24 h post second immunization. Interestingly, MPL presented the lowest level of $CD27^{+}CD11b^{+}$ similarly to either OVA or naïve mice at 24 h post immunizations, but not the population of $CD27^{-}CD11b^{+}$. Correspondence to the release of IFN- γ by NK cell which was the highest production in MPL + Poly I:C mice, following to the production in Poly I:C mice. Meanwhile, Poly I:C and MPL mice groups underwent an effective induction of Granzyme B secretion by immune cells in lung rather than others at day 1 post vaccination. Although MPL indicated a higher level of Granzyme B production than OVA alone and naïve mice, it was not significant. Collectively, we determined $CD27^{+}CD11b^{+}$NK cell might bias to the release of IFN-γ, whereas $CD27^{-}CD11b^{+}$ phenotype might induce the secretion of Granzyme B. Additionally, splenic NK cell in adjuvant-treated mice presented a high cytotoxic activity, especially Poly I:C and combination of MPL + Poly I:C. Consistently, Poly I:C and MPL + Poly I:C can highly induce NK cell isolated from spleen C57BL/6 mice lysis target cells *in vitro*. Further studies are need for better understanding the correspondence of NK cell phenotype with NK cell activation markers expression, NK cytokine productions and NK cytolytic activity.

Addition to the activation of NK cell to lysis target cell, NK cell also interacts with other immune cells for priming and inducing immune responses. The collaboration of NK cell and DC was presented as a crucial mechanism in stimulation of innate immune response and modulation of initial adaptive immunity via the release a

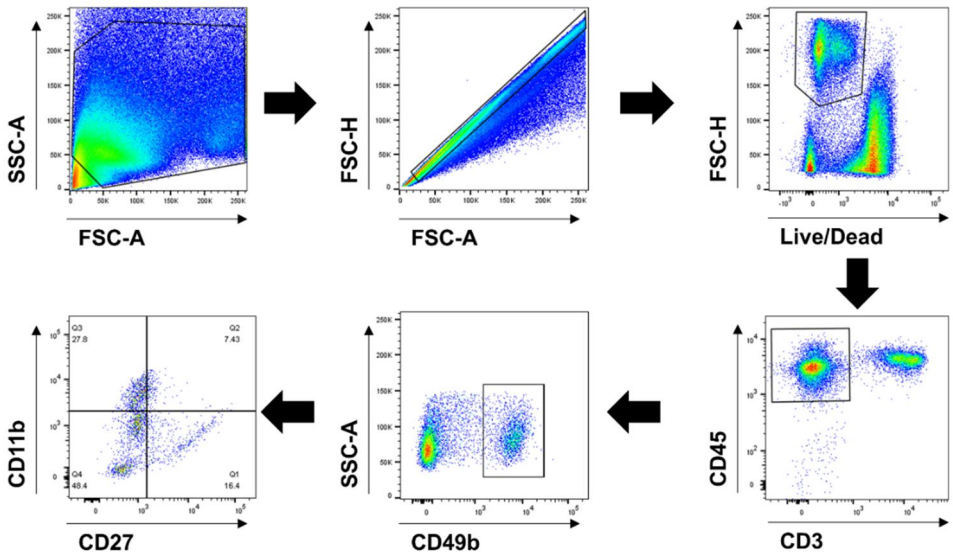
variety of immune-regulatory cytokines including IFN-γ, TNF- α, IL-12, IL-15 and IL-18 [6, 9]. According to the fore-studies, IFN-γ-producing NK cells regulates innate resistance by activating phagocytic cells and priming APCs for IL-12 family of heterodimeric cytokine production mainly IL-12p70 production by DCs which is a major Th1-driving cytokine [30], and optimal production of IL-12 requires. Otherwise, IL-15 was presented as a regulator of NK proliferation, a contributed factor for NK cell IFN-γ production, and a selective factor of memory $CD8^+$ T cells [31-33]. Besides, IL-18 was initially described as an IFN-γ-inducing factor and a potent immune-regulatory cytokine for NK cell activation and cytokine production [9, 10]. Moreover, the cooperation between IL-12 and IL-18 involved in activation and enhancement of the proliferation and cytotoxicity of NK cells [8]. Indeed, our results presented a highly collaboration between NK cell and DC in mice groups receiving vaccination of either Poly I:C or MPL + Poly I:C adjuvanted OVA. As expected, the combination of MPL + Poly I:C adjuvant was highly capable to induce NK cell secreting TNF-α (data not show) and IFN-γ (Figure 3A and D) for priming DC maturation producing the high level of IL-12p40 (which is known as an IL-12p70 heterodimeric subunit playing a critical role in the release of IL-12p70 [34]) and IL-15 at the early time post immunizations. MPL, by contrast, can stimulate the production of IL-12p40 but not IL-15 (Supplementary Figure 2 A, B, D, E). Meanwhile, Poly I:C show an effective to trigger immune cells producing TNF-α (data not show) and NK cell secreting IFN-γ which were essential cytokines for DC activation, but not the production of IL-12p40. Interestingly, whereas Poly I:C and the combined adjuvant indicated an ability to heighten the secretion of IL-15 which was correlated to the production of NK cell IFN-γ and population of NK cell locating at lung, the level of IL-18 measured in lung homogenates was

appeared to be not significantly different between the groups (Supplementary Figure 2C and F). The inconsistent results demonstrated that MPL might be effective in maturation DC but not NK cells, whereas Poly I:C was highly capable to NK cells rather MPL. As result of that, there was a correlation between NK cell and DCs, leading to activated NK cell was not only elicited by adjuvants but also DC maturation-adjuvants, and conversely.

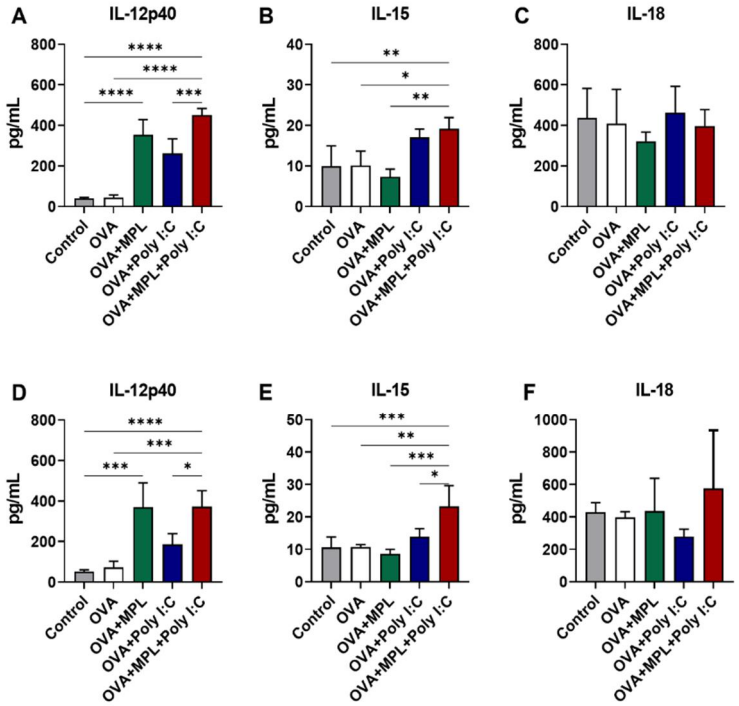
Otherwise, Poly I:C and MPL+ Poly I:C showed an effective induction of NK cell activation and cytokine production *in vivo*, we found the inconsistent results *in vitro*. The combination of MPL + Poly I:C and MPL alone revealed additive effects on activation of NK cell triggering DC maturation up-regulating the expression of MHC II high, CD40 and DC86 activation markers and also the production of IL-12p70 and IL-18, when we co-cultured adjuvant-activated NK cell with immature DC-derived bone marrow (Figure 6). Similarly, when co-culture of adjuvant-matured DC with splenic NK cell isolated from naïve C57BL/6 mice, the highest level of CD69 activation marker expressing on NK and the significant secretion of IFN-γ and Granzyme B was presented in the combined adjuvants of MPL + Poly I:C and MPL alone treatments (Figure 7).

Taken together, Poly I:C adjuvant was effective to NK activation and cytokine production *in vivo* (Figure 1-3), but not *in vitro*. In contrast, MPL appeared to be more effective in activating NK cell and DC maturation *in vitro* treatment rather than *in vivo*. According to the data, we confirmed that combination of MPL + Poly I:C adjuvants might be more effective to stimulation immune responses against pathogens and have broader applications. Also, it remains importantly to investigate the further signaling pathway triggered by MPL and Poly I:C with purpose of addressing to the inconsistent outcomes in *in vivo* and *in vitro*.

Supplementary data



Supplementary Figure 1. Flow cytometry gating strategy. Gating strategy of NK cells ($CD45^{+}CD3^{-}CD49b^{+}$) and NK cell subsets by CD27 and CD11b expressions.



Supplementary Figure 2. Induction of cytokine production after stimulation by MPL and Poly I:C in the lungs of C57BL/6 mice at prime and boost dose. Lung homogenates were collected at day 1 post prime (**A-C**) and boost (**D-F**) immunizations and the levels of IL-12p40, IL-15 and IL-18 were measured by ELISA. The data were show in mean ± SD (n=5). Statistical comparisons between groups were performed in One-way ANOVA and Tukey's multiple comparison test. *; 0.0332, **; 0.0021, ***; 0.0002 and ****; 0.0001.

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