

BASIC IMMUNOLOGY

Prophylactic Vaccination with Adjuvant Monophosphoryl Lipid A Prevents Th2-Mediated Murine Asthmatic Responses

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Objective. Asthma is a chronic respiratory disorder characterized by airway hyperreactivity, eosinophilic infiltration, high titer of allergen-specific IgE, and overproduction of T helper 2 (Th2) cytokines. Antigen combined with an appropriate adjuvant and administered through the proper route can elicit suitable immunological responses to protect humans and animals from diseases. Antigen formulated with monophosphoryl lipid A (MPLA) can produce priming of Th1-mediated immune responses. The purpose of this study was to examine the utility of MPLA as an adjuvant to prevent asthma. **Methods.** BALB/c mice were immunized with ovalbumin (OVA) formulated with or without MPLA by intraperitoneal, footpad, or subcutaneous injection. Vaccinated mice were challenged with OVA aerosol to estimate the protective efficacy of MPLA in comparison to Th2-adjuvant aluminum hydroxide (Alum). Airway hyperresponsiveness to methacholine, eosinophilia in bronchoalveolar lavage fluid (BALF), circulating titers of OVA-specific antibodies, and stimulating levels of IFN- γ and IL-4 cytokines from splenocytes were evaluated. **Results.** Mice immunized by all injection routes with OVA formulated with MPLA increased the ratio of Th1/Th2 responses compared to mice receiving antigen alone. For prophylactic vaccination purpose, MPLA reduced airway responsiveness and eosinophilic inflammation in the lung, decreased serum OVA-specific IgE level, and increased the serum ratio of OVA-specific IgG2a/IgG1 and the ratio of IFN- γ /IL4 from OVA-activated splenocytes compared with mice vaccinated with Alum. **Conclusion.** MPLA may be clinically useful in the vaccination of individuals predisposed to asthma.

Keywords airway inflammation, asthma, MPLA, prophylactic vaccination, Th1 responses

INTRODUCTION

The prevalence of allergic asthma has increased dramatically, particularly in developed countries, and has become a worldwide public health concern (1). Asthma is a chronic inflammatory disorder of the airways associated with enhanced T helper type 2 (Th2) responses to inhaled environmental allergens, such as cat dander, pollen, and house dust mite proteins (2). Th2 cells produce cytokines IL-4, IL-5, and IL-13, and have been shown to drive disease pathology in patients with asthma, producing airway hypersensitivity, pulmonary eosinophilia, and elevated serum allergen-specific IgE (3). The circulating levels of Th2 cytokines in acute asthmatic individuals are significantly increased compared with control or asymptomatic asthmatic subjects (4). The induction of antigen-specific Th1 responses has shown promising efficacy by balancing Th2-mediated allergic diseases (5). Novel preventive strategies capable of modulating Th2 responses might therefore be beneficial.

The treatment of allergic patients is complicated, as it attempts to overcome an already established sensitized response. To prevent the development of allergy, the use of prophylactic vaccination against allergen before first sensitization might be beneficial. This approach is potentially achievable based on the following foundations. There is growing acceptance of a genetic predisposition to allergy (6) and most allergic patients are susceptible to common allergens (7, 8). The field of molecular allergen characterization and recombinant allergens is rapidly progressing (9, 10), and a Th1-inducing adjuvant with US Food and Drug Administration approval is already clinically applicable (11, 12).

Monophosphoryl lipid A (MPLA) is a low inflammatory toxicity derivative of lipopolysaccharide (LPS) (13). Its adjuvant effects also require endotoxin receptor Toll-like receptor 4 (TLR4) to enhance antibody production and T cell priming (14, 15), and only induce strong Toll-interleukin 1 receptor domain-containing adapter inducing interferon- β (TRIF)-biased signaling instead of myeloid differentiation factor 88 (MyD88)-associated responses (16). TRIF-associated gene expression by MPLA correlates with strong interferon regulatory factor 3 activation but weak NF- κ B stimulation (17). Although much has

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been learned over the past decade about Th1-favored immunomodulation of MPLA, little is known about its adjuvant effect through different injection routes. Moreover, its efficacy in prophylactic vaccination against specific allergens has not been extensively evaluated in mice. In the present study, we demonstrate that MPLA enhances strong Th1 responses in mice immunized by various routes. Moreover, we report for the first time that mice prophylactically vaccinated with MPLA adjuvant formulation against chicken ovalbumin (OVA) were more resistant to elicit asthmatic responses when they were challenged with the same antigen.

METHODS

Animals

Four- or five-week-old female BALB/cByJNarl mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Mice were maintained and handled according to the guidelines set by the Animal Care Committee of Chang Gung University (Taiwan) and the US National Institute of Health Guide for the Care and Use of Laboratory Animals. Eight-week-old mice were used.

Immunization and Vaccination Protocols

MPLA, a derivative of lipid A from *Salmonella* Minnesota R595 LPS, was purchased from Invivogen (San Diego, CA, USA). Mice were immunized with emulsified solution of 50 µg chicken OVA (OVA, Sigma-Aldrich Inc., Saint Louis, MO, USA) without or with 50 µg MPLA, being mixed with an equal volume of incomplete Freund's adjuvant (IFA, Sigma-Aldrich Inc.) through intraperitoneal (IP), footpad (FP), or subcutaneous (SC) routes (day 0). After four weeks, the same dosage and formula of OVA was used for boost injection (day 28). Mice were sacrificed one week after boosting (day 35). Control mice were immunized with normal saline emulsified with IFA through each route. In the prophylactic vaccination model, five rounds of inhalation challenge (IH) with three-day intervals were performed one week after boosting through IP or FP routes. For challenging, mice inhaled 2% OVA in normal saline on day 35, 38, 41, 44, and 47 using an atomizer (Pulmo-aide, DeVilbiss Health Care, Somerset, PA, USA) for 20 minutes. Mice, sensitized by IP injection with 50 µg OVA emulsified with 0.8 mg aluminum hydroxide gel (Pierce Biotechnology Inc., Rockford, IL, USA) and subsequently challenged five times by OVA inhalation, were also included as sensitized control. Mice immunized with normal saline formulated with Alum or IFA were challenged with normal saline as nonsensitized control. Mice were analyzed for airway hyper-responsiveness on day 48 and sacrificed on day 49. None of the mice exhibited any overt signs of illness during the experiment.

Airway Hyper-Responsiveness

Airway hyper-responsiveness was measured on day 48 with inhalation of methacholine (Mch, Sigma-Aldrich Inc.),

which is known to induce airway constriction. Mice were first exposed to 0 mg/ml Mch and then to increasing concentrations of Mch (10, 20, 30, 40 mg/ml) for three minutes each. A transducer in each chamber detected the change of respiratory airflow of individual mice. Pulmonary airway obstruction was calculated based on the formulation $Penh = (Te/RT - 1) \times (PEF/PIF)$, where Penh is enhanced pause (dimensionless), Te is expiratory time, RT is relaxation time, PEF is peak expiratory flow (milliliters per second), and PIF is peak inspiratory flow (milliliters per second). Changes were analyzed by BioSystem XA software (all instruments from Buxco Electronics Inc., Wilmington, NC, USA).

Cells in Bronchoalveolar Lavage Fluid (BALF)

The lungs of the mice were lavaged three times with 1 ml of normal saline. BALF cells were centrifuged onto poly-L-lysine-coated slides at 800 rpm for 5 minutes in a Shandon Cytospin 4 (Thermo Shandon, Pittsburgh, PA, USA). Cells were then stained with LiuA dye for 30 seconds and LiuB dye for 90 seconds. Two hundred cells per slide were counted under a microscope (Olympus, Tokyo, Japan) and those with different morphology were noted.

Determination of OVA-Specific IgG1, IgG2a, and IgE

OVA-specific IgG1, IgG2a, and IgE antibody titers in sera were determined by ELISA as described previously with modifications (18). Alterations were the use of 3,3',5,5'-tetramethylbenzidine substrate solution (BD Biosciences) and 2N H₂SO₄ stopping solution. Antibody concentrations were determined according to standard curves.

Cytokine Production from OVA-Stimulated Spleen Cells

Spleen cells were stimulated with 100 µg/ml OVA at a density of 5×10^6 cells/ml in RPMI 1640 medium (Gibco-BRL) with 10% fetal calf serum for 6 days in vitro. Concentrations of IL-4 and IFN-γ in culture supernatants were determined with ELISA kits specific to each cytokine (R&D Systems, Minneapolis, MN, USA). Cytokine concentrations were calculated based on the standards run in parallel with recombinant cytokines. The limits of detection were 15.6 and 31.3 pg/ml for IL-4 and IFN-γ, respectively.

Statistical Analysis

Data were analyzed using the Mann-Whitney test. Results are expressed as mean ± standard error of the mean (SEM). *P* values of < .05 were considered statistically significant.

RESULTS

OVA-Immunization with MPLA Enhances Th1 Immune Responses

The purpose of prophylactic vaccination is to elicit suitable immunity to protect individuals from disease by using proper adjuvant and the appropriate administration route.

To determine which injection routes obtained the best results, mice were immunized with either OVA or OVA/MPLA emulsified with IFA through IP, FP, and SC routes. IFA was used as emulsifying agent because of the advanced Th1 predisposition when MPLA is associated with oil formulation (19). One week after boosting, OVA-specific humoral and cellular responses were determined. As shown in Figure 1A, the titers of OVA-specific IgG2a, a Th1 cytokine-regulated isotype, were greatly enhanced in mice immunized with MPLA formula by all injection routes. Conversely, Th2-related OVA-specific IgG1 and IgE were significantly down regulated in mice injected with MPLA (Figures 1B and 1C). In addition, the amount of IFN- γ in the OVA-stimulated splenocyte supernatant was significantly higher in mice injected with MPLA by the FP route (Figure 1D). Mice immunized with normal saline emulsified with IFA through each route were also included; however, OVA-specific antibodies and cytokines in the

OVA-stimulated splenocyte supernatant were not detected (data not shown). Collectively, MPLA adjuvant proved to augment the Th1 response by stimulating OVA-specific IgG2a and IFN- γ production.

It has been generally believed that MPLA is a Th1-inducing TLR4 agonist. In line with this, the emulsion formula with MPLA induced higher IgG2a/IgG1 antibody and IFN- γ /IL-4 cytokine ratios than the formulation without the TLR4 agonist, especially through the IP and FP routes (Figure 2). In conclusion, Th1-bias responses are detected in mice immunized with Ag + MPLA adjuvant. The IP and FP routes might be applied to vaccinate the mice due to the lower titer of OVA-IgE and notable enhancement of Th1 responses.

MPLA Adjuvant Reduces Pulmonary Inflammation

To evaluate the preventive effect of MPLA-formulated vaccine against asthma, mice were vaccinated with the

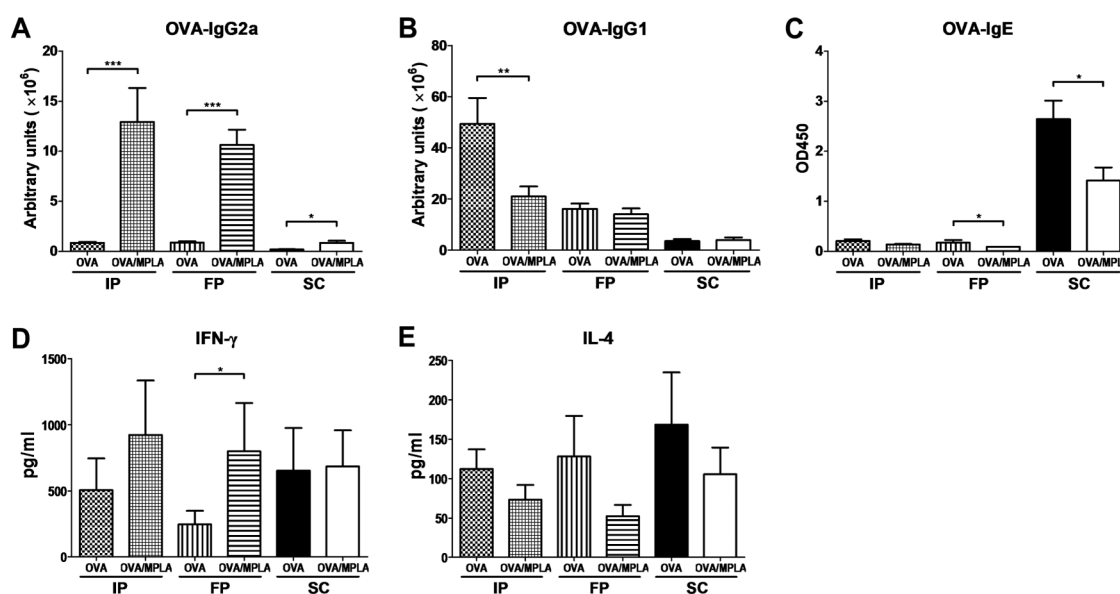


FIGURE 1.—Strong Th1 responses (OVA-IgG2a titer and IFN- γ production) were induced, but Th2 responses (OVA-IgG1 and OVA-IgE titers) were markedly reduced with MPLA compared to OVA control. The immune responses of BALB/c mice immunized through different routes were determined. Titers of OVA-specific IgG2a (A), IgG1 (B), and IgE (C) in sera and the levels of IFN- γ (D) and IL-4 (E) in culture supernatants, in which spleen cells were stimulated by 100 μ g/ml OVA for 6 days, were measured by ELISA. Data are represented as mean $\pm$ SEM from four independent experiments ($n = 11$ –12 mice per group). $p < .05$; ** $p < .01$; *** $p < .001$.

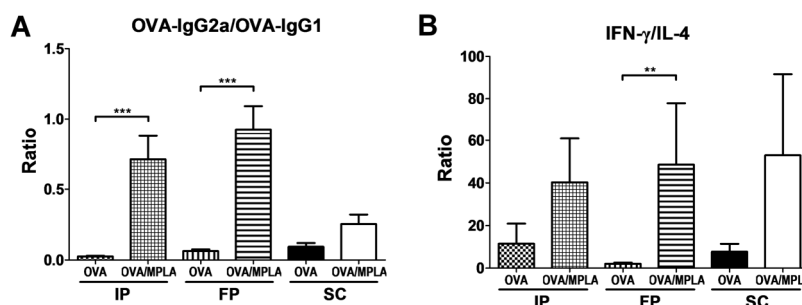


FIGURE 2.—The ratios of Th1/Th2 immunoglobulin and cytokine were largely increased in mice immunized with OVA/MPLA formulation by IP and FP injection compared to OVA controls. (A) OVA-IgG2a vs. OVA-IgG1, (B) IFN- γ vs. IL-4. Data are represented as mean $\pm$ SEM of four independent experiments ($n = 11$ –12 per group). $p < .05$; ** $p < .01$; *** $p < .001$.

same protocol of immunization through IP or FP routes and challenged with OVA aerosol. SC route was excluded in following experiments because high level of OVA-IgE was inappropriate for preventing asthma development. As bronchial asthma is driven by an elevated Th2 response, a strong Th2 adjuvant Alum was used as a parallel control. Mice immunized and challenged with normal saline were included as nonsensitized control. Of note, mean airway hyper-reactivity of mice receiving the OVA/MPLA formulation by IP or FP was significantly decreased than that of the OVA/Alum control group (Figure 3A). The data in Figure 3A also indicate that OVA/MPLA formulation by FP route significantly reduced the Penh value at 20 mg/ml of methacholine challenge compared with OVA-alone group. Consistent with a Th2-inducing adjuvant, the percentage (Figure 3B) and number (Figure 3C) of eosinophils in BALF were much higher in OVA/Alum control mice. However, all IFA-vaccinated mice showed lower extent of eosinophil infiltration (Figures 3B and 3C).

Pulmonary eosinophilia was not observed in all nonsensitized mice (data not shown). Taken together, these results indicate that MPLA-modified vaccine may prevent pulmonary inflammation.

Th1/Th2 Ratio is Significantly Elevated in Mice Vaccinated with OVA/MPLA

To verify whether reduced eosinophilic airway inflammation was paralleled with reduced Th2 responses in mice vaccinated with MPLA adjuvant, we next investigated the OVA-specific Ab and cytokine responses. The levels of OVA-specific antibodies and cytokines were undetectable in nonsensitized control group (data not shown). Levels of OVA-specific IgG2a were notably increased in mice prophylactically vaccinated with MPLA compared to control mice either lacking MPLA or injected with Alum (Figure 4A). OVA-specific IgE, a major mediator in the allergic response, was up-regulated in mice injected with the Alum formula; however, IgE levels in all IFA-vaccinated mice were near the background level

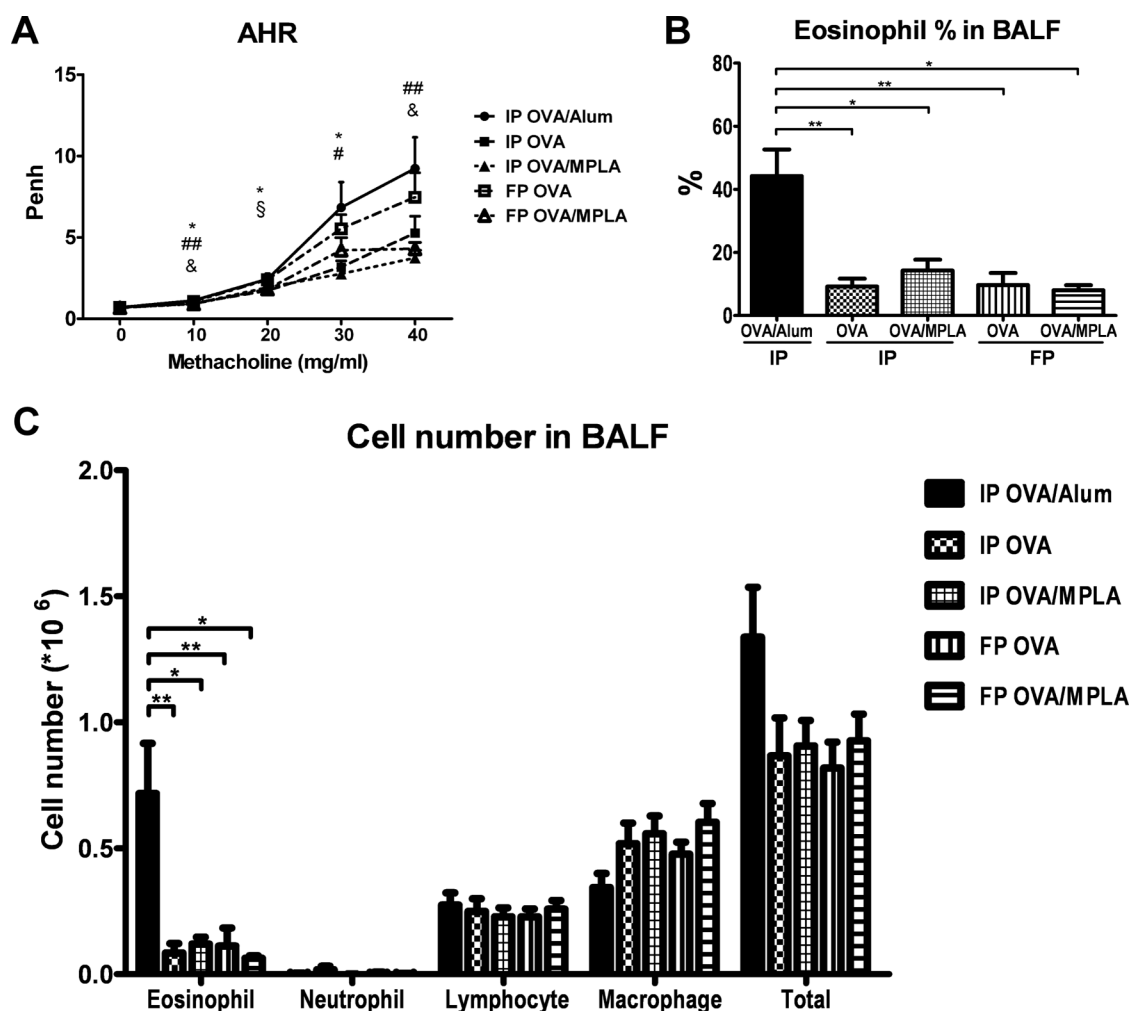


FIGURE 3.—Airway inflammatory responses were reduced in mice vaccinated with MPLA adjuvant. (A) Airway hyperactivity was lower in mice treated with OVA/MPLA formula compared to control mice. Mice were stimulated by increasing concentrations of methacholine, and Enhanced pause (Penh) value was obtained by whole body plethysmography (Buxco). IP OVA/Alum vs IP OVA: $*p < .05$; IP OVA/Alum vs IP OVA/MPLA: $\#p < .05$, $\#\#p < .01$; IP OVA/Alum vs FP OVA/MPLA: $\&p < .05$; FP OVA vs FP OVA/MPLA: $\$p < .05$. Cells in bronchoalveolar lavage fluid were stained by Liu's stain, and the percentage of eosinophils (B) and the number of leukocytes (C) in BALF was calculated. Data are represented as mean $\pm$ SEM ($n = 15$ – 16 mice per group) from five experiments. $*p < .05$; $**p < .01$.

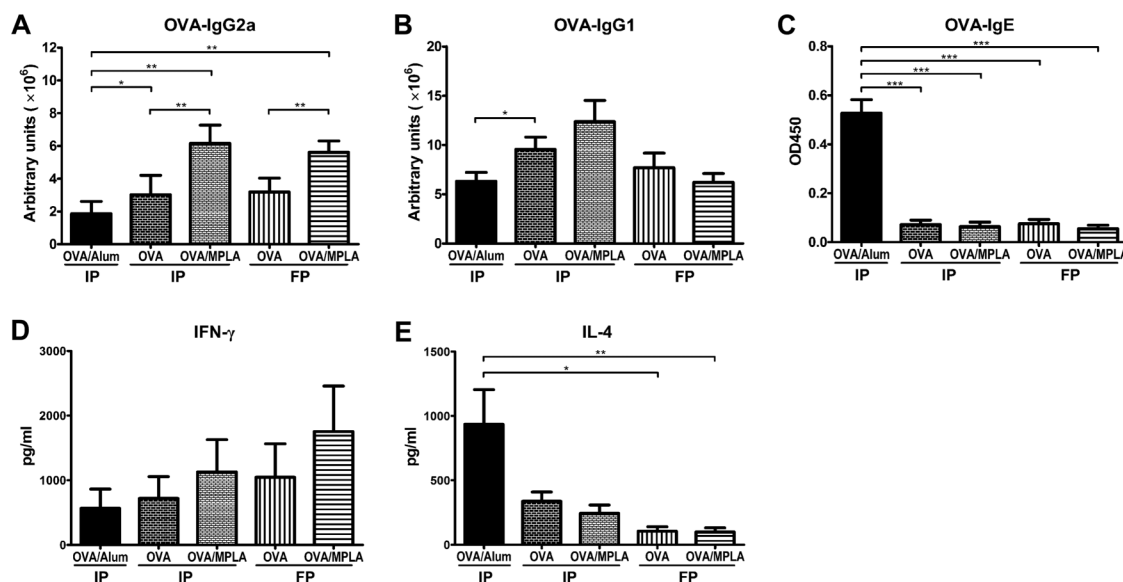


FIGURE 4.—Th2 responses were controlled by MPLA through IP or FP. The immune responses of challenged BALB/c mice immunized with OVA/MPLA formula through IP or FP routes were determined. The levels of OVA-IgG2a (A), OVA-IgG1 (B), OVA-IgE (C) in sera, IFN- γ (D), and IL-4 (E) in culture supernatants were determined by ELISA. Data are represented as mean $\pm$ SEM ($n = 15-16$ mice per group) from five experiments. * $p < .05$; ** $p < .01$; *** $p < .001$.

(Figure 4C). IL-4 has been shown to induce the production of IgE (20). Consistent with this notion, higher IL-4 production was detected in OVA-stimulated spleen cells from OVA/Alum control mice, in contrast to mice vaccinated with the IFA formulation (Figure 4E). The levels of OVA-specific IgG1 in sera and IFN- γ from OVA-stimulated spleen cells were comparable between groups (Figures 4B and 4D).

The ratios of IgG2a/IgG1 antibody and IFN- γ /IL-4 cytokine were analyzed to evaluate Th1/Th2 immune responses. The ratios of IgG2a/IgG1 antibody in the OVA/MPLA group were approximately two- and three-fold increased compared to the OVA control group injected by IP and FP routes, respectively (Figure 5A). Moreover, these increases were three- and six-fold than that of the OVA/Alum control group. Vaccination with the minimally toxic TLR4 agonist also augmented higher IFN- γ /IL-4 cytokine ratios than a formulation with Alum adjuvant (Figure 5B). Collectively, the data demonstrate that mice preventively inoculated Ag with MPLA adjuvant

are less susceptible to allergic asthma when mice are re-challenged with identical Ag. This can be a direct result of increased Th1 phenotype in vaccinated mice.

DISCUSSION

Our findings show that MPLA can act as an adjuvant to augment Ag-specific Th1 responses through different injection routes. Notably, we found that the development of airway hyper-responsiveness and Th2-related humoral and cellular responses were inhibited in mice treated with the MPLA-modulated vaccine prior to allergen challenge. IFA induces a predominantly Th2-based response, and its adjuvant effects are modulated by the intracellular pattern recognition receptor NOD2 (21). Indeed, mice immunized with OVA/IFA emulsion without MPLA produced high amounts of Th2-affiliated isotypes IgG1 or IgE, especially when treated via SC injection; however, both the titers were decreased in the presence of MPLA. The lower IgE

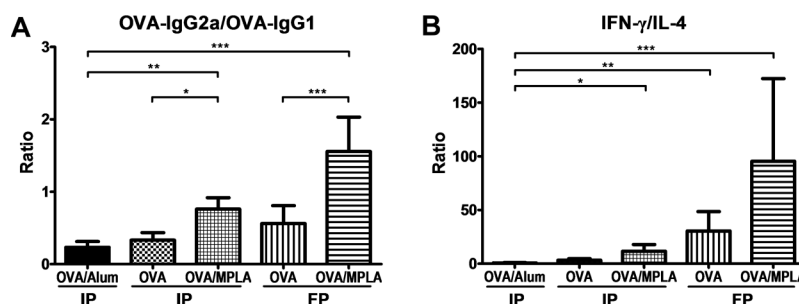


FIGURE 5.—The ratios of Th1/Th2 immunoglobulin and cytokine were greatly increased in mice prophylactically vaccinated with OVA/MPLA compared to its OVA control or OVA/Alum-sensitized control. OVA-IgG2a/OVA-IgG1 (A) and IFN- γ /IL-4 (B) ratios were calculated and depicted. Data are represented as mean $\pm$ SEM of five independent experiments ($n = 15-16$ per group). * $p < .05$; ** $p < .01$; *** $p < .001$.

and higher IgG2a responses induced by MPLA from IP and FP routes provide evidence for the use of these inoculation sites of vaccination against allergy.

TLRs have recently been described as molecules linking innate and adaptive immunity (22). Little is known about the molecular mechanisms through which MPLA-induced TLR4 signaling promotes a predisposition towards Th1 immunity. Using genetic knockout mice, it has been demonstrated that LPS impairs the induction of Th2 immunity via TLR4- and MyD88-dependent signaling and by the Th1 cytokines IL-12 and IFN- γ , but not through a TRIF-dependent pathway (23). However, Mata-Haro and colleagues showed that TRIF-biased activation of TLR4 by MPLA is responsible for its adjuvant effects, and MyD88 is completely non-essential for early T cell priming (16). As shown in our data, one possible explanation for the protective mechanism of MPLA in allergic asthma might be the induction of IFN- γ via TLR4 signaling, with IFN- γ subsequently augmenting IgG2a production, inhibiting IgE synthesis, and reducing pulmonary eosinophilia.

In an attempt to cure allergic symptoms, allergen-specific immunotherapy (SIT) currently represents the primary clinical approach for the treatment of IgE-mediated allergic diseases (24, 25). Patients receive repeated administration of increasing doses of allergen extracts with adjuvant to modulate established allergic responses. SIT-induced immunologic changes include the generation of allergen-specific IgG antibodies, mainly IgG4, a decrease in specific IgE titers, and the induction of peripheral T cell tolerance (5, 26). However, the risk of SIT-induced adverse side effects, especially life-threatening anaphylactic shock and substantial cost, limit its extensive application. In addition, the fact that an already mounted immune response in allergic patients is difficult to cure completely by SIT, highlights the potential utility of a prophylactic vaccine against allergy.

Instead of preventive efficacy, vaccination with recombinant allergen might raise a concern about allergy induction in inoculated individuals. On the basis of recombinant DNA technology and protein engineering, recombinant allergens can be manipulated to modify their allergic portion and to retain epitopes for induction of immune responses (27, 28). In addition, the current experience with vaccination against infectious microorganisms in infancy rarely induces allergic sensitization. Taken together, it might be possible to vaccinate children with a genetic predisposition for developing allergies with hypoallergenic allergen derivatives.

It is well accepted that allergy is associated with the genetic factors; however, its increase in prevalence with time indicate that environmental factors are also involved in allergy induction. Gene-environment interactions have become a key subject of interest in the area of allergic diseases during the last decade, and are likely a critical determinant of allergy susceptibility (29). Pattern-recognition receptors, which can sense environmental factors, play an important role in these interactions (30, 31). The TLR4-agonist LPS, a cell wall component of gram-

negative bacteria, can be readily detected in home dust (32). Early LPS exposure may protect school-age children against the development of allergic diseases (33). High dose of LPS induces a Th1-associated response to control the murine pulmonary inflammation (34). TLR4 mutations lead to decreased NF- κ B activity as MPLA-induced signals were less responsive to LPS (35) and individuals with mutant allele are protected from allergy development at high levels of LPS exposure (36). In addition, new vaccine adjuvant, 3-O-desacyl-4'-monophosphoryl lipid A, is already used in humans, and the safety profiles and potential risks of its clinical application have been extensively evaluated (37). Unwanted adverse side effects, most importantly the induction of allergic sensitization, have not been reported. These findings provide support for utilizing MPLA-modified vaccine to predisposed persons against allergic diseases before allergen sensitization.

CONCLUSIONS/KEY FINDINGS

Our results indicate that MPLA as an adjuvant enhances Ag-specific Th1 responses to inhibit the development of Th2-mediated allergic responses in mice. MPLA-modulated prophylactic vaccine with common allergen might induce high levels of allergen-specific IgG antibodies, and hence probably prevent sensitization and allergen-specific IgE production by natural allergen exposure.

ACKNOWLEDGMENTS

We would like to thank Yu-Pei Chang for technical assistance.

DECLARATION OF INTEREST

All authors declare no conflicts of interest.

This study was supported by grants from National Science Council (NSC98-2320-B-182-020-MY3 and NSC98-2320-B-182-030-MY3) and Chang Gung Memorial Hospital (CMRPD190512 and CMRPG381552), Taiwan.

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