

# Improvement of Th1/Th2 and Th1/Treg Imbalances by Adjuvants CPG, MPLA and BCG in a Model of Acute Asthma Induced By Allergen Derp2 in BALB/c Mice

Vahid Mohammadi-Shahrokhi,<sup>1</sup> Abbas Rezaei,<sup>1</sup> Alireza Andalib,<sup>1</sup> Amir Rahnama,<sup>2</sup> Abdollah

Jafarzadeh,<sup>3,\*</sup> and Nahid Eskandari<sup>1,\*</sup>

<sup>1</sup>Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

<sup>2</sup>Department of Pathology, School of Medicine, Rafsanjan University of Medical Sciences Rafsanjan, Iran

<sup>3</sup>Department of Immunology, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran

\*Corresponding authors: Abdollah Jafarzadeh, Department of Immunology, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran. Tel: +98-3431315000, Fax: +98-343425209, E-mail: jafarzadeh14@yahoo.com; Nahid Eskandari, Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran. Tel: +98-9135697936, Fax: +98-2122066492, E-mail: nesandari@med.mui.ac.ir

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## Abstract

**Background:** The imbalance in the T helper (Th) 1/Th2 and Th1/T regulatory (Treg) cells-related immune responses plays a key role in pathogenesis of allergic asthma.

**Objectives:** The aim of this study was to evaluate the effects of CPG-Oligodeoxynucleotide (CPG-ODN), Monophosphoryl Lipid A (MPLA) and Bacillus Calmette-Guerin (BCG) on the ratio of Th1/Th2 and Th1/Treg cells-related parameters in an animal model of asthma.

**Methods:** This was an experimental study in which female BALB/c mice were divided to five groups and then immunized Subcutaneously (SC) with the allergen Dermatophagoides pteronyssinus 2 (Derp2) on days 1, 15 and 22. Three groups of mice were considered as test groups and pre-treated SC with CPG, CPG + MPLA or CPG + BCG on days 0, 14 and 21. Two groups were also considered as saline-control group and Derp2-sensitized control group that were administrated only saline or allergen Derp2, respectively. The mice (except saline-control group) were challenged intranasally with allergen Derp2 for ten days, from days 28 to 37 of post immunization. Blood samples were obtained from retro-orbital sinus, on days 0, 23 and 40. The serum Interleukin (IL)-4, Interferon (IFN)- $\gamma$ , Immunoglobulin (Ig) E and IgG2a levels were measured using the enzyme linked immunosorbent assay (ELISA) technique. The blood count of Th1- and Treg cells was detected using flow cytometry.

**Results:** At sensitization phase, the serum IFN- $\gamma$ /IL4 ratio was significantly increased in Derp2-sensitized group pre-treated with CPG plus MPLA or CPG plus BCG as compared with Derp2-sensitized control mice ( $P < 0.002$  and  $P < 0.003$ ). The IFN- $\gamma$ /IL4 ratio was also markedly elevated in Derp2-sensitized group pre-treated with CPG as compared with Derp2-sensitized control mice ( $P = 0.07$ ). In the challenge phase, the IFN- $\gamma$ /IL4, IgG2a/IgE and Th1/Treg ratios in Derp2-sensitized mice pre-treated with CPG plus MPLA were significantly higher than in Derp2-sensitized control mice ( $P < 0.003$ ,  $P < 0.006$  and  $P < 0.003$ , respectively). The IFN- $\gamma$ /IL4 and Th1/Treg ratios were also significantly raised in Derp2-sensitized mice pre-treated with CPG plus BCG in comparison with Derp2-sensitized control mice ( $P < 0.001$  and  $P = 0.012$ , respectively). In the challenge phase the Th1/Treg ratio was significantly decreased in Derp2-sensitized group pre-treated with CPG plus BCG in comparison with Derp2-sensitized mice pre-treated with CPG ( $P = 0.01$ ). The Th1/Treg ratio in Derp2-sensitized group pre-treated with CPG plus MPLA was significantly higher than in Derp2-sensitized mice pre-treated with CPG plus BCG ( $P < 0.035$ ).

**Conclusions:** These results showed that BCG, MPLA and CPG improve the Th1/Th2- and Th1/Treg cells imbalances in a mouse model of asthma.

**Keywords:** Asthma, Bacillus Calmette-Guerin, Monophosphoryl Lipid A, CpG Oligodeoxynucleotide, Interferon Gamma, Immunoglobulin G2a, Immunoglobulin E, Interleukin-4

## 1. Background

The prevalence of asthma has increased significantly over the past three decades and about 300 million people have this disease worldwide (1). Immunologically, asthma is characterized by a T helper (Th) 2 profile inflammation with an excess of eosinophils, mast cells, and Th2 lympho-

cytes. The mediators are produced by inflammatory cells, which then cause bronchoconstriction, secretion of mucus and probably air way remodeling (2). In the US, more than half of the asthma patients were dependent on atopy with a strong genetic background. However, atopy is related with either genetic or environmental factors. Accord-

ingly, immunotherapy, medication or reduction in allergen and other environmental exposures have beneficial effects that reduce the symptoms of asthma (3).

House dust mites (HDM; *Dermatophagoides* sp.) are the most popular aeroallergens in the world that induce specific immunoglobulin (Ig) E in up to 85% of asthmatic cases. Recently, there has been a shift from the use of allergen ovalbumin to use of the HDM extract in experimentally murine models of asthma (4). The HDM allergens were arranged within 24 groups, according to their molecular structure and activity (5). However, allergens Derp 1 and Derp 2 are the most important components of HDM (6). The cross-talk between innate elements (such as Toll like receptors (TLRs)) and adaptive immune receptors (including Fc epsilon receptor I (FcεRI)) on dendritic cells (DCs) are illustrated by counter-regulatory mechanisms such that one regulates the other (7).

Allergens such as *Dermatophagoides pteronyssinus* 2 (Derp2) are the activator of T helper 2 (Th2) lymphocytes that produce cytokines such as Interleukin (IL)-4, IL-5 and IL-13 (7). Interleukin-4 is the most important cytokine, which contributes in the development of asthma through several mechanisms such as IgE isotype switching and also induction of mucus secretion in the lung (8). On the other hand, investigations revealed that Th1- and T regulatory (Treg) cell-related cytokines play key roles in the modulation of Th2 cells (9). Furthermore, Th1- and Treg cells utilize several mechanisms to regulate the Th2 cell-related immune responses such as secretion of interferon gamma (IFN-γ) and tumor growth factor (TGF)-β (9). There are some reports showing the imbalances in the Th1/Th2, Treg/Th1 and Treg/Th2-related immune responses in asthmatic patients. Therefore, improvement of the imbalances in the Th1/Th2, Treg/Th1 and Treg/Th2 cells have been considered as suitable immunotherapeutic strategies for treatment of asthma. Due to the strong immunomodulatory effects of TLR agonists, these adjuvants were considered as potent inducers of Th1- and/or Treg cells.

The adjuvant CpG-Oligodeoxynucleotide (CpG-ODN) acts as a synthetic TLR9 that activates both innate and adaptive immunity, induces the tumor necrosis factor (TNF)-α secretion by macrophages, increases the secretion of Th1 type cytokines (IL-12, IFN-γ), and induces the production of pro-inflammatory IL-1, IL-6, IL-18 and TNF-α by B cells, monocytes, macrophages and DCs (10). It was demonstrated that the intra-dermal injection of ragweed pollen together with CpG improved the allergic symptoms of the lungs in murine asthmatic disease (11).

Monophosphoryl Lipid A (MPLA) is a derivative of Lipopolysaccharide (LPS) and acts as a TLR4 agonist and a potent inducer of Th1 cells (12). It enhances the serum levels of IgG1 and IgG2a, increases the expression of MHC and

the co-stimulatory molecules on the surface of antigen presenting cells (APCs) and therefore increases the stimulatory characteristics of APCs (13, 14).

The attenuated *Mycobacterium bovis*, Bacillus Calmette-Guerin (BCG), is a current tuberculosis vaccine that acts as a TLR2 and possibly TLR4 agonist. Ligation of TLR2 and TLR4 via BCG causes macrophages activation and secretion of high amount of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (15). However, BCG also increases the number of Treg lymphocytes in human and animal models and induces the secretion of IL-10 from these cells (16, 17).

As mentioned above, the stimulatory effects of MPLA, CPG-ODN and BCG on the Th1 and/or Treg cells have been demonstrated (18, 19). Therefore, it seems logical that these adjuvants can be used for modulation of immune responses in asthmatic patients. The anti-inflammatory and immunomodulatory effects of MPLA, CPG-ODN and BCG have been investigated separately in animal models of asthma that were usually induced by using ovalbumin as an allergen (20-22). Based on previous investigations, asthma is the main prevalent disorder, which can be prevented by vaccination (23-25), hence, it seems that using suitable adjuvants in combination with appropriate human asthma-inducing allergens can be useful for preventing asthma in a sensitive population. Accordingly, it seems that several investigations on animal models are critically needed to direct the protocols for use in humans. Hence, this study was conducted to evaluate the effects of CPG separately or in combination with MPLA or BCG on the ratios of Th1/Th2 and Treg/Th1 cell-related parameters including IFN-γ/IL-4, IgG2a/IgE and Th1/Treg in an asthma model, which was induced by allergen Derp2 during the early life of BALB/c mice.

## 2. Methods

### 2.1. Mice

The female BALB/c mice (four weeks old) were obtained from Kerman University of Medical Sciences (Kerman, Iran). The mice were housed under standard controlled conditions: temperature  $23 \pm 1^\circ\text{C}$ , humidity  $55 \pm 5\%$  and a 12-hour light/12-hour dark cycle with ad libitum access to normal laboratory mouse food and water. All mice were maintained in a room where the research protocol was performed so as to reduce any stress reaction possibly caused by novel environmental cues. Six to ten animals were allocated to each groups based on previous investigations (1, 20, 26). All experiments were carried out in accordance with the ethics committee on animal experimentation of Rafsanjan University of Medical Sciences and also in

agreement with the national research council guide (NRC, 2011).

## 2.2. Reagents

The used reagents were purchased from the following manufactures: Recombinant Derp2 from IN-DOOR biotechnologies (Cardiff, UK), BCG from Pasteur institute (Tehran, Iran), CPG 1826 ODN vacciGrade (5-TCCATGACGTTCTGACGTT-3) and MPLA-SM VacciGrade (a derivative of the lipid A from Salmonella Minnesota) from InvivoGen (San Diego, CA92121, USA).

## 2.3. Planning of the Research

The mice were randomly classified to five groups (6 - 10 mice in each group (1, 20, 26)) as follows: two groups were considered as saline-administrated control group and Derp2-sensitized control group without treatment with adjuvants, while three groups were considered as Derp2-sensitized group that were treated with CPG, CPG plus MPLA or CPG plus BCG.

## 2.4. Immunization and Challenge Phases

In this experimental study, the mice were divided to five groups by simple randomization. The mice were anesthetized with 4% isoflurane gas at an airflow rate of 3 L/minute in the sensitization phase and then immunized by Subcutaneous injection of 2  $\mu$ g Derp2 (in a total volume of 50  $\mu$ L) on days 1, 15 and 22 (27).

Two groups of mice were regarded as control groups, which were injected subcutaneously (SC) with only 2  $\mu$ g Derp2 or 50  $\mu$ L of normal saline on days 1, 15 and 22.

Three groups were considered as pre-treated mice that were administrated adjuvants one day before sensitization with allergen Derp2. The mice were primarily pre-treated with CPG, CPG + MPLA or CPG + BCG by SC injection on days 0, 14 and 21.

The pre-treated mice were injected 20  $\mu$ g CPG, "20  $\mu$ g CPG + 20  $\mu$ g MPLA" and "20  $\mu$ g CPG +  $4 \times 10^5$  CFU of BCG" on days 0, 14 and 21 and were then immunized with allergen Derp2 one day later on days 1, 15 and 22.

For challenge exposure, the mice were administrated Intranasally (IN) with 1.5  $\mu$ g Derp2 (in a total volume of 30  $\mu$ L, 7.5  $\mu$ L/nosril, two times within five minutes) daily from days 28 to 37. Eventually, the mice were sacrificed 72 hours later last challenge exposure on day 40 (28, 29).

Fifty percent of the mice in the saline-sensitized group were challenged again with saline and regarded as a normal control group without pre-treatment with adjuvants. The remaining in the saline-sensitized group were challenged with Derp2, at the mentioned time points without pre-treatment with the mentioned adjuvants [Tables 1 and 2] [Figure 1(AA)].

## 2.5. Blood Sampling

Blood samples were collected three times on day 0 (before beginning of pre-treatment program), day 23 (24 hours after the last immunization) and eventually 72 hours after termination of allergen challenge, on day 40. The mice were anesthetized with isoflurane gas 4% (at a flow rate of 3 L/minute) and the blood samples were collected from the retro-orbital sinus (30). The blood specimens were incubated at room temperature for 30 minutes and then centrifuged at 14000 rpm for 20 minutes. The serum specimens were isolated and kept at -80°C until use. The concentrations of cytokine (IL-4 and IFN- $\gamma$ ) and immunoglobulins (IgE and IgG2a) were determined using the standard enzyme linked immunosorbent assay (ELISA) (31). The cell pellets were obtained for determination of the blood count of Th1- and Treg cells by using standard flow cytometry method.

## 2.6. Measurement of the Serum Concentrations of Interleukin-4 and Interferon-Gamma

The mice were bled on days 0, 23 and 40, by the retro-orbital method and the serum concentrations of IL-4 and IFN- $\gamma$  were measured using commercial ELISA kits (Boster, Wuhan, China for IL-4 and Eastbiopharm, Hangzhou, China for IFN- $\gamma$ ). The sensitivity levels of the IL-4 and IFN- $\gamma$  kits were < 1 Pg/mL and 2.43 ng/L, respectively. Accordingly, the criteria for measuring serum concentrations of IL-4 and IFN- $\gamma$  were based on the sensitivity of the kits. The ELISA method was calibrated using calibrators provided by the manufacturer.

## 2.7. Measurement of the Serum Concentrations of IgE and IgG2a

The serum concentrations of IgE and IgG2a were also measured using ELISA kits, according to the manufacturers guidelines (Eastbiopharm, Hangzhou, China). The sensitivity levels of the IgE and IgG2a kits were 0.11  $\mu$ g/mL and 2.12  $\mu$ g/mL, respectively. Accordingly, the criteria for measuring serum concentrations of IgE and IgG2a were based on the sensitivity of the kits.

## 2.8. Detection of the Blood Numbers of T Helper 1 Cells by Flow Cytometry

The mice were bled on days 0, 23 and 40, by retro-orbital method and the blood count of Th1- and Treg cells were measured by BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

The blood count of Th1 cells was done using a mouse Th1 multicolor flow cytometry kit (R and D system, Minnea Polis, USA). The kit comprised of conjugated antibodies against T-bet-PerCp, IFN- $\gamma$ -Fluorescein, IL-12R $\beta$ 2-APC and cluster of differentiation (CD) 4-PE. The kit also comprised

**Table 1.** The Protocol of Sensitization Phase<sup>a</sup>

Explanation	1	2	3	4	5
Prescription materials	Normal saline	Der p2	Der p2+, CPG	Der p2+, CPG + MPL	Der p2+, CPG + BCG
Number of mice	10	6	6	6	6
Injection route	SC	SC	SC	SC	SC
Immunization protocol	0 - 14 - 21 days	0 - 14 - 21 days	0-14 - 21 days	0 - 14 - 21 days	0 - 14 - 21 days
Bleeding area	Retro- orbital	Retro- orbital	Retro- orbital	Retro- orbital	Retro- orbital
Bleeding times	0 - 23 days	23 days	23 days	23 days	23 days
Measured parameters	IFN- $\gamma$ /IL-4	IFN- $\gamma$ /IL-4	IFN- $\gamma$ /IL-4	IFN-g/IL-4	IFN- $\gamma$ /IL-4
	IgG2a/IgE	IgG2a/IgE	IgG2a/IgE	IgG2a/IgE	IgG2a/IgE
	TH1/Treg	TH1/Treg	TH1/Treg	TH1/Treg	TH1/Treg

<sup>a</sup>The mice were divided to five groups and after use of isoflurane anesthesia, were immunized by subcutaneous (SC) injection of Derp2 on days 1, 15 and 22. Two groups of mice were considered as control groups that were administrated SC with only Derp2 or normal saline on days 1, 15 and 22. Three groups considered as pre-treated mice were administrated by adjuvants on days 0, 14, and 21 with CPG, CPG + MPLA or CPG + BCG by SC injection. The cytokines IFN- $\gamma$  and IL-4, the immunoglobulines IgG2a and IgE and lymphocytes Th1 and Treg were detected on days 0 and 23 for control group and day 23 for other groups.

**Table 2.** The Protocol of the Challenge Phase<sup>a</sup>

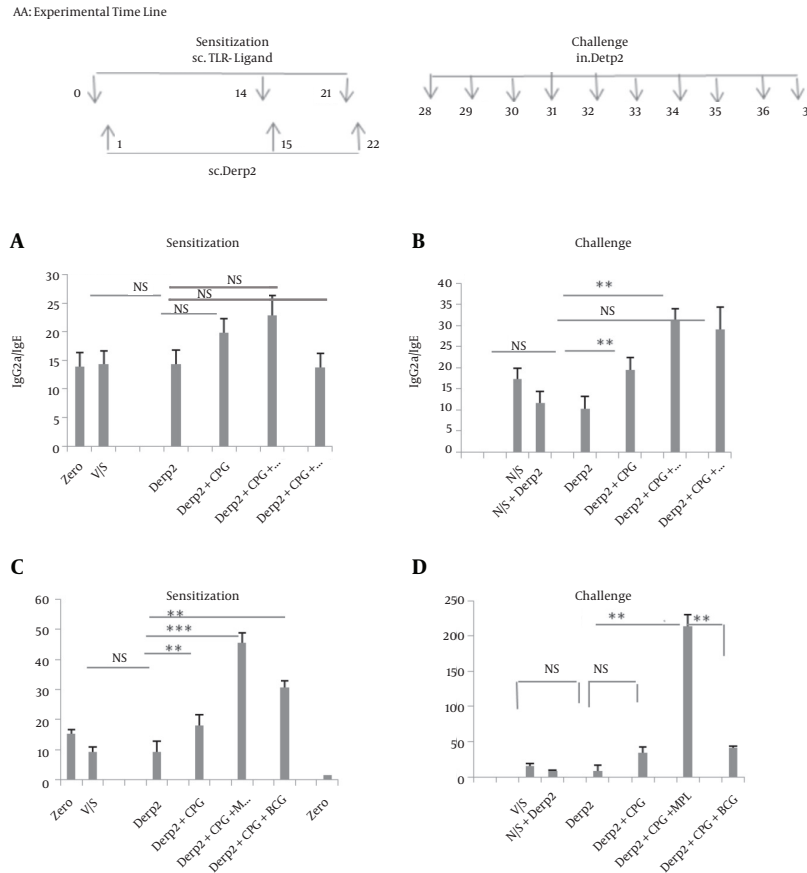
Explanation	1/1	1/2	2	3	4	5
Prescription materials	Normal saline	Der p2	Der p2	Der p2	Der p2	Der p2
Number of mice	5	5	6	6	6	6
Administration	IN	IN	IN	IN	IN	IN
Challenge protocol	28 - 29 - 30 - 31 - 32 - 33 - 34 - 35 - 36 - 37 days	28 - 29 - 30 - 31 - 32 - 33 - 34 - 35 - 36 - 37 days	28 - 29 - 30 - 31 - 32 - 33 - 34 - 35 - 36 - 37 days	28 - 29 - 30 - 31 - 32 - 33 - 34 - 35 - 36 - 37 days	28 - 29 - 30 - 31 - 32 - 33 - 34 - 35 - 36 - 37 days	28 - 29 - 30 - 31 - 32 - 33 - 34 - 35 - 36 - 37 days
Bleeding area	Retro- orbital	Retro- orbital	Retro- orbital	Retro- orbital	Retro- orbital	Retro- orbital
Bleeding times	40 day	40 day	40 day	40 day	40 day	40 day
Time of lung removal	40 day	40 day	40 day	40 day	40 day	40 day
Measured parameters	IFN-g/IL-4	IFN-g/IL-4	IFN-g/IL-4	IFN-g/IL-4	IFN-g/IL-4	IFN-g/IL-4
	IgG2a/IgE	IgG2a/IgE	IgG2a/IgE	IgG2a/IgE	IgG2a/IgE	IgG2a/IgE
	TH1/Treg	TH1/Treg	TH1/Treg	TH1/Treg	TH1/Treg	TH1/Treg
	Lung histology	Lung histology	Lung histology	Lung histology	Lung histology	Lung histology

<sup>a</sup>In the challenge phase, the mice were administrated intranasal (IN) with Derp2 daily from days 28 to 37. Finally, the mice were sacrificed on day 40. The half of the saline-sensitized mice were challenged again with saline and considered as a normal control group. The other half of the mice in the saline-sensitized group were specified as Derp2-sensitized control group and were challenged with Derp2 at mentioned time points without pre-treatment. The cytokines IFN- $\gamma$  and IL-4, the immunoglobulines IgG2a and IgE, lymphocytes Th1 and Treg and lung histology were detected on day 40.

of fixation/permeabilization buffer (1% Formaldehyde, Saponin) and permeabilization/wash buffer (Saponin and 0.05% Sodium azide).

For measurement of the blood count of Th1 cells, the pellet was treated with a lysis buffer for red blood cell (RBC) lysis. Based on the manufacturer's guidelines, the cells were washed twice with PBS and resuspended in fixation/permeabilization buffer and incubated at 2 to 8°C for 30 minutes. The cells were then washed and resus-

ended in permeabilization/wash buffer. After that, 10  $\mu$ L of each antibody or corresponding isotype control antibody was added to the cells. The cells were incubated and washed by permeabilization/wash buffer to remove excess antibody. Eventually, the cells were resuspended in Phosphate Buffered Saline (PBS) for flow cytometric analysis.



**Figure 1.** The Serum Ratios of IgG2a/IgE and Interferon-Gamma/Interleukin-4 in Mice Pre-treated with toll like receptor agonists (CPG, CPG+MPL and CPG+BCG) at sensitization (A and C) and Challenge (B and D) Phases. The ratios were measured on days 0, 23 and 40. The results were presented as mean  $\pm$  Standard Error of the Mean (SEM) for five to six mice/group. NS, \*\*, \*\*\* represent the P values of  $> 0.05$  (non-significant),  $< 0.05$  and  $\leq 0.07$ , respectively.

### 2.9. Detection of Blood Numbers of the Treg Cells by Flow Cytometry

The frequency of the blood Treg cells was also measured by BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The blood count of Treg cells was determined by a mouse Treg multicolor flow cytometry kit (Biolegend, San Diego, CA 2321). The kit contained conjugated immunoglobulins against Treg cell markers (FOXP3-Alexa flour 488, CD4-perCp, CD25-PE), a cell staining buffer and a single color compensation control. Based on the manufacturer's guidelines, the cell pellet was primarily treated with RBC lysis buffer. Then, the cells were washed and suspended in cell-staining buffer. After that, we added the FOXP3 fixation/permeabilization solution to the cells. The cells were incubated and washed with Foxp3 permeabilization buffer. Then the anti-mouse FOXP3-Alexa Fluor,

CD25-PE, CD4-perCp antibody cocktail or Alexa flour 488 rat IgG2b kappa isotype control, CD25-PE, and CD4-perCp antibody cocktail were added to the appropriate tube. The cells were then incubated and washed twice with cell staining buffer. Finally, the cells were suspended in cell staining buffer after and analysis with flow cytometer using appropriate instrument settings. The BD multiset software of flow cytometry system was used for data analysis. The flow cytometry system was calibrated using flow cytometry calibration reagents provided by the manufacturer. The main criteria for evaluation of the cell population were to assess at least 10000 cells.

### 2.10. Lung Preparation

As mentioned, the mice were sacrificed at 72 hours after the last Derp2 challenge, on day 40. The lungs were then



isolated and fixed using 4% formalin for 48 hours. Then the left lobes of the lung were removed vertically from the above of the main bronchus entry. Then formalin was removed and the tissues embedded in the paraffin wax. The lung slices were prepared with a thickness of 2-3 mm from anterior to posterior direction and mounted on the microscopy slides (32).

### 2.11. Lung Staining

The lung slices were baked at 65°C for 45 minutes and then stained using the Hematoxylin and eosin (H and E) reagent (Merck, Darmstadt, Germany). The assessment of goblet cells and mucus secretion was determined using Periodic acid-Schiff (PAS) reagent (Sigma-Aldrich, Germany). The inflammation and mucus production were scored by only one expert pathologist, according the following scales: 0 = without inflammation or mucus, 1 = weak until moderate inflammation or mucus, and 2 = severe inflammation or mucus (32, 33).

### 2.12. Statistical Analysis

Data were presented as mean  $\pm$  standard error of the mean (SEM). Data were examined for normal distribution using non-parametric Kolmogorov-Smirnov test, and statistically no violations were observed from normality assumption ( $P > 0.05$ ). The variables between different groups were compared by one-way Analysis of Variance (ANOVA) or unpaired t-test. One-way analysis of variance together with Tukey's Post hoc test was used for the comparison of variables between pre-treated groups versus the Derp2-sensitized control group. A power of 0.8 was expected for the present study, and  $P$  values of less than 0.05 were considered significant. The Microsoft Office 2007, Microsoft office Excel and SPSS (version 23) software were used for analysis and exhibition of data.

## 3. Results

The effects of adjuvants on inflammatory parameters following the challenge phase: The lung inflammatory score was significantly increased in Derp2-sensitized control group ( $1.14 \pm 0.56$ ) in comparison with saline-administrated control group ( $0.46 \pm 0.50$ ,  $P = 0.030$ ).

The score of inflammation in Derp2-sensitized group pre-treated with CPG-ODN plus MPLA ( $0.29 \pm 0.31$ ) and Derp2-sensitized group pre-treated with CPG-ODN ( $0.34 \pm 0.55$ ) were significantly lower than the Derp2-sensitized control group ( $P < 0.005$  and  $P < 0.009$ , respectively). The inflammatory score was decreased in Derp2-sensitized group pre-treated with CPG-ODN plus BCG ( $0.58 \pm 0.44$ ) as

compared with Derp2-sensitized group ( $P = 0.038$ ) [Figure 2 (chart and images)].

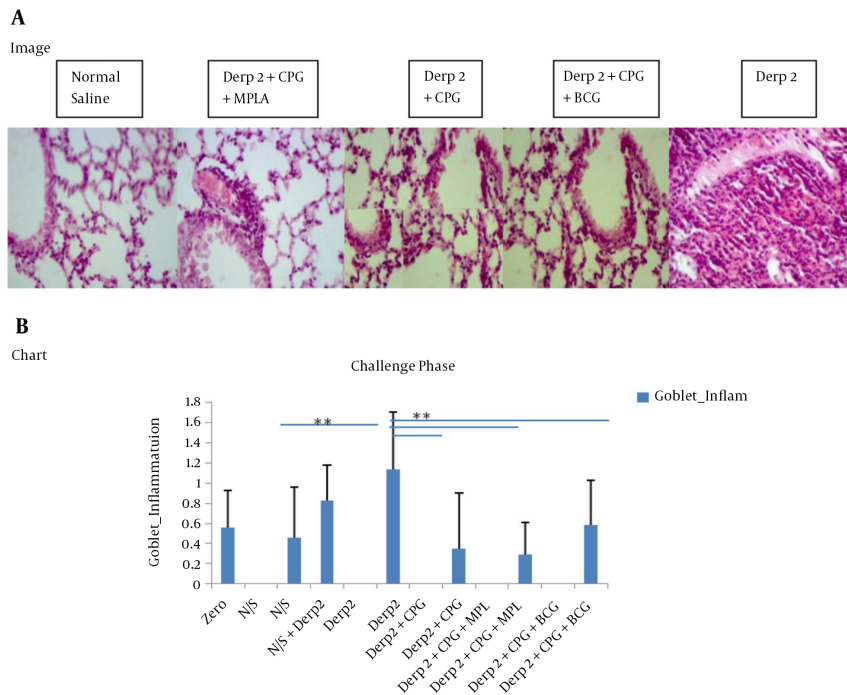
The effects of adjuvants on the IgG2a/IgE ratio in sensitization phase: As demonstrated in Figure 1A, the serum IgG2a/IgE ratio was similarly expressed in Derp2-sensitized control mice ( $14.36 \pm 2.78$ ) and saline-administrated control group ( $14.32 \pm 2.31$ ,  $P = 0.992$ ). The serum IgG2a/IgE ratio in Derp2-sensitized groups pre-treated with CPG plus MPLA, CPG plus BCG or CPG were not significantly altered as compared with Derp2-sensitized control mice ( $P = 0.354$ ,  $P = 0.919$  and  $P = 0.292$ , respectively).

The effects of adjuvants on the IgG2a/IgE ratio in challenge phase: There was no significant difference between Derp2-sensitized control mice and saline-administrated control group regarding the serum ratio of IgG2a/IgE ( $10.22 \pm 2.78$  vs.  $17.36 \pm 2.58$ ,  $P = 0.163$ ). The IgG2a/IgE ratio in Derp2-sensitized groups pre-treated with CPG plus MPLA or CPG were significantly increased as compared with Derp2-sensitized control mice ( $P = 0.006$  and  $P = 0.028$ , respectively). The difference of the IgG2a/IgE ratio between Derp2-sensitized control group pre-treated with CPG plus BCG ( $29.12 \pm 5.18$ ) and Derp2-sensitized control mice was not significant ( $P = 0.216$ ) (Figure 1B).

The effect of adjuvants on the Interferon-Gamma/Interleukin-4 ratio in the sensitization phase: The effects of adjuvants on the IFN- $\gamma$ /IL-4 ratio in the sensitization phase are demonstrated in Figure 1C. The serum IFN- $\gamma$ /IL-4 ratio in Derp2-sensitized mice treated with CPG ( $18.16 \pm 6.97$ ) was markedly increased as compared with Derp2-sensitized control group ( $9.15 \pm 7.15$ ,  $P = 0.066$ ). The IFN- $\gamma$ /IL-4 ratio in Derp2-sensitized group treated with combinations of adjuvants, CPG plus MPLA or CPG plus BCG, was significantly higher than in the Derp2-sensitized control mice ( $P < 0.002$  and  $P < 0.003$ , respectively).

The effect of adjuvants on the Interferon-Gamma/Interleukin-4 ratio in the challenge phase: The difference of the serum IFN- $\gamma$ /IL-4 ratio between Derp2-sensitized mice pre-treated with CPG and Derp2-sensitized control group was not statistically significant, although this parameter was higher in CPG administrated group ( $8.49 \pm 7.06$  vs.  $34.25 \pm 6.88$ ,  $P = 0.070$ ). The IFN- $\gamma$ /IL-4 ratio in Derp2-sensitized groups pre-treated with combinations of adjuvants, CPG plus MPLA or CPG plus BCG, was significantly increased as compared with the Derp2-sensitized control mice ( $P = 0.034$  and  $P = 0.001$ , respectively) (Figure 1D).

The effects of adjuvants on the T helper 1/T regulatory ratio in the sensitization phase: There was no significant difference between Derp2-sensitized control group and saline-administrated control mice regarding Th1/Treg ratio ( $0.25 \pm 0.18$  vs.  $0.44 \pm 0.08$ ,  $P = 0.166$ ). The Th1/Treg ratio in Derp2-sensitized groups pre-treated with CPG plus MPLA,



**Figure 2.** Comparison of the Lung Histopathological changes at challenge phase in Derp2-sensitized control group, saline-administrated control group, and Derp2-sensitized group pre-treatment with CPG + MPL, CPG, and CPG+ BCG. Paraffin-embedded lung slices were prepared 72 hours after the last challenge on day 40 and stained with hematoxylin-eosin (H and E). Data are exhibited by a magnification of 400 × and representative of five randomly selected tissues (Images). The scores of inflammation (mast cells in airway epithelial layer, sub epithelial fibrosis, airway smooth muscle and vascular changes) plus goblet cells (goblet cells hyperplasia and mucus secretion) in mice pre-treated with TLR agonists (CPG, CPG + MPL and CPG + BCG) at challenge phases are indicated. The score of lung inflammation and goblet cells were measured 72 hours after the last challenge on day 40. The results are presented as mean ± SEM for 5-6 mice/group. NS and \*\* represent the P values of > 0.05 (non-significant) and < 0.05, respectively (Chart).

CPG plus BCG or CPG was not significantly altered as compared with Derp2 sensitized control mice ( $P = 0.266$ ,  $P = 0.435$  and  $P = 0.799$ , respectively) (Figure 3A).

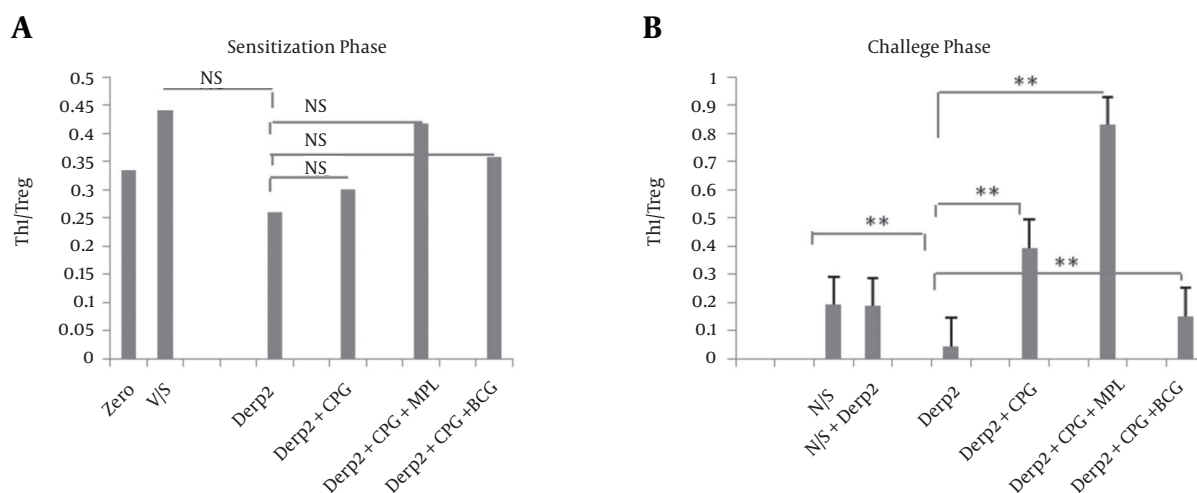
The effects of adjuvants on the T helper 1/T regulatory ratio in the challenge phase: As demonstrated by Figure 3B, the Th1/Treg ratio in Derp2-sensitized control mice ( $0.04 \pm 0.03$ ) was significantly lower than in saline-administrated control group ( $0.19 \pm 0.03$ ,  $P = 0.006$ ). The Th1/Treg ratio in Derp2-sensitized groups pre-treated with CPG plus MPLA, CPG plus BCG or CPG were significantly increased as compared with Derp2-sensitized control mice ( $P = 0.022$ ,  $P = 0.012$  and  $P = 0.003$ , respectively). The Th1/Treg ratio in Derp2-sensitized group pre-treated with CPG plus BCG ( $0.14 \pm 0.02$ ) was significantly lower than in Derp2-sensitized groups pre-treated with CPG plus MPLA or CPG ( $P = 0.035$  and  $P = 0.011$ , respectively).

#### 4. Discussion

The modulatory effects of adjuvants CPG, MPLA and BCG on the Th1/Th2 cell polarization have been demon-

strated in a number of investigations. The immunomodulatory influences of these adjuvants on allergic reaction have been also evaluated in a number of investigations with respect to the inflammatory cytokines, immunoglobulins, leukocytes and lung inflammatory parameters (28, 31, 34).

In agreement with our findings, in a mouse cockroach extract-induced asthma model, it was demonstrated that intranasal pre- and post-treatment with CpG-ODN results in a significant decrease in airway inflammation, reduction of IL-13, IL-5 and serum IgE, cockroach-specific IgE and IgG1/IgG2a ratio. The beneficial effects of CpG-ODN have been attributed to its ability to modulate Th2 cells and induce regulatory T cells or Th1 responses (10). In another study, the BALB/c mice were immunized with Ovalbumin (OVA) formulated with MPLA, and then were challenged with OVA aerosol. In immunized mice with OVA formulated with MPLA, lung inflammation and OVA-specific IgE levels were reduced, whereas the ratio of OVA-specific IgG2a/IgG1 and the ratio of IFN- $\gamma$ /IL-4 were increased (35). However, it has been suggested that IL-17 also plays a



**Figure 3.** Comparison of Th1/Treg ratio in mice pre-treated with toll like receptor agonists. The ratio of CD4+T-bet+ IFN $\gamma$ +Th1/CD4+CD25+FOXP3+ Treg mice pre-treated with TLR agonists in sensitization (A) and challenge phases (B). The results were presented as mean  $\pm$  SEM for 5-6 mice/group. NS and \*\* represent the P values of  $> 0.05$  (non-significant) and  $< 0.05$ , respectively.

pivotal role in neonatal airway inflammation, and anti-asthma effects of BCG neonatal vaccination occur via the down-regulation of IL-17 (3). It has also been demonstrated that the inhibition of IFN- $\gamma$ , enhances the anti-asthma effects of BCG neonatal vaccination in an OVA-induced murine asthma model. Therefore, it seems that BCG vaccination may have different effects in the neonatal period and young adulthood (36).

In the present study, we investigated the effects of CPG, MPLA and BCG on the ratios of Th1/Th2 and Treg/Th1 cell-related parameters including IFN- $\gamma$ /IL4, IgG2a/IgE and Th1/Treg in a mouse model of asthma. The adjuvants were used prior to sensitization with allergen Derp2 and then the mice were challenged with allergen Derp2 alone by intra-nasal administration.

Ovalbumin was used for induction of allergy in most animal models of asthma, yet this allergen is not considered as a common allergen in human atopic asthma (4, 37). Therefore, the allergen Derp2 was used in the present study (31, 38). The allergen Derp2 promotes TLR4 aggregation, which is necessary for TLR signal transduction. In addition to its allergenic role, Derp2 may also act as an auto-adjuvant (39).

As mentioned, imbalances of Th1/Th2 and Treg/Th2 cells play an important role in the development of asthma and allergic diseases. Therefore, the correction of the Th1/Th2 and Treg/Th2 imbalances is the basis of most immunotherapeutic strategies for treatment of allergic diseases. The adjuvants CPG, MPLA and BCG act as TLR agonists that transduce signal through the TLR9, TLR4 and

TLR4 plus TLR2, respectively, and induce Th1 cell-related cytokines and dependent immunoglobulin production (15, 20, 28). In contrast to other investigations, this study was performed during the early period of life in mice, which resulted in the unexpected results, possibly, due to the immaturity of the immune system during this time (40).

The cytokines IFN- $\gamma$  and IL-4, and immunoglobulins IgG2a and IgE are Th1 and Th2 cell-related parameters, respectively (41). In the present study, for the first time, the IFN- $\gamma$ /IL-4, IgG2a/IgE and Th1/Treg ratios and the effects of CPG, MPLA and BCG on these ratios were measured in both immunization and challenge phases in a mouse model of acute asthma. Ovalbumin was used for induction of allergy in most animal models of asthma, but this allergen is not considered as a common allergen in human atopic asthma (4, 37).

Therefore, the novelties of the current study are as follows: 1, The current study was designed for preventive properties of TLR agonists combination to vaccinations against Derp2; 2, Derp2 has been used instead of ovalbumin for vaccination of mice prior to induction of asthma; 3, This study evaluated the ratio of Th1/Th2 cytokines instead of cytokines alone, which resulted in better understanding of the effects of the adjuvants on immune responses. Therefore, based on the novelties of the current study, it seems that the results can direct future investigations toward using Derp2 as a new antigen for vaccination against asthma. Moreover, it seems that the novelties of the study shed light on understanding the main mechanisms used by TLR agonist's combination to shift the immune responses to Th1/T



reg responses.

We observed that the differences of the IgG2a/IgE ratios between Derp2-sensitized groups pre-treated with CPG-, CPG plus MPLA- or CPG plus BCG- and Derp2-sensitized control mice were not statistically significant at sensitization phase. However, at challenge phase, the IgG2a/IgE ratio in Derp2-sensitized group pre-treated with CPG-, and CPG plus MPLA was significantly increased as compared with Derp2-sensitized control mice. These results indicated that the application of adjuvants CPG or CPG plus MPLA might differentially influence the IgG2a/IgE ratio at sensitization and challenge phases. The adjuvants have no effects on the IgG2a/IgE ratio at sensitization phase probably due to the immaturity of the immune system in this period (40). Indeed, the age-dependent changes in the immune system of Balb/c mice have been demonstrated (42). Moreover, age-dependent alterations have been demonstrated in the expression and function of human toll like molecules as receptors of used adjuvants (43). Similar alterations may occur in the TLRs of mice in an age-related manner.

The results of the present study showed that the IFN- $\gamma$ /IL-4 ratio in Derp2-sensitized groups pre-treated with CPG plus MPLA and CPG plus BCG was significantly higher than in Derp2-sensitized control mice. These results indicate that adjuvants BCG, CPG and MPLA might influence the balance of Th1/Th2 cells and deviate it toward Th1 cells, which in turn result in higher IFN- production or lower IL-4 synthesis and therefore higher IFN-g/IL-4 ratio, which may be a reason for the higher IgG2a/IgE ratio in adjuvant-treated groups.

The results of this study also demonstrated that in the sensitization phase, the differences of the Th1/Treg ratios between Derp2-sensitized groups pre-treated with CPG, CPG plus MPLA or CPG plus BCG, and Derp2-sensitized control mice were not statistically significant. At challenge phase, the Th1/Treg ratios in Derp2-sensitized group pre-treated with CPG, CPG plus MPLA and CPG plus BCG were significantly increased as compared with Derp2-sensitized control mice. These results indicated that application of adjuvants CPG, CPG plus MPLA or CPG plus BCG might differentially influence the Th1/Treg ratio at sensitization and challenge phases. The adjuvants had no effects on the Th1/Treg ratio at sensitization phase probably due to the immaturity of the immune system in this period (40). Indeed, some age-associated changes have been demonstrated in the immune system of Balb/c mice (42). Moreover, some age-related changes have been reported on toll like molecules expression and activation, which act as receptors for used adjuvants (43). Similarly, age-related changes may also happen in the TLRs of mice.

The strong points of this study were as follows; firstly, using allergen Dep2 instead allergen ovalbumin; the data

form ovalbumin-induced asthma in mice may not be translatable to humans, whereas allergen Dep2 is a component of many allergens of the human respiratory system. Secondly, immunological parameters were measured in a time period at both sensitization and challenge phases of asthma. Thirdly, using of an inbred strain of mice (Balb/c) may exclude many genetically interfering factors on data. Moreover, one of the most important novelties of this study was the evaluation of the combinational effects of MPLA, CPG-ODN and BCG on some major immunological parameters in an animal model of asthma. Although, in a number of studies using animal models of asthma, the effects of these adjuvants on some immunological parameters were investigated, however, there has been no report regarding the combinational effects of the mentioned adjuvants.

It should be noted that our study had several limitations: the first was the absence of immunohistochemical studies on airway tissues, nevertheless, the histopathological examination of lung tissues provided valuable findings. Second, we used only one strain of mice therefore the results may not translatable to humans or even different strains of mice. Third, the measurement of some other important immunological parameters such as Th17 cells, chemokines and toll like receptors was not a part of our protocol that should be investigated in future studies.

In conclusion, the results of the present study showed that the BCG, MPLA and CPG improve the Th1/Th2- and Th1/Treg imbalances in a mouse model of asthma. Therefore, it seems that the combination of adjuvants, especially CPG and BCG, should be considered in more studies as preventive or therapeutic adjuvants against atopic asthma in humans.

Based on previous studies, ovalbumin is the most studied allergen in animal investigations, while, it is not considered as a common allergen in human atopic asthma, so, the strong points of our study was to use Derp2 as the most common inducer of asthma in humans. Additionally, combined adjuvants were used in the current study, which was associated with a good vaccination against Derp2.

No evaluation of Th17 population was the main weakness of this study.

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## Footnote

**Declaration of Interest:** The authors had no conflicts of interest.

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