

ORIGINAL ARTICLE

Immunomodulatory Effects of Adjuvants CPG, MPLA, and BCG on the Derp2-Induced Acute Asthma at Early Life in an Animal Model of BALB/c Mice

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Abstract—The Th1- and Treg cell-related immune responses play key roles in the modulation of Th2 cell-related allergic disorders. The aim was to evaluate the effects of CPG, MPLA, and BCG on the number of Th1-, Th2-, and Treg cell-related parameters in an animal model of asthma. BALB/c mice were divided into five groups and immunized subcutaneously (SC) on days 1, 15, and 22 with allergen Derp2. Three groups of mice were pretreated SC on days 0, 14, and 21 with CPG, CPG + MPLA, or CPG + BCG. All mice were then challenged intranasally with Derp2 on days 28–37. Blood samples were collected from the retro-orbital sinus, on days 0, 23, and 40. The serum levels of IL-4, IFN- γ , IgE, and IgG2a were measured using ELISA technique. The blood number of Th1 and Treg cells was determined using flow cytometry. At the sensitization phase, the number of Th1 and the serum levels of IFN- γ and IgG2a were significantly increased in the Derp2-sensitized group pretreated with CPG plus MPLA, and the number of Treg cells was significantly elevated in Derp2-sensitized mice pretreated with CPG or CPG plus MPLA as compared with that in Derp2-sensitized control mice. At the challenge phase, the number of Th1 was significantly elevated in Derp2-sensitized mice pretreated with CPG plus MPLA, CPG plus BCG, or CPG; the count of Treg cells was significantly increased in Derp2-sensitized mice pretreated with CPG plus BCG group; and the levels of IFN- γ and IgG2a were significantly enhanced in the Derp2-sensitized group pretreated with CPG plus MPLA in comparison with those in Derp2-sensitized control mice. The scores of inflammation and mucus secretion in the lung were significantly decreased in the Derp2-sensitized group pretreated with CPG, BCG, and CPG plus MPLA in comparison with those in the Derp2-sensitized control group. These results showed that BCG, MPLA, and CPG modulate Th1-, Th2-, and Treg-related parameters and ameliorate lung inflammatory parameters in a mouse model of asthma.

KEY WORDS: asthma; BCG; MPLA; CPG-ODN; inflammation; mice.

INTRODUCTION

Asthma is the most prevalent chronic disorder which belongs to type 1 hypersensitivity and affects 10% of the population [1]. Approximately, 70% of asthma is atopic and 30% is nonatopic [2]. Thus, it seems that allergens play key roles in the induction of asthma. Accordingly, it has been hypothesized that environmental (or epigenetic) parameters as well as genetic factors have important effects on the pathogenesis of the disease [3]. The house dust mite (HDM) is the most inducing factor for allergies including

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asthma, which consists of several components specially allergens Derp1 and Derp2 [4]. It has been demonstrated that allergen Derp2 can induce allergic asthma in susceptible individuals [4]. Allergens, including Derp2, are the inducer of T helper 2 (Th2) lymphocytes to produce type 1 hypersensitivity-related cytokines such as IL-4, IL-5, and IL-13 [5]. IL-4 is the most important cytokine which participates in several mechanisms which are taking place during asthma, such as IgE isotype switching and also production of mucus in the lung [6]. On the other hand, investigations revealed that Th1 cell-related cytokines play key roles in the regulation of Th2 cells [7]. Hence, their induction can be considered for immunotherapy against asthma. Th1 cells utilize several mechanisms to regulate the Th2 cell-related immune responses such as secretion of interferon gamma (IFN- γ) [7]. Additionally, it has been documented that regulatory T (Treg) lymphocytes also modulate immune responses via establishment of a balance between Th1/Th2 cell-related responses [8]. Thus, immunotherapeutic agents which lead to increase the number and/or functions of Th1/Treg cells may be considered as a suitable candidate for the treatment of asthma. At the present time, a number of adjuvants [especially toll like receptor (TLR) agonists] are introduced as main inducers of Th1 and/or Treg cells.

CpG oligodeoxynucleotide (CpG-ODN) was defined as a synthetic TLR9 that stimulates both innate and adaptive immunity, induces the tumor necrosis factor (TNF)- α expression in macrophages, and augments the IFN- γ and IL-12 expression [9]. Furthermore, CpG-ODN induces B cells, monocytes, macrophages, and dendritic cells (DCs) to produce proinflammatory (such as IL-1, IL-6, IL-18 and TNF- α) and Th1 type cytokines (IL-12, IFN- γ), resulting in the induction of a type 1-biased immune response [10]. It was documented that intradermal deliver of ragweed pollen linked to CpG reversed the established allergic responses in the murine lung [11].

Monophosphoryl lipid A (MPLA), a TLR4 agonist, is the first TLR ligand which is used in licensed human vaccines and acts as a potent stimulator of Th1 cells and antibody responses [12]. MPLA also enhances the serum titers of both IgG1 and IgG2a antibodies when administered subcutaneously or mucosally, represents a mixed Th1 and Th2 immune responses, increases the surface expression of the major histocompatibility complex (MHC) and the co-stimulatory molecules on the APCs, and therefore increases the stimulatory properties of DCs, macrophages, and B lymphocytes [13, 14].

The current tuberculosis vaccine, Bacillus Calmette-Guérin (BCG), acts as a TLR2 and possibly TLR4 agonist and is

widely used as an adjuvant in some clinical and experimental situations. Ligation of TLR2 and TLR4 through the BCG results in the activation of macrophages and production of high levels of TNF- α , IL-1 β , and IL-6 [15]. Furthermore, BCG administration induces the IL-10 secretion by Treg cells [16]. However, BCG is also increasing the number of Treg lymphocytes in human and animal models [17].

MPLA, CPG-ODN, and BCG are the main adjuvants whose effects on the induction of Th1 cells have been documented [18, 19]. Therefore, it seems that these adjuvants can be used as preventive agents in high-risk susceptible individuals or for therapeutic purpose in asthmatic patients. There are a number of studies which used the mentioned adjuvants, separately, as anti-inflammatory agents following induction of asthma in animal models, but they have usually used ovalbumin as an allergen [20–22]. Based on the principal mechanisms which are involved in the pathogenesis of asthma and the immunomodulatory effects of adjuvants in the deviation of balances of Th1/Th2 cells toward Th1 cells, the main aim of this study was to evaluate the effects of CPG separately or in combination with MPLA or BCG through subcutaneous administration on the number of immunologic parameters including T cell subsets (Th1 and Treg cells), cytokines (IL-4 and IFN- γ), immunoglobulins (IgG2a and IgE), and lung inflammatory characteristics in an asthma model which was induced by allergen Derp2 during the early life of BALB/c mice.

MATERIALS AND METHODS

Mice

Female (4 weeks old) BALB/c mice were purchased from Kerman University of Medical Science (Kerman, Iran). Mice were maintained at temperature-controlled conditions (23 ± 1 °C), humidity ($55 \pm 5\%$), and lighting (a 12-h light/12-h dark cycle) and were administered standard laboratory mouse food and water *ad libitum*. All mice were housed in a room where the investigation protocol was performed so as to minimize any stress reaction potentially caused by novel environmental cues. All experiments were carried in accordance with the recommendations for the care and use of laboratory animals of the Ethics Committee on Animal Experimentation of Isfahan University of Medical Science and were also in agreement with the National Research Council Guide (2011).

Reagents

CPG 1826 ODN vacciGrade (5-TCCATGACGTTTCCT GACGTT-3) and MPLA-SM VacciGrade (a derivative of the lipid A from *Salmonella minnesota*) were purchased from InvivoGen (San Diego, CA 92121, USA). Recombinant Derp2 was obtained from INDOOR Biotechnologies (Cardiff, UK). BCG was purchased from the Pasteur Institute (Tehran, Iran).

Planning of Research

Five BALB/c groups were enrolled in the study. The control group consisted of 10 mice and the other groups consisted of six mice.

Immunization and Challenge Phases

The mice were classified into five groups and after anesthesia (with 4% isoflurane gas at an airflow rate of 3 L/min in the sensitization phase) were immunized by subcutaneous (SC) delivery on days 1, 15, and 22 with 2 µg Derp2 in a total volume of 50 µL [23].

For the pretreatment study, three groups of mice were pretreated on days 0, 14, and 21 with CPG, CPG + MPLA, or CPG + BCG, by SC delivery. Two groups were also considered as control groups that were administrated by SC delivery on days 1, 15, and 22 with only 2 µg Derp2 or with 50 µL normal saline. Groups III, IV, and V were immunized at the mentioned time points and pretreated SC with 20 µg CPG, “20 µg CPG + 20 µg MPLA,” and “20 µg CPG + 4×10^5 CFU of BCG,” respectively. All mice were then challenged with 1.5 µg Derp2 intranasally (in a total volume of 30 µL, 7.5 µL/nostril, 2 times within 5 min) on days 28–37. Finally, all mice were sacrificed 72 h later on day 40 [24, 25]. It should be noted that the 50% of the mice in normal saline-sensitized mice were challenged with saline and considered as a normal control group without pretreatment with any adjuvants. The remaining of the normal saline-sensitized mice were challenged with Derp2 at the mentioned time points and considered as Derp2 control group without pretreatment with any adjuvants (Tables 1 and 2; Fig. 1(AA)).

Blood Sampling

After anesthesia with isoflurane gas 4% at a flow rate of 3 L/min, blood samples were collected from the retro-orbital sinus, on day 0 (before beginning of the pretreatment program), on day 23 (24 h after the last immunization), and

finally 72 h after termination of the allergen challenge, on day 40 [26]. Blood samples were incubated for 30 min at room temperature for clotting and then centrifuged at 14,000 rpm for 20 min. The serum samples were separated and stored at -80°C until analysis. The levels of cytokines (IL-4 and IFN- γ) and antibodies (IgE and IgG2a) were measured using a standard ELISA technique [27]. The pellets were collected for determination of the blood number of Th1 and Treg cells by using the standard flow cytometry technique.

Detection of the Serum Levels of IL-4 and IFN- γ

On days 0, 23, and 40, the mice were bled by retro-orbital method and the serum levels of IL-4 and IFN- γ were quantitated by using commercial enzyme-linked immunosorbent assay (ELISA) kits (from Boster, Wuhan, China, for IL-4 and from Eastbiopharm, Hangzhou, China, for IFN- γ). The levels of sensitivity of the IL-4 and IFN- γ kits were <1 Pg/mL and 2.43 ng/L, respectively.

Detection of the Serum Levels of IgE and IgG2a

The serum concentrations of IgE and IgG2a were also measured by using ELISA kits according to the manufacturer's instruction (Eastbiopharm, Hangzhou, China). The levels of sensitivity of the IgE and IgG2a kits were 0.11 and 2.12 µg/mL, respectively.

Detection of Th1 by Flow Cytometry

The mice were bled on days 0, 23, and 40 by retro-orbital method, and the blood numbers of Th1 and Treg cells were determined by counting 10,000 cells using BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and BD CellQuest PRO software.

The blood number of Th1 cells was determined using a mouse Th1 multicolor flow cytometry kit (R&D Systems, Minneapolis, USA). The kit contained the conjugated antibodies to T-bet-PerCp, IFN- γ -fluorescein, IL-12R β 2-APC, and cluster of differentiation (CD)4-PE. The kit also contained fixation/permeabilization buffer (1% formaldehyde, saponin) and permeabilization/wash buffer (saponin and 0.05% sodium azide).

The blood number of Th1 cells was measured after treatment of whole blood with a red blood cell (RBC) lysis buffer. According to the manufacturer's guides, the cells were washed twice in PBS and resuspended in fixation/permeabilization buffer and incubated at $2-8^\circ\text{C}$ for 30 min. The cells were then centrifuged and resuspended in

Table 1. Sensitization Phase

Explanation	Groups				
	1	2	3	4	5
Prescription materials	Normal saline	Derp2	Derp2 + CPG	Derp2 + CPG + MPL	Derp2 + CPG + BCG
Number of mice	10	6	6	6	6
Injection	SC	SC	SC	SC	SC
Immunization protocol (days)	0–14–21	0–14–21	0–14–21	0–14–21	0–14–21
Bleeding times (days)	0–23	0–23	0–23	0–23	0–23
Experimental conducted	IFN- γ /IL-4	IFN- γ /IL-4	IFN- γ /IL-4	IFN- γ /IL-4	IFN- γ /IL-4
	IgG2a/IgE	IgG2a/IgE	IgG2a/IgE	IgG2a/IgE	IgG2a/IgE
	Th1/Treg	Th1/Treg	Th1/Treg	Th1/Treg	Th1/Treg

permeabilization/wash buffer. After that, 10 μ L of each antibody or each corresponding isotype control antibody was added to the cells. Following the incubation, any excess antibody was removed by washing the cells in permeabilization/wash buffer. Finally, the cells were resuspended in PBS for flow cytometric analysis.

Detection of Treg Cells by Flow Cytometry

The numbers of Treg cells were also determined by BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

The mouse Treg multicolor flow cytometry kit was obtained from BioLegend (San Diego, CA 3231)

and contained FOXP3 (Forkhead box P3)-Alexa Fluor 488, CD4-perCp, CD25-PE, a cell staining buffer, and a single color compensation control. According to the manufacturer's manual, after treatment of the pellet with RBC lysis buffer and washing, the cells of interest were suspended in cell staining buffer. Then, the FOXP3 fixation/permeabilization solution was added to the cells, and following incubation, the cells were centrifuged and washed with FOXP3 permeabilization buffer. Following incubation, the anti-mouse FOXP3-Alexa Fluor, CD25-PE, CD4-perCp antibody cocktail or Alexa Fluor 488 rat IgG2b kappa isotype control, CD25-PE, and CD4-perCp antibody cocktail were added into an

Table 2. Challenge Phase

Explanation	Groups					
	1/1	2/1	2	3	4	5
Prescription materials	Normal saline	Derp2	Derp2	Derp2	Derp2	Derp2
Number of mice	5	5	6	6	6	6
Injection	IN	IN	IN	IN	IN	IN
Sensitization protocol (days)	28–29–30–31–32–	28–29–30–31–32–	28–29–30–31–32–	28–29–30–31–32–	28–29–30–31–32–	28–29–30–31–32–
	33–34–35–36–	33–34–35–36–	33–34–35–36–	33–34–35–36–	33–34–35–36–	33–34–35–36–
	37	37	37	37	37	37
Bleeding area	Retro-orbital	Retro-orbital	Retro-orbital	Retro-orbital	Retro-orbital	Retro-orbital
Bleeding times (days)	40	40	40	40	40	40
Lung removed (day)	40	40	40	40	40	40
Experimental conducted	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

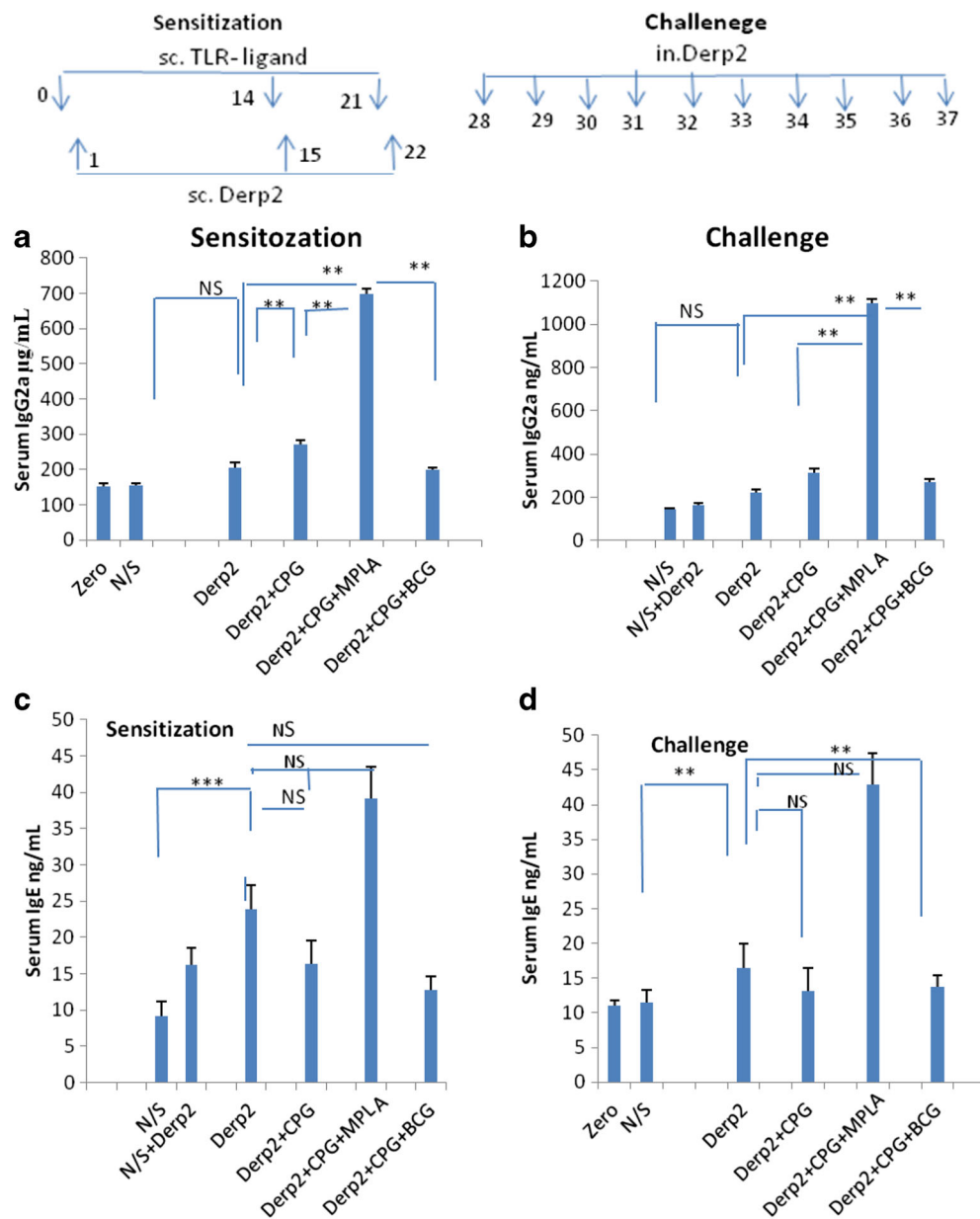


Fig. 1. AA section shows the time line of sensitization and challenge phases. The serum levels of immunoglobulins (IgE and IgG2a) in mice pretreated with TLR agonists (CPG, CPG + MPL, and CPG + BCG) at the sensitization (**a, c**) and challenge (**b, d**) phases. The serum levels of immunoglobulins were measured on days 0, 23, and 40. The results are presented as mean $\pm$ SEM for 5–6 mice/group. NS, **, and *** represent the *P* values of >0.05 (nonsignificant), <0.05 , and ≤ 0.01 , respectively. **a, b** The serum levels of IgG2a in the Derp2-sensitized group pretreated with CPG, CPG plus MPLA, and CPG plus BCG group were significantly higher than Derp2-sensitized control mice in sensitization and challenge phases, respectively. **c, d** The serum levels of IgE in Derp2-sensitized mice were higher than saline-administrated control group in sensitization and challenge phases, respectively. **d** The serum levels of IgE in the Derp2-sensitized group pretreated with CPG plus BCG were significantly decreased in comparison with Derp2-sensitized control mice.

appropriate tube. After incubation, the cells were washed twice with cell staining buffer and then were suspended in cell staining buffer after and analyzed

with flow cytometer using an appropriate instrument setting. The system uses BD multiset software for data analysis.

Lung Preparation

The mice were sacrificed 72 h after the last Derp2 challenge on day 40. The lungs were then separated and were fixed in 4% formalin for 48 h. Then the left lobes of the lung were cut vertically directly above the main bronchus entry, and after that, the formalin was removed and the tissues were embedded in paraffin wax. The tissue slices were prepared in a thickness of 2–3 mm from the anterior to posterior direction and placed on microscopy slides [28].

Lung Staining

The lung slices were stained after the baking of slices at 65 °C for 45 min. Hematoxylin and eosin (H&E) reagent (Merck, Darmstadt, Germany) was used for the evaluation of inflammatory changes. Periodic acid-Schiff (PAS) reagent (Sigma-Aldrich, Germany) was used for the assessment of goblet cells and mucus production. The degree of inflammation and mucus production was scored by the following scale: 0 = no inflammation or mucus, 1 = mild inflammation or mucus, 2 = moderate inflammation or mucus, and 3 = strong inflammation or mucus [28, 29].

Statistical Analysis

Data are presented as mean $\pm$ SEM. The variables between different groups were evaluated by one-way ANOVA or unpaired *t* test. One-way analysis of variance together with Tukey's *post hoc* test was used for comparison of variables between the pretreated groups *versus* the Derp2 control group. *P* values less than 0.05 were considered significant. The software such as Microsoft Office 2007, Microsoft office Excel, and SPSS version 23 were used for analysis and presentation of data.

RESULTS

The Effects of Adjuvants on the IgG2a Levels in the Sensitization Phase

As demonstrated in Fig. 1a, the serum levels of IgG2a in Derp2-sensitized mice (205.87 ± 16.54 $\mu\text{g/mL}$) were not significantly changed in comparison with those in the saline-administrated control group (154.58 ± 6.9 $\mu\text{g/mL}$, $P = 0.07$). The serum levels of IgG2a in the Derp2-sensitized group pretreated with CPG plus MPLA (700.06 ± 13.04 $\mu\text{g/mL}$) and in the Derp2-sensitized group pretreated with CPG (269.60 ± 16.03 $\mu\text{g/mL}$) were significantly higher than in the Derp2-sensitized control mice

(205.87 ± 16.54 $\mu\text{g/mL}$, $P < 0.03$). The serum levels of IgG2a in the Derp2-sensitized group pretreated with CPG plus MPLA were also significantly higher than in the Derp2-sensitized mice pretreated with CPG ($P < 0.004$). Moreover, the serum levels of IgG2a in the Derp2-sensitized group pretreated with CPG plus MPLA were significantly increased as compared with those in the Derp2-sensitized group pretreated with CPG plus BCG (198.63 ± 7.54 $\mu\text{g/mL}$, $P < 0.002$).

The Effects of Adjuvants on the IgG2a Levels in the Challenge Phase

After the challenge phase, the serum levels of IgG2a in Derp2-sensitized mice (219.80 ± 16.28 $\mu\text{g/mL}$) were not significantly changed as compared with those in the saline-administrated control group (144.07 ± 6.93 $\mu\text{g/mL}$, $P = 0.10$) (Fig. 1d). The serum levels of IgG2a in the Derp2-sensitized group pretreated with CPG plus MPLA (1098.08 ± 19.00 $\mu\text{g/mL}$) were significantly higher than in the Derp2-sensitized control group and Derp2-sensitized mice pretreated with CPG (314.96 ± 15.81 $\mu\text{g/mL}$, $P < 0.003$ and $P < 0.01$, respectively). The serum levels of IgG2a in the Derp2-sensitized group pretreated with CPG plus MPLA were significantly increased as compared with those in Derp2-sensitized mice pretreated with CPG plus BCG (270.41 ± 13.28 $\mu\text{g/mL}$, $P < 0.006$) (Fig. 1b).

The Effects of Adjuvants on the IgE Levels in the Sensitization Phase

The serum levels of IgE in Derp2-sensitized mice (16.41 ± 3.43 $\mu\text{g/mL}$) were higher than in the saline-administrated control group (11.51 ± 1.76 $\mu\text{g/mL}$), but the difference was not statistically significant ($P = 0.07$). Moreover, the serum levels of IgE in Derp2-sensitized groups pretreated with adjuvants were not significantly changed in comparison with those in Derp2-sensitized mice ($P \geq 0.08$) (Fig. 1c).

The Effects of Adjuvants on the IgE Levels in the Challenge Phase

The serum levels of IgE in Derp2-sensitized mice (23.84 ± 3.38 $\mu\text{g/mL}$) were significantly higher than in the saline-administrated control group (9.19 ± 1.99 $\mu\text{g/mL}$, $P = 0.01$). Moreover, the serum levels of IgE in the Derp2-sensitized group pretreated with CPG plus BCG (12.7 ± 1.87 $\mu\text{g/mL}$) were significantly decreased in comparison with those in Derp2-sensitized control mice ($P = 0.04$) (Fig. 1d).

The Effects of Adjuvants on the IFN- γ in the Sensitization Phase

As demonstrated in Fig. 2a, the difference of the serum levels of IFN- γ between Derp2-sensitized control mice and saline-administrated control group was not significant (97.60 ± 11.61 vs 122.16 ± 6.77 ng/L, $P = 0.40$). The serum levels of IFN- γ in the Derp2-sensitized group pretreated with CPG plus MPLA (460.76 ± 14.60 ng/L) were also significantly increased in comparison with those in the Derp2-sensitized control group ($P < 0.05$).

The Effects of Adjuvants on the IFN- γ in the Challenge Phase

The serum levels of IFN- γ were similarly expressed in Derp2-sensitized control mice and saline-administrated control group (115.3 ± 11.43 vs 117.37 ± 7.47 ng/L, $P = 0.90$). The serum levels of IFN- γ in the Derp2-sensitized group pretreated with CPG plus MPLA (494.96 ± 15.50 ng/L) were significantly increased in comparison with those in the Derp2-sensitized control group ($P < 0.003$) (Fig. 2b).

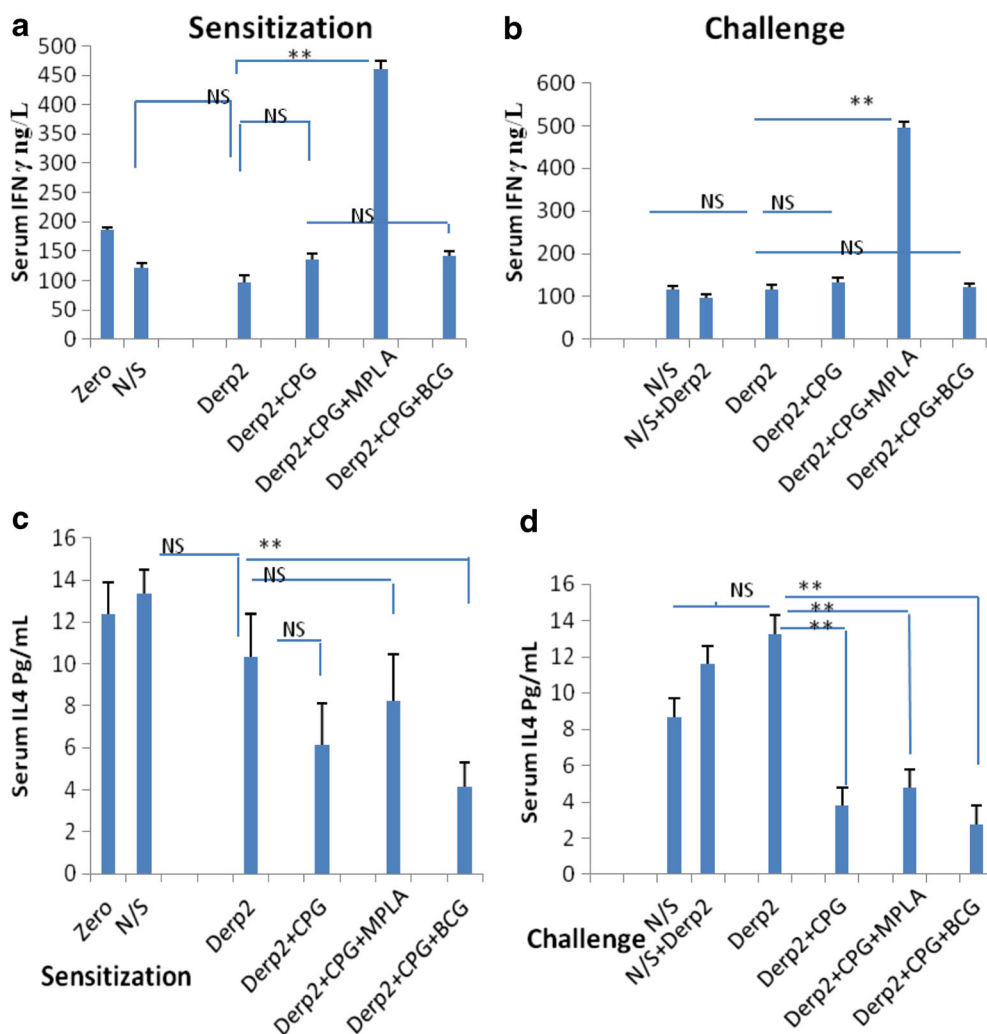


Fig. 2. The serum levels of cytokines (IL-4 and IFN- γ) in mice pretreated with TLR agonists (CPG, CPG + MPL, and CPG + BCG) at the sensitization (a, c) and challenge (b, d) phases. The serum levels of cytokines were measured on days 0, 23, and 40. The results are presented as mean $\pm$ SEM for 5–6 mice/group. NS, **, and *** represent the P values of >0.05 (nonsignificant), <0.05 , and ≤ 0.07 , respectively. a, b The serum levels of IFN- γ in the Derp2-sensitized group pretreated with CPG plus MPLA were significantly increased in comparison with the Derp2-sensitized control group in sensitization and challenge phases. c, d In the sensitization phase, the serum levels of IL-4 in the Derp2-sensitized group pretreated with CPG plus BCG were significantly decreased only, while in the challenge phase, the serum levels of IL-4 in the Derp2-sensitized group pretreated with adjuvants in the three groups were significantly decreased in comparison to the Derp2-sensitized control group.

The Effects of Adjuvants on the IL-4 in the Sensitization Phase

The serum levels of IL-4 in Derp2-sensitized control mice (10.32 ± 2.02 Pg/mL) were higher than in the saline-administrated control group (13.36 ± 1.11 Pg/mL), but the difference was not statistically significant ($P = 0.4$). However, the serum levels of IL-4 in the Derp2-sensitized group pretreated with CPG plus MPLA (8.22 ± 2.23 Pg/mL) were lower than those in Derp2-sensitized control mice ($P = 0.50$). The serum levels of IL-4 in the Derp2-sensitized group pretreated with CPG plus BCG (4.16 ± 1.10 Pg/mL) were significantly decreased as compared with those in the Derp2-sensitized control group ($P < 0.02$) (Fig. 2c).

The Effects of Adjuvants on the IL-4 in the Challenge Phase

As demonstrated in Fig. 2d, the serum levels of IL-4 in Derp2-sensitized control mice (13.28 ± 2.00 Pg/mL) were higher than in the saline-administrated control group (8.68 ± 2.22 Pg/mL), but the difference was not statistically significant ($P = 0.10$). However, the serum levels of IL-4 in the Derp2-sensitized group pretreated with CPG (3.80 ± 1.97 Pg/mL) and in the Derp2-sensitized group pretreated with CPG plus MPLA (4.78 ± 1.82 Pg/mL) were significantly decreased in comparison with those in Derp2-sensitized control mice ($P < 0.01$). The serum levels of IL-4 in the Derp2-sensitized group pretreated with CPG plus BCG (2.77 ± 1.02 Pg/mL) were significantly decreased as compared with those in the Derp2-sensitized control group ($P = 0.001$).

The Effects of Adjuvants on Th1 and Treg Cells in the Sensitization Phase

Figure 3a shows examples of whole blood cells stained for CD4 positive surface and IFN- γ and T-bet intracellular expression. Figure 3b also illustrates examples of whole blood cells stained for CD4 and CD25 positive surface and FOXP3 intracellular expression. As demonstrated in Fig. 3(A), the peripheral blood counts of Th1 cells in Derp2-sensitized mice (0.67 ± 0.76) were lower than in the saline-administrated control group (1.33 ± 0.45), but the difference was not statistically significant ($P = 0.20$). The peripheral blood counts of Th1 cells in Derp2-sensitized mice pretreated with CPG plus BCG (1.26 ± 0.32) or CPG (1.64 ± 0.75) were not significantly changed in comparison with those in the Derp2-sensitized control group ($P = 0.10$ and $P = 0.20$, respectively). The peripheral blood

Fig. 3. The flow cytometry plots showing the percentages of Th1 cells and Treg cells in mice pretreated with TLR agonists. **a** Examples of whole blood cells stained for anti-CD4⁺ conjugated with PE surface and anti-IFN- γ conjugated with FITC and anti-T-bet conjugated with PerCP intracellular expression. **b** Examples of whole blood cells stained for anti-CD4⁺ conjugated with PerCP and anti-CD25⁺ conjugated with PE surface and anti-FOXP3 conjugated with Alga Fluor 488 intracellular expression. The figures contain numbers in quadrants indicating percentage of CD4⁺ T-bet⁺ IFN- γ ⁺ Th1 and CD4⁺ CD25⁺ FOXP3⁺ cells. **A, B** The blood number of CD4⁺ T-bet⁺ IFN- γ ⁺ Th1 cells in mice pretreated with TLR agonists in sensitization and challenge phases. The sections illustrate that the peripheral blood counts of Th1 cells in Derp2-sensitized mice pretreated with CPG plus MPLA were significantly higher than Derp2-sensitized control mice in the sensitization phase and the peripheral blood counts of Th1 cells in Derp2-sensitized mice pretreated with CPG, CPG plus MPLA, or BCG were significantly increased in comparison with Derp2-sensitized control mice in the challenge phase. **C, D** The blood number of and CD4⁺ CD25⁺ FOXP3⁺ Treg cells in mice pretreated with TLR agonists in the sensitization and challenge phases. The **C** section shows that the peripheral blood counts of Treg cells in Derp2-sensitized mice pretreated with CPG and CPG plus MPLA were significantly higher than in Derp2-sensitized control mice in the sensitization phase. The **D** section reveals that the peripheral blood counts of Treg cells in Derp2-sensitized mice pretreated with CPG plus MPLA or BCG groups were significantly lower than Derp2-sensitized control mice in the challenge phase. **E** The blood number of CD4⁺ IL-12R⁺ Th1 cells in mice pretreated with TLR agonists in the sensitization and challenge phases. The results are presented as mean $\pm$ SEM for 5–6 mice/group. NS and ** represent the P values of >0.05 (nonsignificant) and <0.05 , respectively.

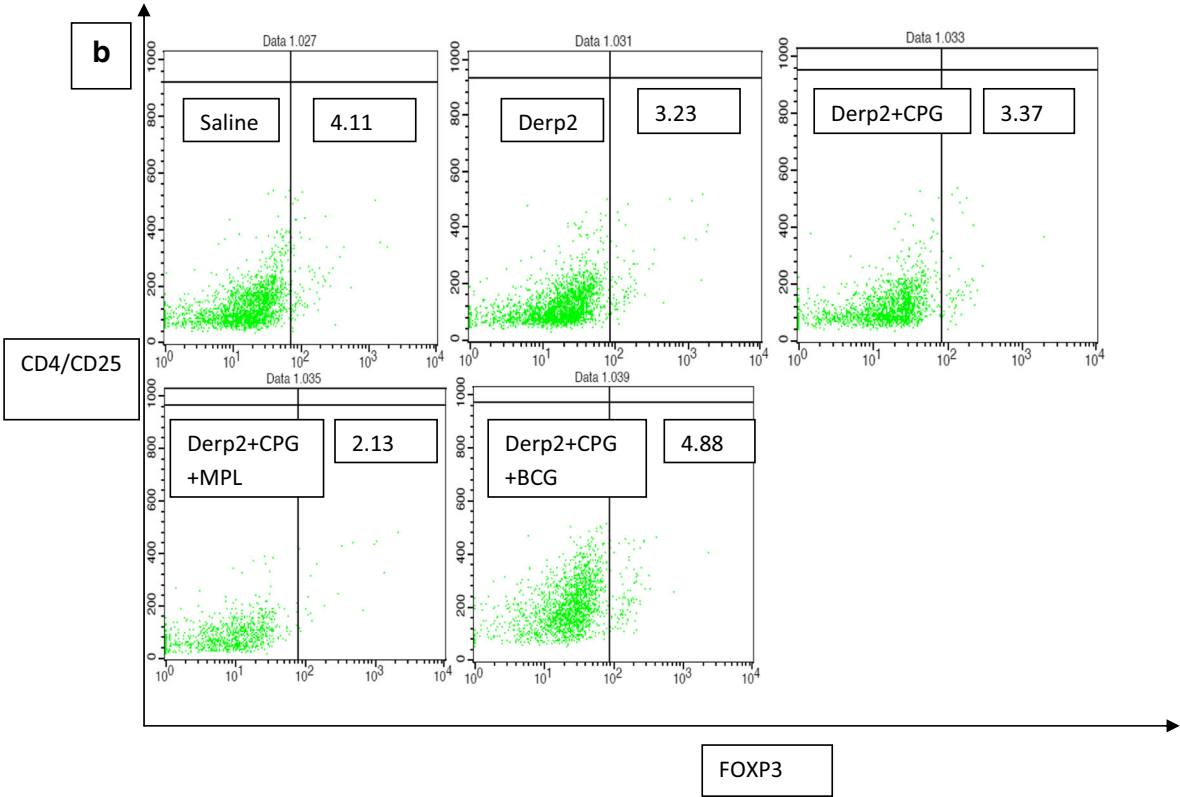
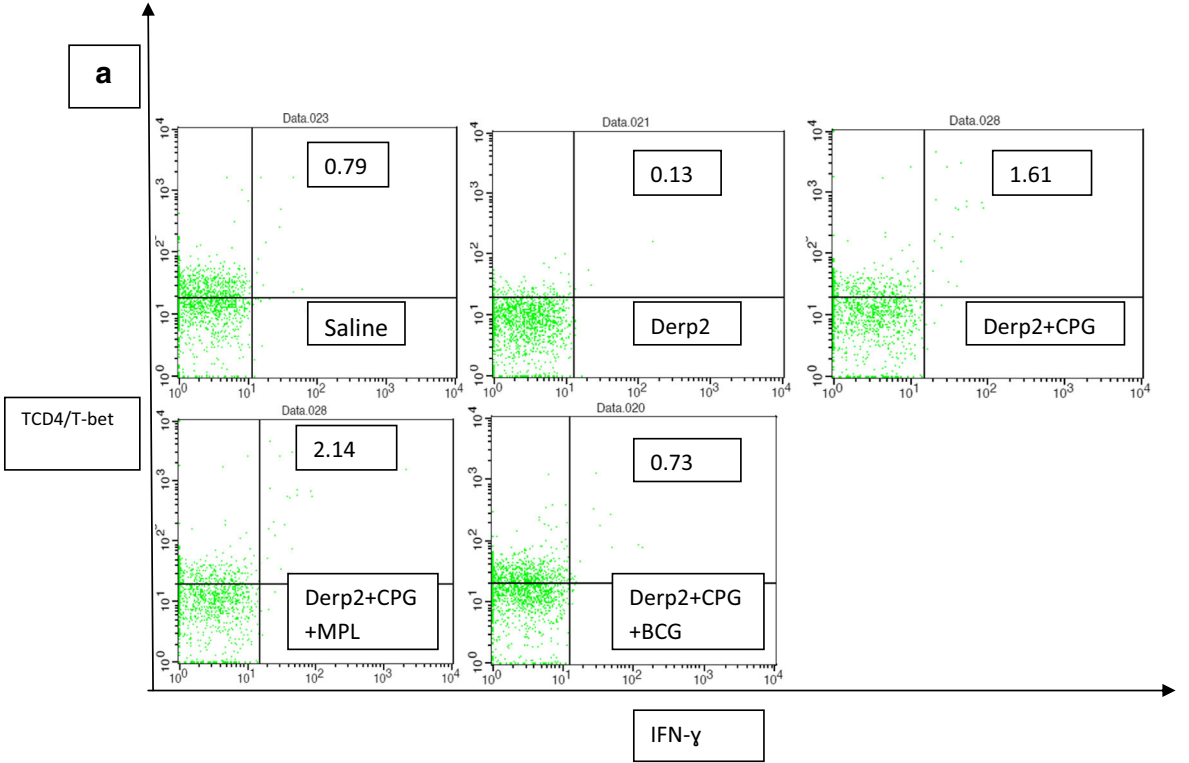
counts of Th1 cells in Derp2-sensitized mice pretreated with CPG plus MPLA (1.81 ± 0.75) were significantly higher than in Derp2-sensitized control mice ($P < 0.05$).

The peripheral blood counts of Treg in Derp2-sensitized mice (2.53 ± 1.29) were lower than in the saline-administrated control group (3.02 ± 0.65), but the difference was not statistically significant ($P = 0.20$). The peripheral blood counts of Treg cells in Derp2-sensitized mice pretreated with CPG plus MPLA (4.31 ± 0.64) were significantly higher than in Derp2-sensitized control mice ($P < 0.05$). Moreover, the peripheral blood frequency of Treg cells in Derp2-sensitized mice pretreated with CPG (5.40 ± 1.27) was significantly elevated in comparison with that in Derp2-sensitized control mice ($P < 0.001$) (Fig. 3(C)).

The Effects of Adjuvants on Th1 and Treg Cells in the Challenge Phase

The peripheral blood counts of Th1 in Derp2-sensitized mice (0.13 ± 0.75) were significantly lower than in the saline-administrated control group (0.79 ± 0.45 , $P < 0.009$). The peripheral blood counts of Th1 cells in

Effects of CPG, MPLA, and BCG in a Mouse Model of Asthma



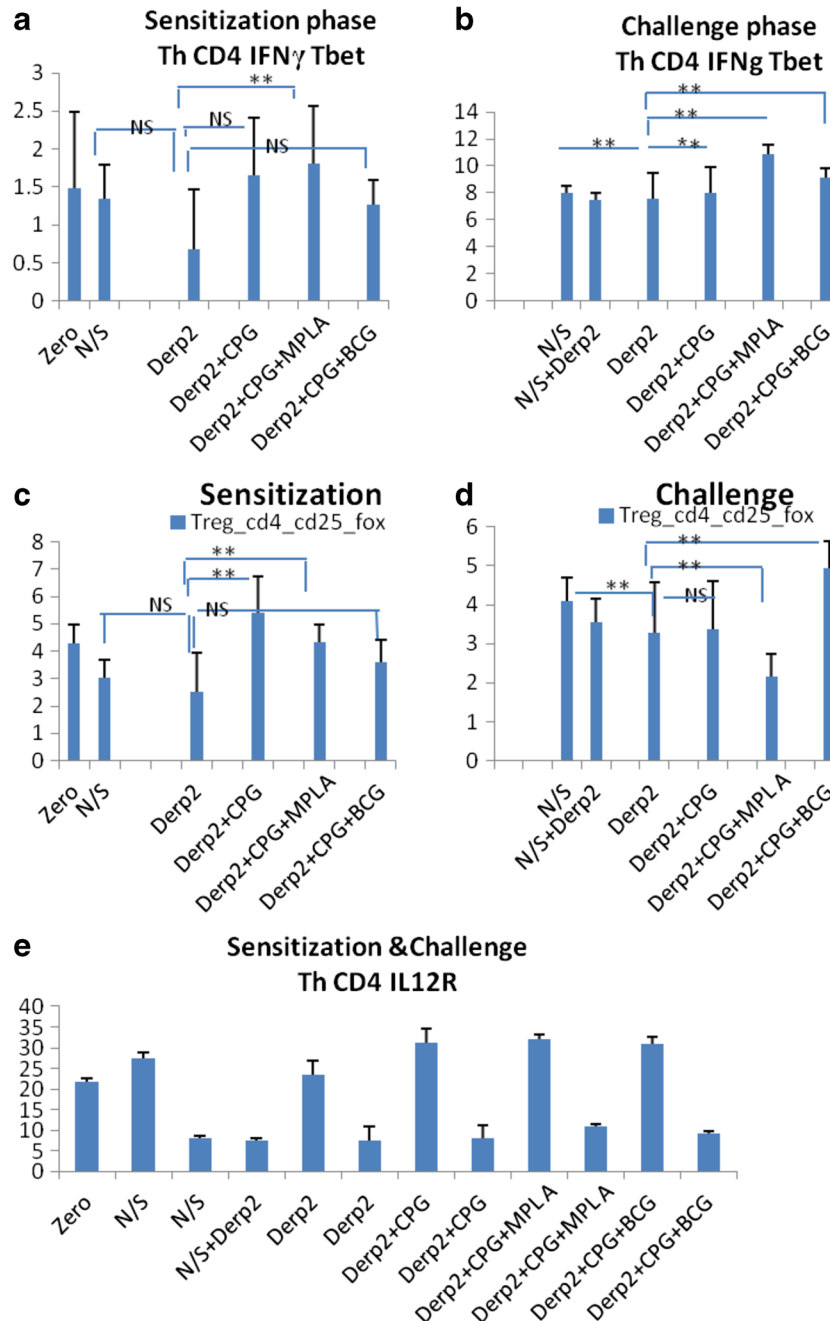


Fig. 3. (continued)

Derp2-sensitized mice pretreated with CPG plus BCG (0.73 ± 0.30) or CPG (1.31 ± 0.74) were significantly increased in comparison with those in Derp2-sensitized control mice ($P < 0.002$ and $P < 0.004$, respectively). The peripheral blood counts of Th1 cells in Derp2-sensitized mice pretreated with CPG plus MPLA (1.75 ± 0.84) were

significantly higher than in Derp2-sensitized control mice ($P < 0.01$) (Fig. 3(B)).

The peripheral blood counts of Treg in Derp2-sensitized mice (3.28 ± 1.28) were significantly lower than in the saline-administrated control group (4.08 ± 0.61 , $P < 0.02$). The peripheral blood counts of Treg cells in

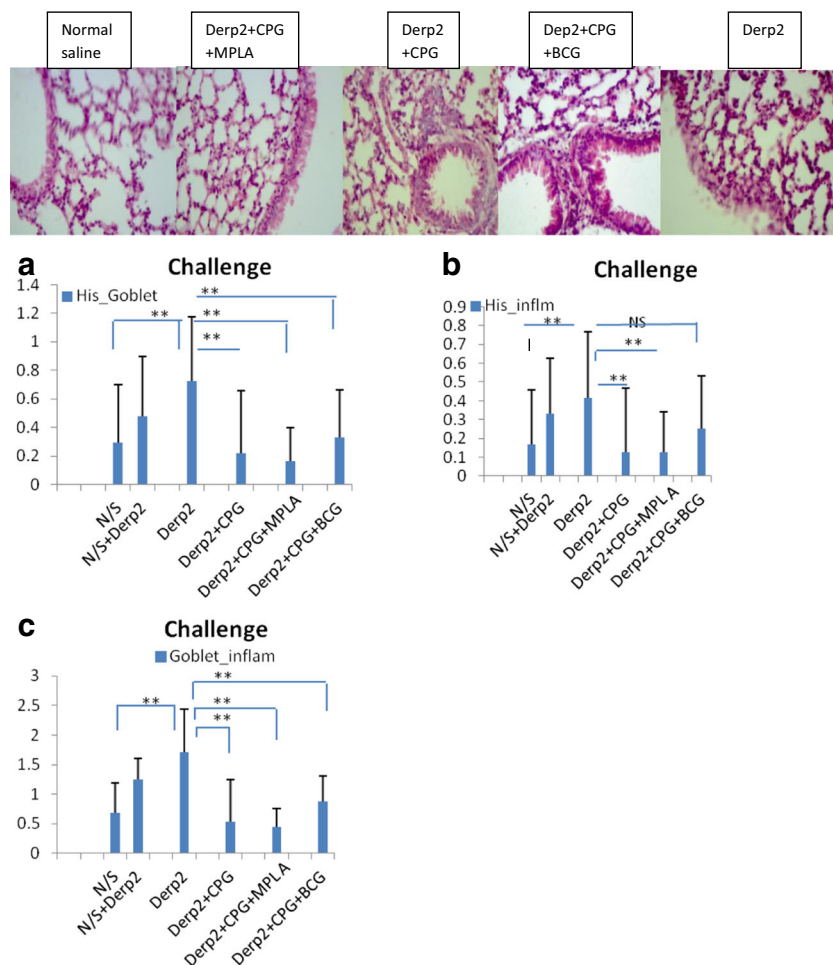


Fig. 4. Comparison of the histological evaluation of lung inflammation at the challenge phase in the Derp2-sensitized control group, saline-administrated control group, and those pretreated with CPG + MPL, CPG, and CPG + BCG. Paraffin-embedded lung sections were prepared 72 h after the last Derp2 challenge and stained with hematoxylin-eosin (H&E). Data are exhibited at a magnification of $\times 400$ and representative of five randomly selected tissues. The score of goblet cells (goblet cell hyperplasia and mucus secretion) and inflammation (mast cells in airway epithelial layer, subepithelial fibrosis, airway smooth muscle and vascular changes) and also goblet cell score plus inflammation score in mice pretreated with TLR agonists (CPG, CPG + MPL, and CPG + BCG) at the challenge phases (a–c). The score of goblet cells and inflammation of the lung were measured on day 40. The figure shows that the differences between groups regarding the score of goblet cells, inflammation, and goblet cells plus inflammation were significant except for the difference between Derp2-sensitized mice pretreated with CPG plus BCG with Derp2-sensitized control mice which was not significant. The results are presented as mean $\pm$ SEM for 5–6 mice/group. NS and ** represent the P values of >0.05 (nonsignificant) and <0.05 , respectively.

Derp2-sensitized mice pretreated with CPG plus MPLA group (2.16 ± 0.57) were significantly lower than in Derp2-sensitized control mice ($P < 0.01$). Moreover, the peripheral blood frequency of Treg cells in Derp2-sensitized mice pretreated with CPG (3.36 ± 1.26) was elevated in comparison with that in the Derp2-sensitized control group, but the difference was not statistically significant ($P = 0.70$). The peripheral blood frequency of Treg cells in Derp2-sensitized mice pretreated with CPG plus BCG ($4.93 \pm$

0.70) was significantly elevated in comparison with that in Derp2-sensitized control mice ($P < 0.005$) (Fig. 3(D)).

The Effects of Adjuvants on the $CD4^+$ IL-12R $^+$ T Cells in the Sensitization Phase

In the Derp2-sensitized group, the frequency of $CD4^+$ IL-12R $^+$ T cells (23.51 ± 3.44) was significantly reduced as compared with that in the saline-administrated control

group (27.47 ± 1.46 , $P < 0.05$). The peripheral blood frequency of $CD4^+$ IL-12R $^+$ T cells in Derp2-sensitized mice pretreated with CPG plus BCG (30.99 ± 1.58) was slightly lower than in Derp2-sensitized mice pretreated with CPG plus MPLA (31.87 ± 1.24 , $P = 0.63$) (Fig. 3(E)).

The Effects of Adjuvants on the IL-12R $^+$ CD4 $^+$ T Cells in the Challenge Phase

In the Derp2-sensitized group, the frequency of $CD4^+$ IL-12R $^+$ T cells (7.54 ± 3.38) did not significantly change as compared with that in the saline-administrated control group (8.02 ± 0.51 , $P = 0.1$). The peripheral blood frequency of $CD4^+$ IL-12R $^+$ T cells in Derp2-sensitized mice pretreated with CPG plus BCG (9.1 ± 0.70) was significantly lower than in Derp2-sensitized mice pretreated with CPG plus MPLA (10.86 ± 0.74 , $P < 0.01$). Our results also demonstrated that the frequencies of $CD4^+$ IL-12R $^+$ T cells in Derp2-sensitized mice pretreated with adjuvants were significantly decreased as compared with those in the equal groups in the sensitization stages ($P < 0.009$) (Fig. 3(E)).

The Effects of Adjuvants on the Inflammatory Parameters Following the Challenge Phase

The inflammation and mucus secretion scores were increased significantly in the Derp2-sensitized group (1.14 ± 0.56 and 1.71 ± 0.84 , respectively) in comparison with those in the saline-administrated control group (0.46 ± 0.50 and 0.69 ± 0.75 , $P < 0.02$ and $P < 0.03$, respectively).

The scores of inflammation and mucus secretion were significantly decreased in the Derp2-sensitized group pretreated with CPG-ODN plus MPLA (0.29 ± 0.31 and 0.43 ± 0.45 , respectively) in comparison with those in the Derp2-sensitized control group ($P < 0.006$ and $P < 0.005$, respectively). The scores of inflammation and mucus secretion were also significantly decreased in the Derp2-sensitized group pretreated with CPG-ODN (0.34 ± 0.55 and 0.51 ± 0.70 , respectively) in comparison with those in the Derp2-sensitized control group ($P < 0.006$ and $P < 0.01$, respectively). The inflammation and mucus secretion scores were decreased in the Derp2-sensitized group pretreated with CPG-ODN plus BCG (0.58 ± 0.44 and 0.77 ± 0.66 , respectively) in comparison with those in the Derp2-sensitized group ($P = 0.07$ and $P < 0.02$, respectively) (Fig. 4a–c).

DISCUSSION

The effects of adjuvants CPG, MPLA, and BCG on Th1 cell polarization have been demonstrated in various studies. The inhibitory role of these adjuvants on allergic reactions has been also evaluated in a number of investigations concerning cytokines, immunoglobulins, immune cells, and lung inflammatory parameters [24, 27, 30].

In the present study, we have investigated the effects of the mentioned adjuvants on atopic asthma and related immunological parameters in a mouse model. Accordingly, CPG, MPLA, and BCG were used prior to sensitization with allergen Derp2 and then mice were subjected to the challenge phase by administration of allergen Derp2 alone.

In most animal models of asthma, ovalbumin was used for the induction of allergy; however, this allergen was not considered as a common allergen in human atopic asthma [31, 32]. Therefore, the allergen Derp2 was used in this study, because it is a major allergen for the induction of atopic asthma in humans [27, 33].

The allergen Derp2 facilitates the aggregation of TLR4 which is necessary for signal transduction from this TLR. Indeed, allergen Derp2 may act either as an allergen or as an auto-adjuvant [34]. Accordingly, it seems that the using allergen Derp2 gives new insights for better interpretation of adjuvant effects.

Based on clinical and experimental immunotherapeutic observations, the deviation of immunologic responses from Th2 to Th1 response is the basis of most therapeutic strategies. However, the risk of allergic disease relapse is almost 55% during the 3 years after immunotherapy, due to the persistence of the memory cells and their contribution in the pathogenesis of atopic asthma [35, 36]. Hence, this investigation was designed to induce a specific adaptive immune response and establish a continuous immunological memory from early life that confers protection against asthma disease by using a very young susceptible animal model of healthy mice.

The induction of adaptive immune responses is dependent on the signal from the innate immune responses [37]. The adjuvants CPG, MPLA, and BCG act as TLR agonists that stimulate the TLR9, TLR4, and TLR4 plus TLR2, respectively, and induce the Th1 cell-associated cytokines and related immunoglobulin production [15, 24, 38].

In contrast to other studies, this study was carried out during early life in mice which presented unexpected results, probably due to the immaturity of the immune system at this time of life [39].

In the present study, for the first time, the immunological parameters including cytokines, immunoglobulins, and T lymphocyte subsets (Th1 and Treg cells) were assessed in both immunization and challenge phases of acute asthma.

Similar to type 1 hypersensitivity reactions, IgG2a that reflects the Th1 cell-related responses was increased in the immunization period, whereas IL-4 (as Th2-type cytokine) was decreased in the challenge phase in Derp2-sensitized mice pretreated with CPG compared with the Derp2-sensitized control group [40].

The serum levels of IgE were significantly increased in the Derp2-sensitized control group as compared with those in the saline-administrated control group at the challenge phase, whereas the serum levels of IgG2a, IFN- γ , and IL-4 did not significantly change between the Derp2-sensitized and saline-administrated control groups. This phenomenon probably indicates either an immaturity of the immune system during time points of investigation or may be attributed to the administration of Derp2 alone without using any adjuvant.

The result of this study also demonstrated that the serum levels of IgG2a in the Derp2-sensitized group pretreated with CPG plus MPLA or CPG were significantly higher than in Derp2-sensitized mice at the sensitization and/or challenge phases. Moreover, the IgG2a levels in the Derp2-sensitized group pretreated with CPG plus BCG group were lower than in the Derp2-sensitized group pretreated with CPG at the sensitization phase ($P = 0.07$). These observations represent that the adjuvant CPG has synergistic effects with MPLA, regarding the induction of IgG2a synthesis. Furthermore, BCG has inhibitory effects on the IgG2a production in the sensitization phase. BCG acts as a TLR2 and TLR4 agonist that may activate Treg cells and in turn cause the suppression of Th1 cells that results in the diminished IgG2a production during the sensitization phase. In accordance with our finding, it has been demonstrated that pretreatment with BCG increases Treg cell function and decreases airway inflammatory reactions in an animal model of asthma [41].

The result of the present study also demonstrated that the serum levels of IgE did not alter between Derp2-sensitized mice pretreated with CPG, CPG plus MPLA, and CPG plus BCG compared with those in the Derp2-sensitized control group at the sensitization phase. However, the serum levels of IgE in the Derp2-sensitized group pretreated with CPG plus BCG were significantly decreased as compared with the Derp2-sensitized control group at the challenge phase. These observations may be due to the prolonged effects of BCG on IgE production due

to the high persistence of bacteria. It has been demonstrated that BCG bacilli persisted long-time postvaccination that perpetuate CD4⁺ T effector memory cells and protection against tuberculosis [42].

The result of this investigation also indicated that the serum levels of IFN- γ in the Derp2-sensitized group pretreated with CPG plus MPLA were significantly higher than those in Derp2-sensitized mice pretreated with CPG at both the sensitization and challenge phases. These observations represent that the adjuvant CPG has synergistic effects with MPLA, regarding the induction of IFN- γ synthesis. In agreement with our results, it has been demonstrated that stimulation of macrophages by the combination of anti-CD40, CpG, and MPLA results in the higher production of IL-12, IFN- γ , and MCP-1 in comparison to the combinations of any two of these reagents [43].

In this study, there were no significant differences between Derp2-sensitized mice pretreated with CPG plus MPLA or CPG and Derp2-sensitized control group regarding serum IL-4 levels at the sensitization phase. However, the serum levels of IL-4 were significantly decreased in the Derp2-sensitized group pretreated with CPG plus BCG in comparison with those in Derp2-sensitized control mice at both the sensitization and challenge phases. These alterations in IL-4 levels can be attributed to the BCG effects rather than CPG and MPLA. The downregulation of Th2 cell-related immune responses (such as inhibition of IL-4, IgE, and IgG1 production) by treatment with BCG has been demonstrated in ovalbumin-induced airway inflammation in BALB/c mice which is in agreement with our results [44].

The result of the present investigation also indicated that the blood Treg cell frequencies in Derp2-sensitized mice pretreated with CPG plus MPLA were significantly increased and decreased in the sensitization and challenge phases compared with those in the Derp2-sensitized control group, respectively. The reason for these observations is not exactly clear. In the periphery, antigenic stimulation of naive CD4⁺ T cells in the presence of TGF- β and IL-2 results in the differentiation of naive cells into Treg cells [45]. At the sensitization phase, pretreatment with CPG and MPLA may directly influence Treg cell differentiation through the induction of TGF- β and IL-2 production. At the challenge phase, Treg cell frequencies were reduced. This phenomenon may be due to the migration of Treg cells into allergic sites in response to the inflammatory signals [46]. In the pretreated group with CPG plus BCG, the reduction of Treg cells during the challenge phase did not occur. In contrast to the pretreated group with CPG plus MPLA, in the pretreated group with CPG plus BCG, the

count of Treg cells remains at a high point during the challenge phase. Therefore, the adjuvant BCG may have very stronger effects on Treg cell differentiation than CPG and MPLA.

The result of the present study also demonstrated that the differences of blood frequencies of Th1 cells were not significant between groups pretreated with CPG and CPG plus BCG compared with the Derp2-sensitized control group at the sensitization phase; however, the frequencies of Th1 cells were significantly increased in the mentioned groups in comparison with the Derp2-sensitized control group at the challenge phase. Th1 cells are induced in response to IFN- γ and IL-12, which are secreted primarily by the dendritic cells (DCs) and natural killer (NK) cells, respectively [47]. Therefore, the used adjuvants may directly influence Th1 differentiation through induction of IFN- γ and IL-12 production at the challenge phase. MPLA, CPG, and BCG have no influence on the Th1 cell count at the sensitization phase, which may be due to the immaturity of the immune system during the early life of mice.

As it was expected, we have observed that the blood number of CD4⁺ IL-12R⁺ T cells in the pretreated mice with CPG plus MPLA and CPG plus BCG was significantly higher than in the Derp2-sensitized control group at the sensitization phase. The blood number of CD4⁺ IL-12R⁺ T cells in the pretreated group with CPG, CPG plus MPLA, or CPG plus BCG was significantly decreased at the challenge phase in comparison with that in the sensitization phase. This phenomenon may be due to the migration of Th1 cells into allergic sites or draining lymph nodes in response to the inflammatory signals [48]. The number of blood CD4⁺ IL-12R⁺ T cells in the pretreated group with CPG plus MPLA was significantly increased compared with that in pretreated mice with CPG at the challenge phase. Therefore, it seems that the adjuvant CPG may have synergistic effects with MPLA, regarding the induction of CD4⁺ IL-12R⁺ T cell differentiation.

The result of the present study also demonstrated that the hyperplasia of goblet cells and mucus secretion in the lung tissues of pretreated mice with CPG, CPG plus MPLA, or CPG plus BCG were significantly lower than those of the Derp2-sensitized control group. Both goblet cell hyperplasia and mucus secretion are Th2 cell-dependent responses [27, 49]. These observations may be due to the inhibitory effects of adjuvants on Th2 cell differentiation.

The result of this investigation also showed that the scores of lung inflammation in the pretreated group with CPG or CPG plus MPLA were significantly decreased in comparison to those in Derp2-sensitized control mice at the challenge phase. The scores of lung inflammation in

Derp2-sensitized mice pretreated with CPG plus BCG were markedly lower than in the Derp2-sensitized control group, but the difference statistically did not reach a significant value ($P = 0.07$). These data represent that the combination of CPG plus MPLA and the combination of CPG plus BCG may have a similar influence regarding the inhibition of lung inflammation. It should be noted that CPG and MPLA may have some side effects such as induction of autoimmune reactions. Therefore, it seems that the CPG and BCG combination can be a good preventive adjuvant candidate against atopic asthma for more consideration in future investigations [50, 51].

In conclusion, the results of the present study showed that BCG, MPLA, and CPG modulate Th1-, Th2-, and Treg cell-related immune responses and ameliorate the lung inflammatory parameters in a mouse model of asthma. Moreover, the adjuvant CPG had synergistic effects with MPLA, regarding the induction of Th1 cell-related responses, and the combination of CPG plus BCG may also similarly inhibit lung inflammation. The blood number of Treg cells was also significantly increased using the combination of CPG plus BCG. Therefore, it seems that the combination of CPG and BCG may be a good preventive adjuvant candidate against atopic asthma to consider in future investigations.

ACKNOWLEDGEMENTS

The authors wish to thank the authorities in the research council of Isfahan University of Medical Sciences and Iran National Science Foundation for financial support.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflicts of interest.

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