

ORIGINAL ARTICLE

***Bacillus*-derived poly- γ -glutamic acid attenuates allergic airway inflammation through a Toll-like receptor-4-dependent pathway in a murine model of asthma**

K. Lee¹, S.-H. Kim², H. J. Yoon², D. J. Paik³, J. M. Kim⁴ and J. Youn^{1,3}¹Department of Biomedical Sciences, ²Department of Internal Medicine, ³Department of Anatomy & Cell Biology and ⁴Department of Microbiology, College of Medicine, Hanyang University, Seoul, Korea

Clinical & Experimental Allergy

Summary

Background Asthma is an inflammatory disease of the airways that is mediated by Th2 responses. Poly- γ -glutamic acid (γ -PGA) is an extracellular polymeric compound that is synthesized by *Bacillus* cells. Previously, we found that γ -PGA promoted Th1 cell development in a manner dependent on antigen-presenting cells, but inhibited Th2 cell development.

Objective To investigate the effect of γ -PGA on dendritic cells (DCs), and its potential for treating Th2-mediated allergic asthma.

Methods Wild-type, Toll-like receptor (TLR)-2 deficient, and TLR-4-defective mice were used. DCs derived from the bone marrow and extracted from the lung were stimulated with γ -PGA and assayed for the expression of signalling molecules, costimulatory molecules, and cytokines. Mice were sensitized and challenged with ovalbumin (OVA) to induce asthma. They were repeatedly injected intranasally with γ -PGA before and during the challenge period, and inflammation and structural remodelling of the airways were examined.

Results γ -PGA selectively signalled conventional DCs to activate NF- κ B and mitogen-activated protein kinase, leading to the up-regulation of CD86, CD40, and IL-12, but not IL-10 and IL-6. These effects of γ -PGA were dependent on TLR-4 and independent of TLR-2. Importantly, the intranasal administration of γ -PGA to OVA-sensitized/challenged mice reduced the airway hyperresponsiveness and allergic inflammation such as leucocyte influx, goblet cell hyperplasia, eosinophilia, and Th2 cytokine production. In addition to lowered IgE titres, the treatment of mice with γ -PGA significantly reduced the multiplication and Th2 polarization of mediastinal lymph node T cells upon allergen-specific restimulation. These anti-asthmatic effects of γ -PGA were also abolished in TLR-4-defective mice.

Conclusions and Clinical Relevance Our data indicate that γ -PGA activates DCs to favour Th1 cell induction through a TLR-4-dependent pathway and alleviates pathologic symptoms in a Th2-biased asthmatic model. These findings highlight the potential of γ -PGA for the treatment of asthma and other allergic disease in which Th2 polarization plays an important role.

Keywords airway inflammation, asthma, dendritic cells, poly- γ -glutamic acid, Th2 responses, Toll-like receptor-4

Submitted 31 January 2011; revised 3 May 2011; accepted 4 May 2011

Correspondence:

Dr Jeehee Youn, Department of Anatomy and Cell Biology, College of Medicine, Hanyang University, 17 Haengdang-dong, Sungdong-gu, Seoul 133-791, Korea. E-mail: jhyoun@hanyang.ac.kr

Introduction

Asthma is a chronic inflammatory disorder of the airways characterized by episodic airway obstruction and airway hyper-responsiveness (AHR). The prevalence and morbidity caused by asthma have increased drastically during the

past several decades despite the expanding repertoire of medications [1, 2]. There is thus a need for therapeutic strategies that more effectively control this disease.

It is generally accepted that asthma is caused by an inappropriate, Th2-biased immune response to environmental allergens in genetically predisposed individuals [3,

4]. Allergen-specific Th2 cells develop in response to antigen-presenting cells (APCs), which are pulsed with allergens, and help B cells to produce IgE antibodies (Abs). The early phases of asthmatic responses are triggered by IgE-mediated degranulation of mast cells. During the late phase of asthmatic reactions, respiratory tissue infiltration by leucocytes becomes prominent and accelerates the inflammatory response. Infiltrated Th2 cells secrete IL-4, IL-5, IL-9, and IL-13, all of which are important for IgE production, eosinophilia, and pathogenic remodelling of the airways [4, 5]. Therefore, Th2 responses to allergens play a central role in both the induction and the orchestration of inflammatory responses in asthmatic airways. In view of the developmental and functional antagonism between Th1 and Th2 cells [6], rebalancing Th1 and Th2 responses has been proposed as a strategy for controlling asthma.

Factors impinging on the selective developmental pathways of Th1 and Th2 cells include pathogen-associated molecular patterns (PAMPs). PAMPs signal APCs, such as dendritic cells (DCs), to express costimulatory molecules and cytokines, which can then drive the polarization of naïve CD4⁺ T cells towards Th1 and Th2 cells. One of the PAMPs, bacterial DNA containing unmethylated CpG motifs bound to Toll-like receptor (TLR)-9 of APCs, promotes Th1 responses and inhibits Th2 responses [7, 8]. The therapeutic effects of CpG motifs have been well established in mouse models of asthma and in the human peripheral blood mononuclear cells from atopic patients [9–11]. The immunomodulatory effects of the TLR-4 ligand and lipopolysaccharide (LPS) are diverse, depending on the extent of LPS exposure. DCs activated with high- and low-dose LPS drive the development of allergen-specific Th1 and Th2 cells, respectively [12, 13]. Therefore, not only the type of PAMP but also the dose can shape aspects of adaptive immunity.

Murine DCs can be divided into two major subsets, classical or conventional DCs (cDCs) and plasmacytoid DCs (pDCs), depending on their phenotypes and functions [14]. cDCs retain the CD11c⁺CD11b⁺B220[−] phenotype, preferentially express TLR-2 and TLR-4, and play a canonical role in T cell priming. In contrast, pDCs secrete large amounts of IFN- α in response to viral infection [15]; they have the CD11c⁺CD11b[−]B220⁺ phenotype, preferentially express TLR-7 and TLR-9, and are weaker T cell stimulators and more potent regulatory T cell inducers than cDCs [16, 17]. Therefore, cDCs and pDCs seem to recognize different arrays of PAMPs and are responsible for different immunological outcomes. In other studies, lung cDCs were further subdivided into CD11c^{hi}CD11b^{lo} and CD11c^{lo}CD11b^{hi} cells, and were likely to promote Th1 and Th2 cells, respectively [18, 19]. The therapeutic effect of the FLT3 ligand was associated with increased Th1-prone DCs and decreased Th2-prone DCs in a model of allergic airway inflammation. In this context, agents that

alter the balance between DC subsets may have potential for treating established asthma.

Poly- γ -glutamic acid (γ -PGA) is an anionic polymer composed of D- and L-glutamic acid units connected by γ -amide linkages between α -amino and γ -carboxylic acid groups [20]. It is mainly produced by strains of Gram-positive bacilli during the fermentation of soybeans and is not present in mammals. We have shown previously that the presence of γ -PGA during priming promotes the development of Th1 cells and inhibits the development of Th2 cells [21]. The mechanism by which γ -PGA affects the Th1/Th2 balance involves a TLR-4-dependent action on antigen-pulsed DCs. Thus, these data led us to hypothesize that γ -PGA might have potential for treating Th2-dominated diseases such as allergic asthma.

In the present study, we further characterized the functional features of DCs signalled by γ -PGA and examined the potential of γ -PGA to suppress allergic airway disease in a murine model of asthma. We found that γ -PGA acted as a TLR-4 agonist to activate the conventional subset of bone marrow (BM)-derived DCs (BMDCs) and lung DCs, and that intranasally administered γ -PGA was capable of reducing Th2-dominated responses in the airways.

Methods

Mice

BALB/c and C57BL/6 mice (6–8 weeks old) were purchased from Orient Co. (Seongnam-si, Korea). The C.C3-*Tlr4*^{lps-d}/J strain, a BALB/c strain bearing the *Tlr4*^{lps-d} congenic interval from C3H/HeJ mice, was purchased from the Jackson Laboratory (Bar Harbor, ME, USA). C3H/HeN and C3H/HeJ mice were purchased from SLC (Shizuoka, Japan). TLR2^{−/−} and wild-type NOD mice were provided by Dr M.-S. Lee (Sungkyunkwan University, Korea). All mice were maintained in a specific pathogen-free barrier facility at Hanyang University. The study was approved by our Institutional Animal Care and Use Committee.

Reagents

γ -PGA (Bioleaders, Daejeon, Korea) was dissolved in a neutral pH buffer and diluted in PBS. All experiments presented here were carried out with 50 kDa γ -PGA. The level of endotoxin contained in a 20 μ M γ -PGA solution was <0.01 EU/mL when measured by Limulus amoebocyte lysate assays using E-Toxate kits (Sigma-Aldrich, St Louis, MO, USA). Ovalbumin (OVA), methacholine, aluminium hydroxide (alum), and LPS were purchased from Sigma-Aldrich. CpG oligodeoxynucleotides (5'-TCC ATG ACG TTC CTG ACG TT-3') were manufactured by Bioneer (Daejeon, Korea). Pam3CSK4 was from InvivoGen (San

Diego, CA, USA), and purified anti-mouse CD40 Ab was from BioLegend (San Diego, CA, USA).

Preparation and culture of DCs

BM cells were isolated by flushing femurs with 5 mL RPMI-1640 containing 10% fetal bovine serum (WelGene, Daegu, Korea). RBC-depleted BM cells were cultured in the presence of 10 ng/mL GM-CSF (Peprotech, Rocky Hill, NJ, USA) or 100 ng/mL FLT3 ligand (Peprotech) for 6 or 9 days, respectively, to generate immature BMDCs. Lungs were collected from BALB/c mice, cut into small fragments, and digested with 1 mg/mL collagenase VIII (Sigma-Aldrich) in RPMI-1640 containing 1 mg/mL DNase (Sigma-Aldrich) at 37 °C for 1 h. The digested lung tissue was filtered through a 70 μ m cell strainer (SPL, Pocheon-si, Korea) to obtain a single-cell suspension. After washing, these cells were stimulated with 1 μ g/mL LPS, 2 μ g/mL CpG oligodeoxynucleotides, 10 μ g/mL Pam3CSK4, 5 μ g/mL anti-CD40 Ab, or 1–20 μ M γ -PGA for 24–48 h, followed by FACS, Western blotting, and ELISA assays.

Western blot analysis

BMDCs (10^7 cells) were lysed in 100 μ L of ice-cold cell lysis buffer (iNtRON, Seongnam-si, Korea) for 10–20 min. The lysates were assayed using standard Western blotting methods. Horse radish peroxidase-conjugated Abs to I-kB α , phosphorylated p38, phosphorylated Erk, and β -actin (all from Cell Signaling Technology, Boston, MA, USA) were used at 1:5000 dilutions. Immunoreactive bands were visualized by ECL (Amersham Biosciences, Uppsala, Sweden).

FACS

BMDCs were stained with an appropriate mixture of monoclonal Abs (mAbs) as described [22]. Data acquisition was performed on BD FACS_{Calibur} or FACS_{Canto}. The mAbs used for FACS were as follows: anti-CD11c-FITC (BD Biosciences, San Jose, CA, USA), anti-B220-APC, anti-CD80-PE, anti-CD86-PE, anti-CD40-PE, and anti-CD11b-PerCp-Cy5.5 (all from eBioscience, San Diego, CA, USA).

Asthma induction

Wild-type BALB/c and C3 $\times$ B6-*Tlr4*^{lps-d}/J mice were sensitized intraperitoneally with 75 μ g OVA combined with 2 mg alum on days 0 and 7. OVA-sensitized mice were intranasally challenged with 50 μ g OVA in PBS on days 14, 15, 21, and 22. γ -PGA was intranasally injected at 50 or 250 mg/kg body weight/day in 50 μ L PBS on days 12, 14, 15, 19, 21, and 22. Vehicle control mice were intrana-

sally injected with PBS. Twenty four hours after the final OVA challenge on day 23, AHR was measured by enhanced pause (Penh) using a whole-body plethysmograph, as described [23]. All *ex vivo* assays were carried out 24 h after AHR measurement on day 24.

Leucocyte count in bronchoalveolar lavage fluid

Twenty-four hours after AHR measurement, bronchoalveolar lavage (BAL) fluid was collected by cannulation of the trachea and flushing of the airways with a total of 1 mL PBS. Recovered cells were treated with RBC lysis buffer, collected on cytospin slides, and stained with Diff-Quik (Sysmex Corp, Kobe, Japan) to count the leucocyte subsets.

Lung histology

The lavaged lung was subjected to a histopathologic examination using standard methods [24]. In brief, the lung tissue was fixed with 4% paraformaldehyde, embedded in paraffin, sectioned at 6 μ m, and stained with haematoxylin and eosin or haematoxylin and periodic acid-Schiff.

ELISAs

Cytokine concentrations in the culture supernatants were measured by sandwich ELISA, according to the manufacturer's instructions (BD Biosciences). Serum levels of whole IgE and OVA-specific IgE were also measured by sandwich ELISA (BD Biosciences), with modifications. Serum was collected from asthma-induced mice 48 h after the final OVA challenge. Serum collected from a severely asthmatic mouse was serially diluted and used as a standard to determine the relative levels of OVA-specific IgE in serum samples, expressed in arbitrary units/mL.

RT-PCR

Total RNA was extracted from lung tissue using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed and cDNA was amplified using specific primers for β 2 microglobulin (5'-TGA CCG GCT TGT ATG CTA TC-3' and 5'-CAG TGT GAG CCA GGA TAT AG-3'), IL-13 (5'-GAC CCA GAG GAT ATT GCA TG-3' and 5'-CCA GCA AAG TCT GAT GTG AG-3'), RANTES (5'-TGC CCT CAC CAT CAT CCT CA-3' and 5'-GGA CCG GAG TGG GAG TAG GG-3'), MCP-1 (5'-AAG CCA GCT CTC TCT TCC TC-3' and 5'-CCT CTC TCT TGA GCT TGG TG-3'), and IL-5 (5'-CTC AAG TAT TCC AGG CTG AC-3' and 5'-CTT CTC TCT CCC CAA GAA AT-3').

Culture of mediastinal lymph node cells

Mice were killed 48 h after the final OVA challenge and mediastinal lymph nodes (MLNs) were removed. They were ground and a single-cell suspension was prepared. MLN cells (10^6 cells/mL) were cultured with or without 50 µg/mL OVA, and their proliferative responses were measured as ^3H -thymidine incorporation during the last 16 of 72 h culture. Cytokine levels in the supernatants collected from 2-day cultures were measured by ELISA.

Statistical analysis

Results are presented as means $\pm$ SD in histograms and Student's *t*-test was used to determine levels of significance. Numbers of animals are given in 'Results' and figure legends. Unless indicated otherwise, three independent experiments were performed and one representative experiment is shown in the figures to represent the results.

Results

Selective γ -PGA inducibility of costimulatory molecules and cytokines in DCs

Previously, we found that γ -PGA regulated the choice of the developmental pathway of naïve CD4^+ T cells via a DC-dependent pathway [21]. To further characterize the action of γ -PGA on DCs, we examined the production of costimulatory molecules and cytokines after the stimulation of DCs with γ -PGA. Immature BMDCs that had been stimulated with either GM-CSF or the FLT3 ligand (referred to hereafter as GMDCs and FLTDCs, respectively) were further cultured in the presence or absence of γ -PGA or well-established DC activators such as LPS and CpG, followed by FACS and ELISA assays. γ -PGA increased the expression of CD86 and CD40 on the surface of GMDCs and FLTDCs in a concentration-dependent manner, although it was less potent than LPS or CpG (Fig. 1a). It did not affect the expression of CD80 significantly (data not shown). Therefore, signalling of DCs by γ -PGA appears to preferentially enhance CD86 and CD40 expression at the cost of CD80.

Activated DCs produce diverse cytokines, depending on the conditions of stimulation. Of these cytokines, the relative activities of IL-12 and IL-10 appear to be important in determining the nature of the adaptive immune

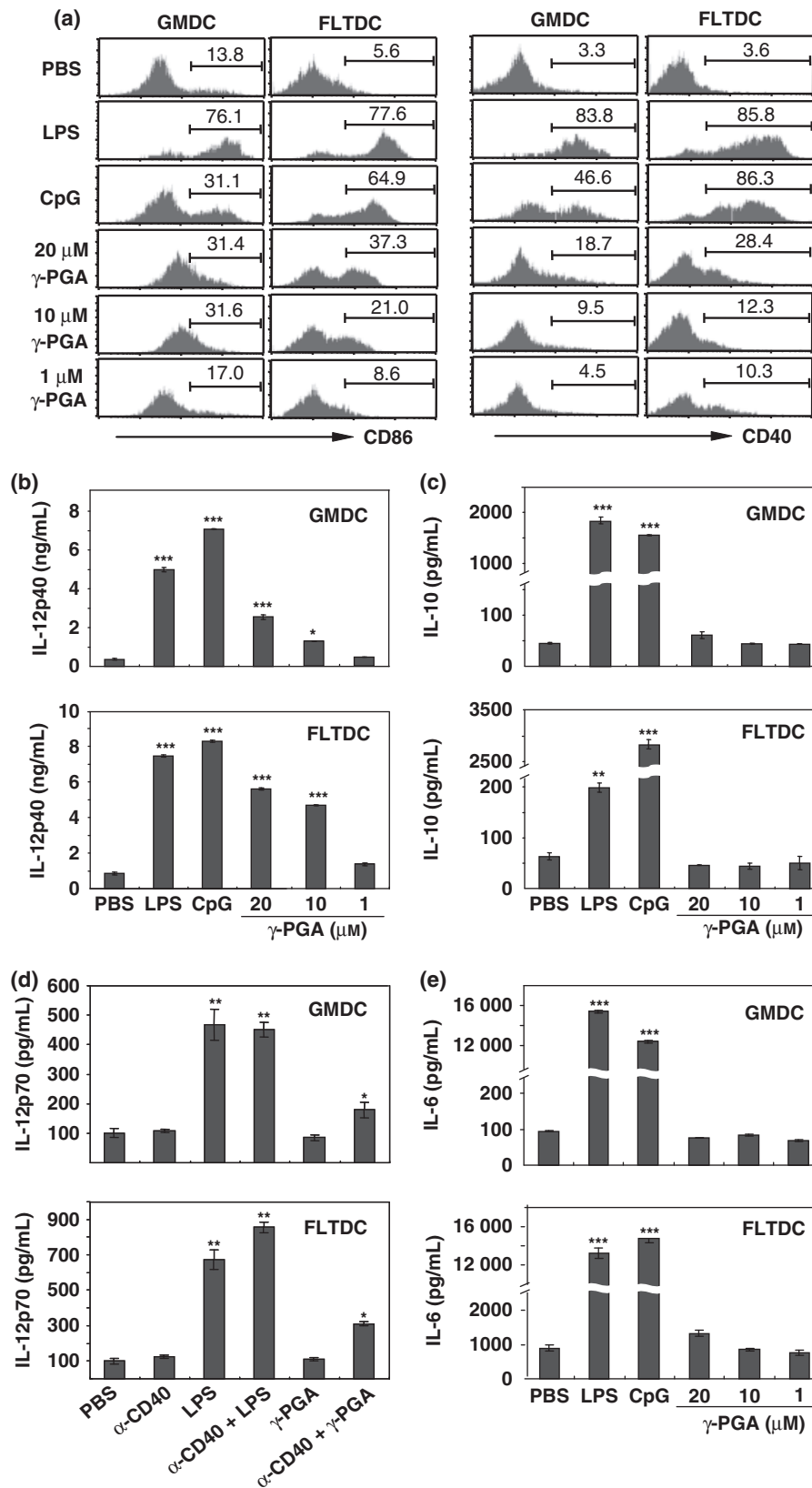
response, because IL-12 and IL-10 are critical for driving Th1 and Th2 cell development, respectively [25, 26]. In addition, IL-6 has been shown to direct Th2 cell development and to impede Th1 responses [27, 28]. We found that LPS and CpG induced DCs to produce both IL-12p40 and IL-10, but that the levels of IL-12p40 were three- to 35-fold higher than those of IL-10, confirming that these PAMPs preferentially drive Th1 polarization. γ -PGA induced both GMDCs and FLTDCs to produce IL-12p40, albeit to a lesser extent than LPS and CpG, whereas it did not induce the production of IL-10 and IL-6 (Figs 1b, c, and e). Interestingly, γ -PGA induced IL-12p70 production only when given together with anti-CD40 agonistic Ab (Fig. 1d). This synergism between γ -PGA and CD40 was not seen in the case of IL-10 and IL-6 production (data not shown). Taken together, these results demonstrate that γ -PGA signals DCs to induce cytokines that drive naïve CD4^+ T cells towards the Th1 rather than the Th2 pathway.

cDCs, but not pDCs, from the BM and lung respond to γ -PGA

Murine DCs are divided into two major subsets, cDCs and pDCs, depending on their phenotypes and functions. To determine whether γ -PGA signals discriminate between these two DC subsets, we examined the induction of costimulatory molecules by *in vitro* generated BMDCs. As reported previously [29], the FLTDCs consisted predominantly of $\text{CD11c}^+\text{CD11b}^+\text{B220}^-$ cDCs (approx. 66% of the CD11c^+ cells) and a minority of $\text{CD11c}^+\text{CD11b}^-\text{B220}^+$ pDCs (approx. 27% of the CD11c^+ cells), while nearly all the GMDCs were cDCs (Fig. 2a). Therefore, the results shown in Fig. 1 reflect the properties of cDCs rather than of pDCs. In agreement with this, cDCs responded strongly to γ -PGA as well as to LPS and CpG by expressing CD86 and CD40. In contrast, the basal levels of these molecules on unstimulated pDCs were much lower than on unstimulated cDCs, and the pDCs responded most strongly to CpG and to a lesser extent to LPS. Importantly, the pDCs barely expressed any CD86 and CD40 in response to γ -PGA (Fig. 2b). This result suggests that γ -PGA interacts with the TLRs expressed on cDCs rather than those expressed on pDCs.

Airway DCs forming a dense network in the lung sample antigens migrate to draining lymph nodes to stimulate naïve T cells [30]. Therefore, lung DCs play an important role not only in initiating specific Th2

Fig. 1. Poly- γ -glutamic acid (γ -PGA) activates bone marrow-derived dendritic cells in a dose-dependent manner. GMDCs and FLTDCs derived from C57BL/6 mice were cultured for 24 and 48 h, respectively, in the presence of 1 µg/mL LPS, 2 µg/mL CpG, or 1–20 µM γ -PGA as indicated, and assayed by fluorescence-activated cell sorting (FACS) and cytokine ELISA. (a) Representative FACS profiles gated on CD11c^+ cells. Numbers indicate the percentages of CD86^+ (left) and CD40^+ (right) cells among the CD11c^+ cells. (b, c, and e) Culture supernatants were assayed by ELISA to measure the concentrations of IL-12p40 (b), IL-10 (c), and IL-6 (e). (d) GMDCs and FLTDCs were cultured for 24 and 48 h, respectively, in the presence of 1 µg/mL LPS, 5 µg/mL anti-CD40 Ab, and/or 20 µM γ -PGA as indicated, and IL-12p70 concentrations were measured by ELISA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the PBS-treated control.



responses leading to asthma but also in restimulating effector cells during ongoing airway inflammation. To determine whether the activities of lung DCs are affected by γ -PGA, cells extracted from the lung of normal mice were stimulated with γ -PGA and assayed by FACS. γ -PGA-mediated induction of CD86 and CD40 expression was more evident in lung $\text{CD11c}^+\text{CD11b}^+\text{B220}^-$ cDCs than in lung $\text{CD11c}^+\text{CD11b}^-\text{B220}^+$ pDCs (Fig. 2c). This

result suggests that γ -PGA can modulate pulmonary immunity by targeting lung cDCs.

γ -PGA-induced DC activation is dependent on TLR-4 but not TLR-2

Although our previous work pointed to the involvement of TLR-4 in γ -PGA signalling [21], the possibility that

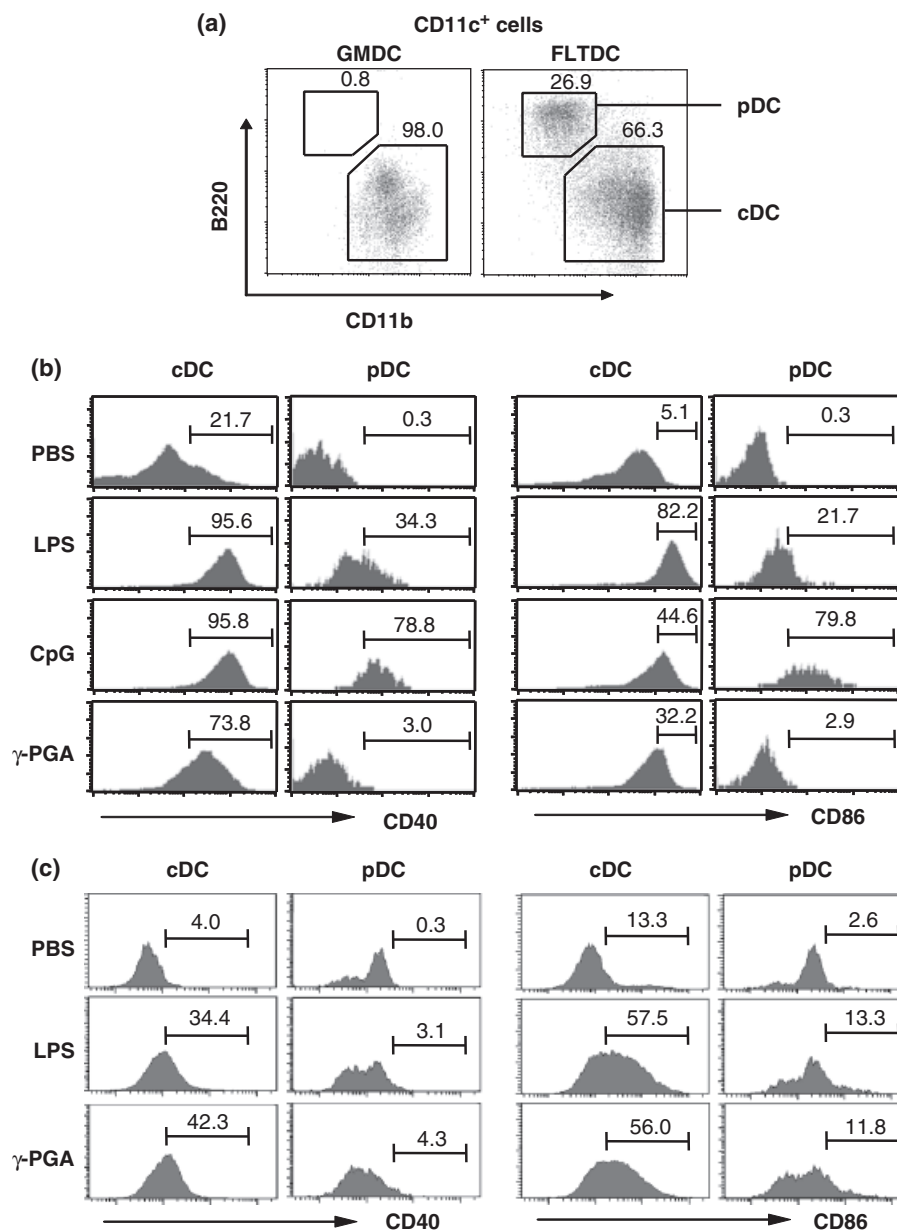


Fig. 2. Conventional DCs (cDCs), but not plasmacytoid DCs (pDCs), are activated by poly- γ -glutamic acid (γ -PGA). (a) GMDCs and FLTDCs derived from C57BL/6 mice were stained for B220, CD11c, and CD11b and assayed by fluorescence-activated cell sorting (FACS). Plots are representative FACS profiles gated on CD11c^+ cells, yielding the percentages of the indicated cells. (b) FLTDCs were cultured for 48 h in the presence of 1 $\mu\text{g}/\text{mL}$ LPS, 2 $\mu\text{g}/\text{mL}$ CpG, or 20 μM γ -PGA. The cells were stained with Abs and assayed by FACS. Representative FACS profiles gated on $\text{CD11c}^+\text{CD11b}^+\text{B220}^-$ cells (cDC) and $\text{CD11c}^+\text{CD11b}^-\text{B220}^+$ cells (pDC) with percentages of the indicated cells are shown. (c) Lung cells were treated using the same methods as (b). Representative FACS profiles gated on $\text{CD11c}^+\text{CD11b}^+\text{B220}^-$ cells (cDC) and $\text{CD11c}^+\text{CD11b}^-\text{B220}^+$ cells (pDC) with percentages of the indicated cells are shown.

γ -PGA was also recognized by TLR-2 could not be ruled out for several reasons. First, γ -PGA activates cDCs, which preferentially express TLR-2 in addition to TLR-4. Second, γ -PGA is derived from Gram-positive bacilli, which produce many TLR-2 ligands. Third, some TLR-4 ligands, such as HSP 70 and *Mycoplasma arthritidis* mitogen, are also recognized by TLR-2 [31, 32]. Therefore, we tested whether γ -PGA signals DCs via TLR-4 only or also via TLR-2.

As expected, γ -PGA, like LPS, failed to stimulate BMDCs derived from C3H/HeJ mice bearing a loss-of-function mutation in the TLR-4 gene to produce CD86, CD40, and IL-12p40, but did activate control BMDCs derived from wild-type C3H/HeN mice (Figs 3a–c). In contrast, BMDCs derived from TLR-2 knockout mice that failed to respond to Pam3CSK4, a well-known TLR-2

ligand, responded to γ -PGA essentially like TLR-2 wild-type mice (Figs 3d–f). These results demonstrate that γ -PGA signals to BMDCs through TLR-4 and not through TLR-2.

γ -PGA induces TLR-4-dependent activation of NF- κ B and MAPKs in DCs

TLR-4-triggered activation of DCs is mediated by diverse intracellular signalling transducers, including nuclear factor-kappa B and mitogen-activated protein kinase B (MAPKs), which are critical for the expression of costimulatory molecules and cytokines [33]. To determine whether γ -PGA transduces signals to DCs through these downstream molecules, we measured the levels of I- κ B α and phosphorylated MAPKs in γ -PGA-treated DCs by

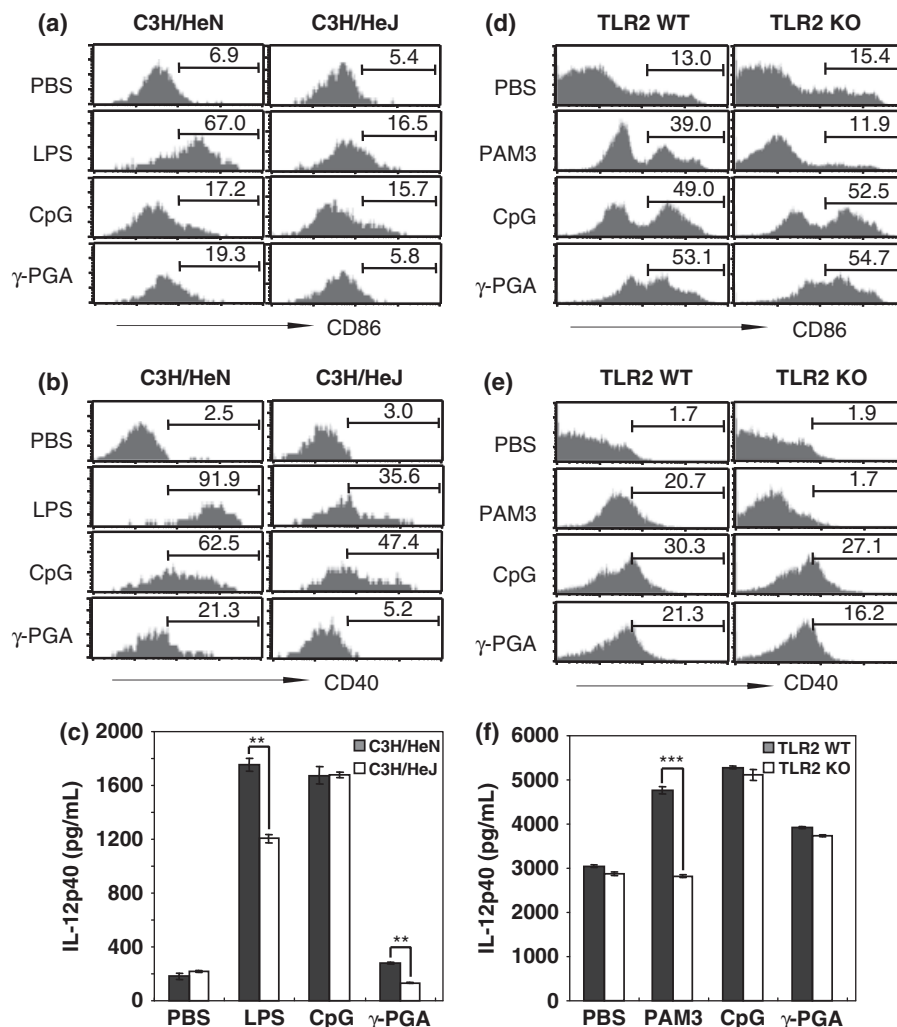


Fig. 3. Poly- γ -glutamic acid (γ -PGA) effects on bone marrow-derived dendritic cells are Toll-like receptor (TLR)-4 dependent and TLR-2 independent. GMDCs from the indicated mice were stimulated with 1 μ g/mL LPS, 2 μ g/mL CpG, 10 μ g/mL Pam3CSK4 (PAM3), or 20 μ M γ -PGA for 48 h and assayed by FACS and ELISA. (a, b, d, and e) Representative FACS profiles gated on CD11c⁺ cells. Numbers indicate the percentages of the indicated cells among CD11c⁺ cells. (c and f) Culture supernatants were assayed by ELISA to measure the concentrations of IL-12p40. ** P < 0.01, *** P < 0.001 by Student's t -test.

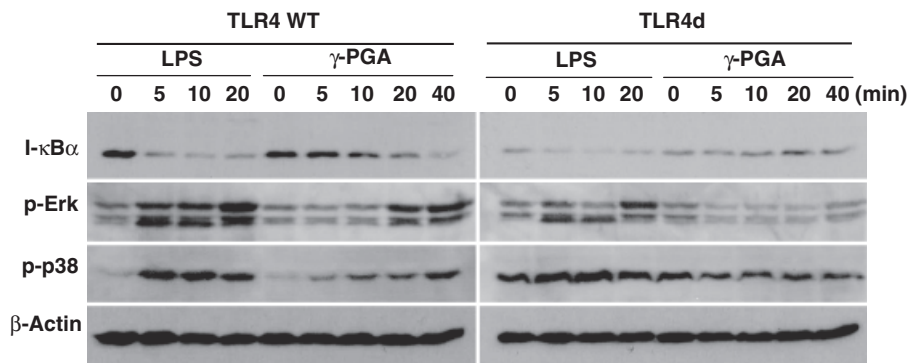


Fig. 4. Poly- γ -glutamic acid (γ -PGA) induces NF- κ B and MAPKs activation in GMDs. GMDs derived from BALB/c (TLR4 WT) or C.C3-*Tlr4*^{lps-d/J} (TLR4d) mice were stimulated with 1 μ g/mL LPS or 20 μ M γ -PGA for the indicated times. Cell lysates were analysed by Western blotting using Abs to I- κ B α and the phosphorylated form of p38 and Erk.

Western blot, using LPS as a positive control (Fig. 4). I- κ B α degradation and MAPK (Erk and p38) phosphorylation were evident after 5 min of LPS treatment of BMDCs derived from wild-type mice but not from TLR-4-defective BALB/c mice. γ -PGA also induced I- κ B α degradation and MAPK phosphorylation in a TLR-4-dependent manner, although with slower kinetics than LPS. Thus, these results confirm that γ -PGA engages TLR-4 on the surface of DCs, triggering the activation of TLR-4-specific signalling pathways.

γ -PGA attenuates the key features of AHR and allergic inflammation

Given that γ -PGA has the potential to elicit TLR-4-dependent DC activation, as shown above, and to bias Th1/Th2 differentiation, as we reported previously [21], we hypothesized that γ -PGA might be useful for treating Th2-mediated allergic disorders such as asthma. To test this idea, we used the OVA-sensitized/challenged model of allergic airway asthma. Compared with the vehicle-treated mice, OVA-sensitized/challenged mice exhibited significantly increased AHR in response to methacholine (a muscarinic receptor agonist), as measured using the enhanced pause method (Fig. 5a). OVA challenge of OVA-sensitized mice induced dramatic increases in leucocyte infiltration in the peribronchovascular areas and in mucous-secreting goblet cells within the bronchiolar epithelium (Figs 5b and c). Numbers of whole BAL cells, which consisted mainly of macrophages and eosinophils, significantly increased in the OVA-sensitized/challenged mice (Fig. 5d). All these pathophysiologic manifestations were significantly attenuated by intranasally administered γ -PGA at a dose of 50 mg/kg body weight/injection. A higher dose (250 mg/kg body weight/injection) of γ -PGA was less effective against airway leucocyte influx and goblet cell hyperplasia than a lower dose of γ -PGA, whereas the effect on AHR was not significantly influenced by dosage.

γ -PGA reduces Th2 responses in asthmatic mice

Because Th2 responses are central to the pathogenesis of allergic airway asthma, we tested whether γ -PGA reduced Th2 responses in mice established asthma. γ -PGA treatment reduced the numbers of eosinophils and macrophages among BAL influx; the former reached statistical significance but the latter did not (Fig. 6a). The serum titres of whole IgE and OVA-specific IgE in lung tissue were lowered by γ -PGA treatment (Fig. 6b), as were the levels of Th2 cytokines and chemokines, such as IL-5, IL-13, and MCP-1 (Fig. 6c). Interestingly, γ -PGA also reduced the transcripts of RANTES, a Th1-type cytokine acting as an eosinophil chemoattractant, in lung tissue. Except for its effects on the production of IL-5 and IL-13, low-dose γ -PGA was more effective in inhibiting airway Th2 responses than high-dose γ -PGA.

Lung cDCs are subdivided into Th1-prone CD11c^{hi}CD11b^{lo} DCs and Th2-prone CD11c^{lo}CD11b^{hi} DCs [18, 19]. We found that, of these two populations, CD11c^{hi}CD11b^{lo} DCs predominated in the lungs of the non-sensitized controls, whereas CD11c^{lo}CD11b^{hi} DCs predominated in the lungs of the OVA-sensitized/challenged mice. γ -PGA treatment restored the ratio of CD11c^{hi}CD11b^{lo} DCs to CD11c^{lo}CD11b^{hi} to a level equivalent to that in unsensitized mice (Fig. 6d).

As asthmatic adaptive immune responses are initiated in peripheral lymphoid tissues adjacent to the airways, such as the MLNs, we examined allergen-specific T cell responses in the MLNs obtained from asthmatic mice. MLN cells from OVA-sensitized/challenged mice multiplied rapidly in response to *in vitro* stimulation with OVA, whereas those from unsensitized/challenged mice did not. γ -PGA administration inhibited this proliferative response (Fig. 7a). Moreover, the production of the Th2 cytokines IL-4, IL-5, and IL-13, but not the Th1 cytokine IFN- γ , was reduced in the γ -PGA-treated MLN cells upon *in vitro* stimulation with OVA (Fig. 7b). These results demonstrate that γ -PGA inhibits allergen-specific T cell expansion and

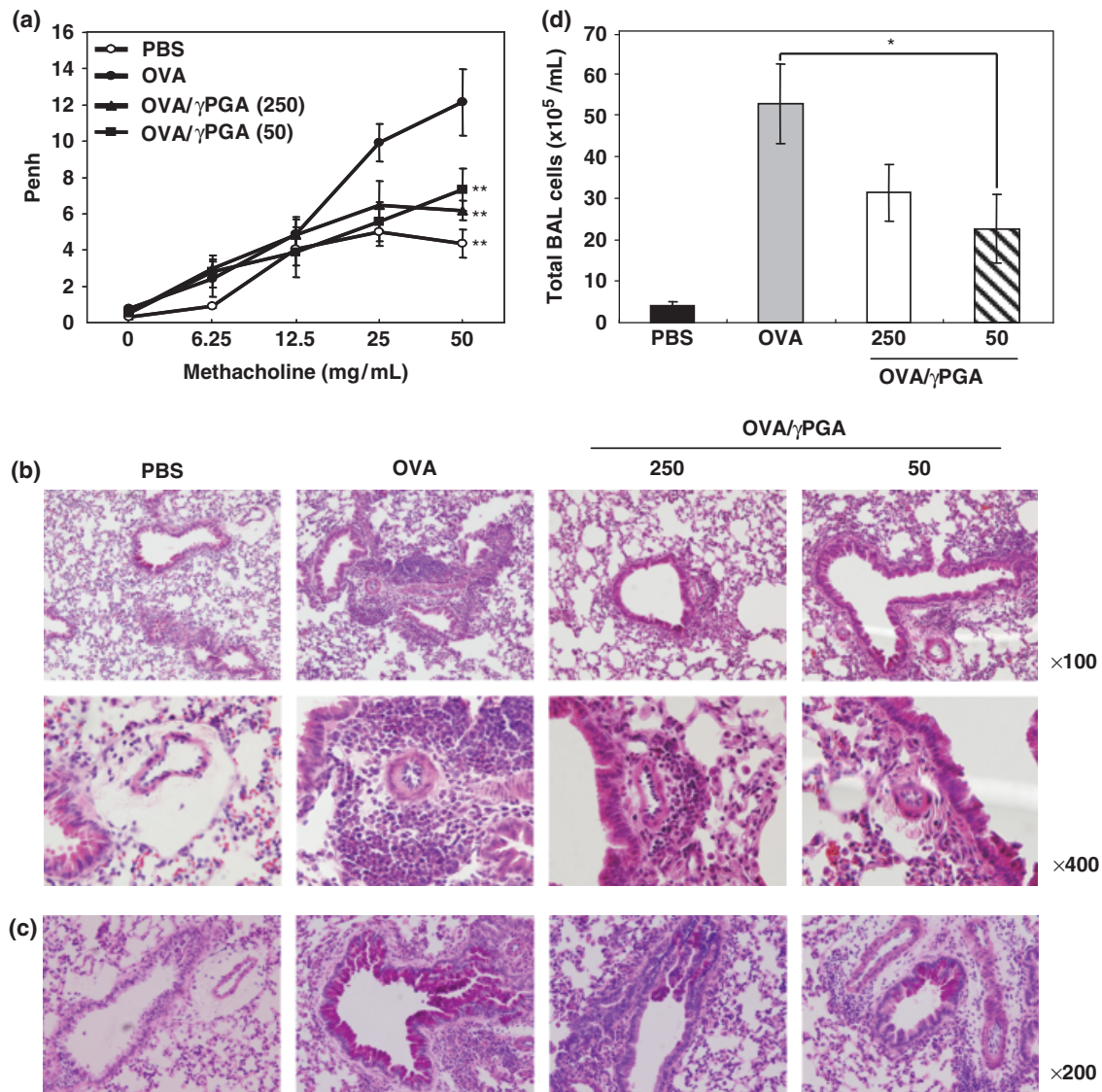


Fig. 5. Intranasal administration of poly- γ -glutamic acid (γ -PGA) attenuates the pathophysiologic features of allergic airway disease. BALB/c mice were ovalbumin (OVA)-sensitized/challenged and intranasally injected with PBS or γ -PGA. PBS, PBS-sensitized/challenged and PBS-treated; OVA, OVA-sensitized/challenged and PBS-treated; OVA/ γ -PGA 250, OVA-sensitized/challenged and γ -PGA-treated (250 mg/kg body weight per injection); OVA/ γ -PGA 50, OVA-sensitized/challenged and γ -PGA-treated (50 mg/kg body weight per injection). (a) AHR measured as enhanced pause (Penh). * P < 0.05, ** P < 0.01 when exposed to 25 and 50 mg/mL methacholine. (b) Lung tissue sections stained with haematoxylin and eosin. (c) Lung tissue sections stained with periodic acid-Schiff and haematoxylin. (d) Total numbers of bronchoalveolar lavage cells. * P < 0.05, ** P < 0.01 when compared with the OVA group by Student's t -test. n = 7 mice per group.

selectively suppresses Th2 responses in lymph nodes draining the airways.

The anti-asthmatic activity of γ -PGA is TLR-4 dependent

As we had shown that the activity of γ -PGA *in vitro* was TLR-4 dependent, we tested whether the anti-asthmatic effect of γ -PGA *in vivo* also relied on TLR-4 signalling. To this end, we evaluated the efficacy of γ -PGA in C.C3-*Tlr4*^{lps-d/J} (TLR-4-defective BALB/c congenic) mice subjected to OVA sensitization and challenge. We were able to

induce airway inflammation in TLR-4-defective mice, although disease severity was slightly lower than that in wild-type mice, indicating that TLR-4 signalling *per se* is not necessary for asthma induction in this system, as shown previously [13]. However, γ -PGA did not inhibit the symptoms of the TLR-4-defective mice, such as AHR to methacholine, leucocyte influx into lung tissue and BAL fluid, eosinophilia, and goblet cell hyperplasia (Figs 8a–c and not shown). Consistent with this, OVA challenge induced enhanced Th2 responses, such as serum IgE, IL-5, and IL-13, independent of treatment with γ -PGA (Figs

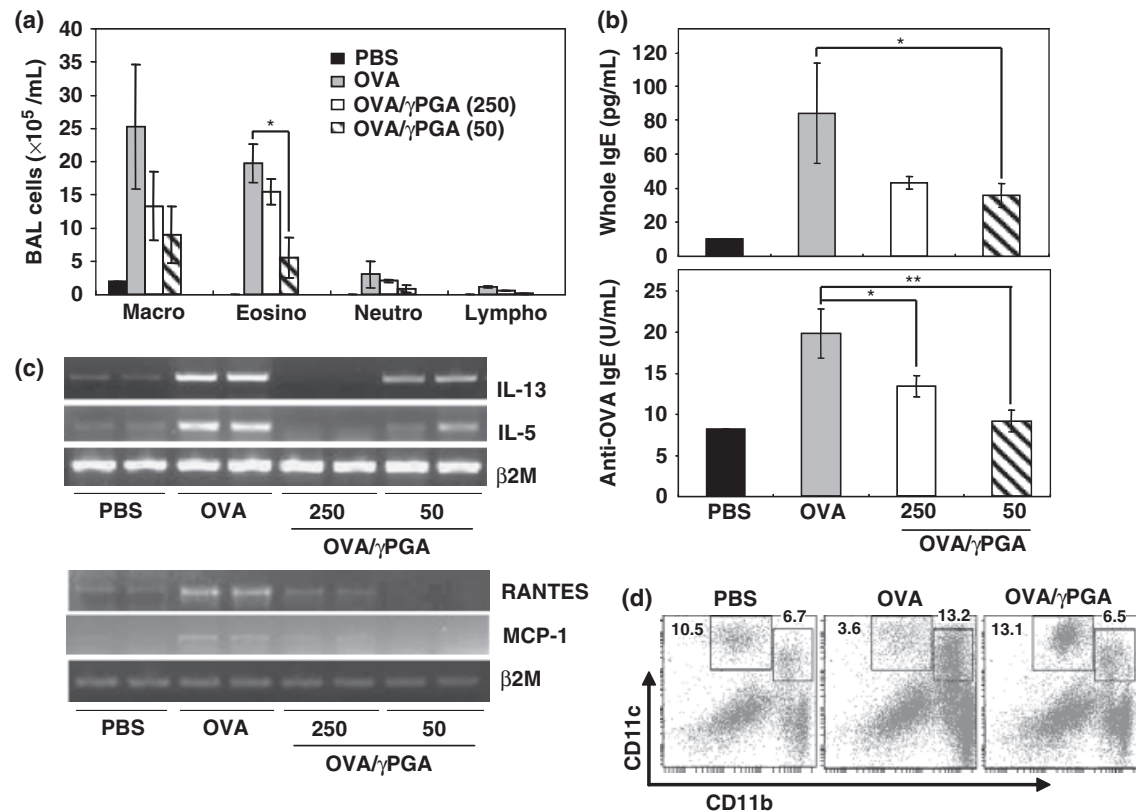


Fig. 6. Intranasal administration of poly- γ -glutamic acid (γ -PGA) reduces Th2 responses in ovalbumin (OVA)-sensitized/challenged mice. BALB/c mice were OVA-sensitized/challenged and intranasally injected with PBS or γ -PGA. PBS, PBS-sensitized/challenged and PBS-treated; OVA, OVA-sensitized/challenged and PBS-treated; OVA/ γ -PGA 250, OVA-sensitized/challenged and γ -PGA-treated (250 mg/kg body weight per injection); OVA/ γ -PGA 50, OVA-sensitized/challenged and γ -PGA-treated (50 mg/kg body weight per injection). (a) Leucocyte subsets among the bronchoalveolar lavage (BAL) cells. (b) Serum levels of whole IgE and OVA-specific IgE determined by ELISA. (c) Levels of IL-13, IL-5, RANTES, and MCP-1 transcripts from lung tissues determined by semiquantitative RT-PCR. (d) Lung cells were stained for CD11b and CD11c, followed by FACS. Representative FACS profiles with the percentages of CD11c^{hi}CD11b^{lo} DCs and CD11c^{lo}CD11b^{hi} DCs are shown. * $P < 0.05$, ** $P < 0.01$ when compared with the OVA group by Student's *t*-test. $n = 7$ mice per group.

8d–f). These results demonstrate that the inhibition of asthmatic responses by γ -PGA is mediated by TLR-4 signalling.

Discussion

We found previously that γ -PGA was able to affect the balance between Th1 and Th2 cells by promoting Th1 cell development and inhibiting Th2 cell development [21]. The present study demonstrates that the mechanism by which γ -PGA biases the outcome of Th cell polarization includes an action on the DCs. We concluded that γ -PGA signalling promoted the selective formation of Th1 cells for several reasons. First, upon conventional Ag challenge, it selectively activated cDCs, which are highly effective in priming T cells, rather than pDCs, which are relatively weak T cell stimulators [16]. Second, γ -PGA, like LPS, favoured Th1 cell development when present at a substantial dose during priming [12, 13]. Finally, γ -PGA induced cDCs to produce IL-12, a Th1 inducer, but not IL-10 and IL-6, which are Th2 inducers [25–28]. The γ -PGA

inducibility of CD40 seems to contribute to Th1 polarization, because IL-12p70 induction by γ -PGA required a costimulatory signal through CD40.

This effect of γ -PGA on Th cell development was confirmed in a model of Th2-mediated allergic asthma. We found that the intranasal administration of γ -PGA alleviated AHR and airway inflammation upon allergen provocation, suggesting that the anti-Th2 activity of γ -PGA is sufficient to suppress asthmatic symptoms. This is further supported by the finding that γ -PGA treatment increased Th1-prone DCs and decreased Th2-prone DCs in the lungs of OVA-sensitized/challenged mice. Of the leucocyte populations infiltrated into bronchoalveolar tracks, eosinophils were the most severely affected by γ -PGA, presumably due to a γ -PGA-mediated reduction in the levels of various eosinophil chemoattractants, such as IL-5, MCP-1, and RANTES [34]. IL-13 is known to induce more diverse symptoms than IL-5, such as goblet cell metaplasia, contraction of airway smooth muscle, and IgE production [35], all of which were significantly reduced by γ -PGA administration. We failed to detect IL-4

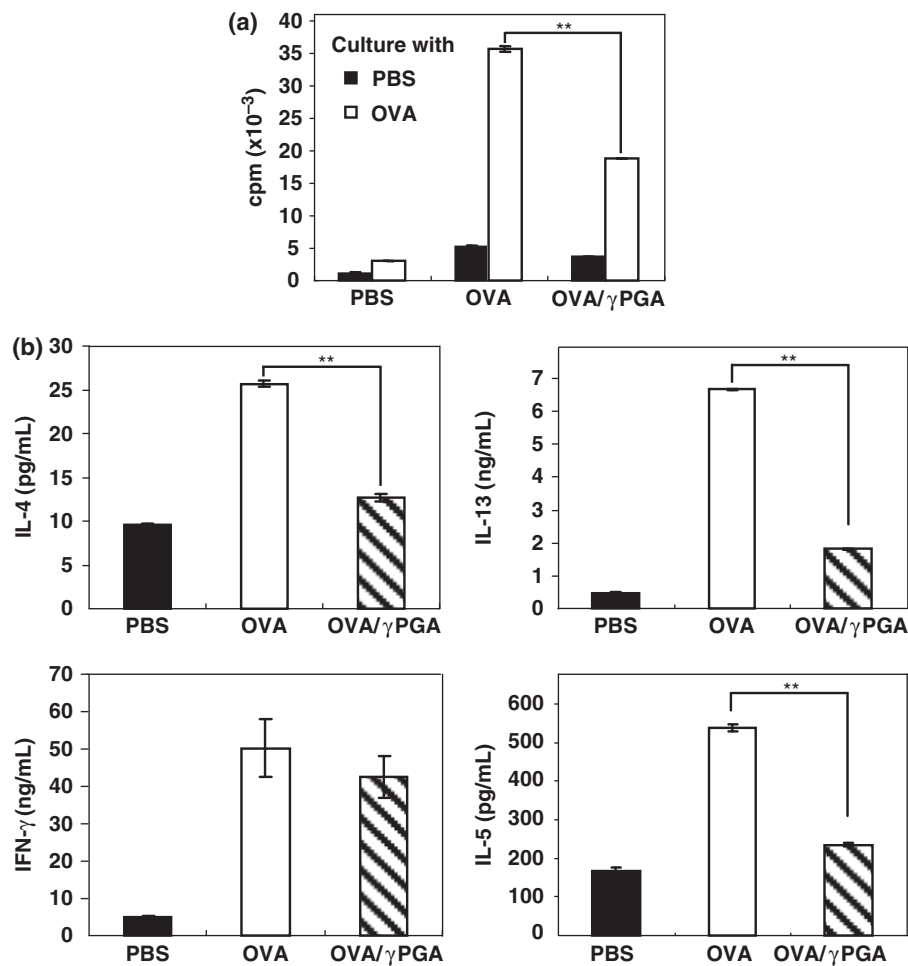


Fig. 7. Poly- γ -glutamic acid (γ -PGA) administration reduces the proliferation of ovalbumin (OVA)-specific T cells in mediastinal lymph node (MLNs) from OVA-sensitized/challenged mice and decreases their IL-4 production. BALB/c mice were OVA-sensitized/challenged and intranasally injected with PBS or γ -PGA. PBS, PBS-sensitized/challenged and PBS-treated; OVA, OVA-sensitized/challenged and PBS-treated; OVA/ γ -PGA, OVA-sensitized/challenged and γ -PGA-treated (50 mg/kg body weight per injection). Two days after the last OVA challenge, MLNs were restimulated *in vitro* with OVA for 3 days. (a) Proliferative responses assessed by ^{3} H-thymidine incorporation. (b) Culture supernatants assayed by ELISA. ** $P < 0.01$ by Student's *t*-test.

expression in lung tissue, possibly because IL-4 was produced earlier by the Th2 cells than IL-5 and IL-13 [36]. Furthermore, the data obtained from MLNs clearly demonstrate that γ -PGA reduced the activities of allergen-specific Th2 cells. It should be noted, however, that, although aspects of the effects on Th2 responses observed *in vivo* were consistent with those observed *in vitro*, γ -PGA treatment *in vivo* did not enhance the activity of allergen-specific Th1 cells as it did *in vitro*. This difference may be due to differences in the immune processes exposed to γ -PGA in the two cases. In the *in vivo* intervention experiments, the mice were exposed to γ -PGA just before, or even during, the effector phase of the asthmatic reaction to allergen challenge, and this may not have been effective in converting cells that had already become committed to the Th2 lineage during the phase of sensitization to Th1 cells. We also found that the intraperitoneal delivery of γ -PGA elicited an effect similar to that

of intranasal delivery, further supporting the view that the anti-asthmatic effects of γ -PGA are due to systemic, adaptive immunity rather than to innate effectors restricted to the respiratory mucosal airways.

γ -PGA has different immunomodulatory effects depending on its molecular weight. 50 kDa γ -PGA was more effective in terms of DC activation and Th cell polarization [21], whereas 2000 kDa γ -PGA was more potent in eliciting antitumour immunity by enhancing the activities of NK cells [37, 38]. Therefore, it is likely that low-molecular-weight γ -PGA preferentially regulates CD4⁺ T cells while high-molecular-weight γ -PGA targets NK cells, although the reason for the difference is unknown. This size-dependent difference in γ -PGA activity was also evident in our *in vivo* system: unlike 50 kDa γ -PGA, which had anti-asthmatic activity (Figs 5 and 6), high-molecular-weight (500–2000 kDa) γ -PGA was not able to suppress inflammatory processes in the airways

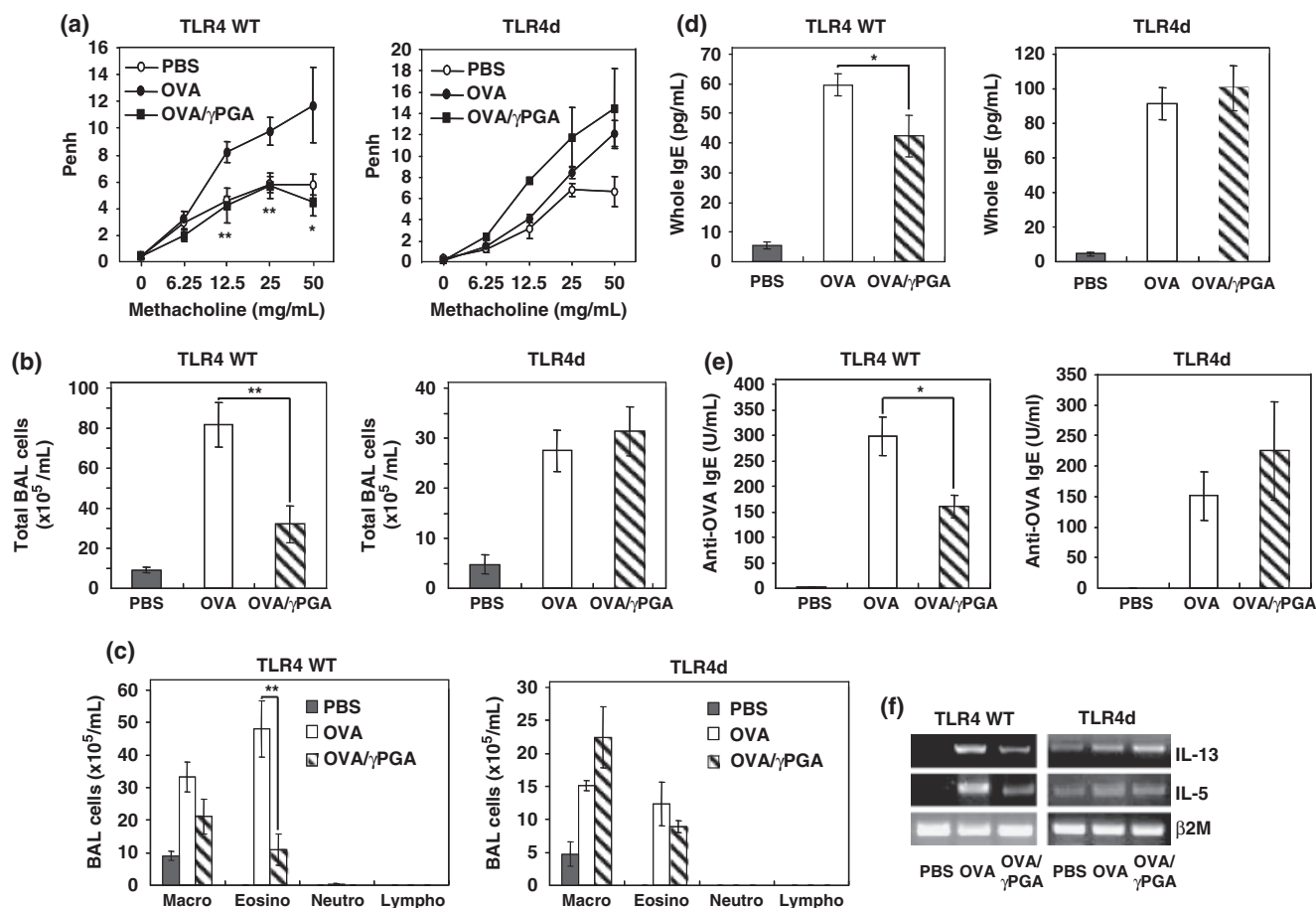


Fig. 8. The anti-asthmatic effect of poly- γ -glutamic acid (γ -PGA) is dependent on toll-like receptor (TLR)-4. BALB/c (TLR4 WT) and C.C3-*Tlr4*^{lps-d/J} (TLR4d) mice were ovalbumin (OVA)-sensitized/challenged and intranasally injected with PBS or γ -PGA. PBS, PBS-sensitized/challenged and PBS-treated; OVA, OVA-sensitized/challenged and PBS-treated; OVA/ γ -PGA, OVA-sensitized/challenged and γ -PGA-treated (50 mg/kg body weight per injection). (a) Twenty-four hours after the last challenge, the indicated mice were exposed to methacholine, and AHR was measured as enhanced pause (Penh). (b and c) Numbers of total bronchoalveolar lavage (BAL) cells and of leucocyte subsets, among the BAL cells. (d and e) Serum levels of whole IgE and OVA-specific IgE determined by ELISA. (f) Levels of IL-13 and IL-5 transcripts in lung tissues determined by semiquantitative RT-PCR. * $P < 0.05$, ** $P < 0.01$ when compared with the OVA group by Student's *t*-test. $n = 7$ mice per group for each experiment.

(data not shown). This *in vivo* result supports the view that the anti-asthmatic effect of γ -PGA is due to its effect on Th2 responses.

We also found that, in addition to its molecular weight, γ -PGA dosage was critical for its efficacy. *In vivo* administration of 50 kDa γ -PGA to asthmatic mice elicited a bell-shaped dose-response curve in the range of 10–6250 mg/kg body weight/day. In contrast to its anti-asthmatic effect at doses of 50–250 mg/kg body weight/day, γ -PGA, at higher doses, aggravated asthmatic symptoms (data not shown). Although the mechanism underlying this paradoxical effect remains to be studied, we suspect that it involves down-regulation or desensitization of TLR-4 at higher doses of γ -PGA. Indeed, it has been shown that high doses of LPS, a TLR-4 agonist, induce rapid down-regulation of surface TLR-4 expression and failure to transmit LPS-mediated signals to the cell [39]. This type of dose-response appears not to be unique to γ -

PGA but to be a common feature of many drugs, including erythropoietin and endothelial-monocyte activating polypeptide II [40, 41]. In relation to the potential use of γ -PGA as an anti-asthmatic agent, further clinical studies are necessary to determine the minimal therapeutic dose and to identify additional dose-dependent side-effects.

Several biological modifiers that inhibit Th2 responses have been developed as part of a rational strategy for treating Th2-mediated diseases. Representative examples include anti-IL-5 mAbs, a mutant form of IL-4, and TLR-9 vaccines. Anti-IL-5 therapy counteracted certain symptoms of asthma, such as eosinophilia, but it was not effective in moderate asthmatics, and even exacerbated a severe form of asthma [42–46]. In addition, it had serious adverse effects, including heart failure. Whereas the selective inhibition of IL-4 alone did not reduce asthmatic symptoms, simultaneous blocking of both IL-4 and IL-13 signalling with a mutant form of IL-4 produced a clinical

benefit [47, 48]. This underscores the message that combinatorial targeting of multiple Th2 cytokines is more effective than selective targeting of a single cytokine. However, the use of mutant cytokines needs to be weighed against the risk of untoward effects arising from their potential to elicit anti-idiotypic Ab circuits. On the other hand, non-protein therapeutics are relatively unaffected by immunogenic issues. TLR-9 vaccines using CpG oligodeoxynucleotides have been designed to inhibit Th2 responses with the parallel enhancement of Th1 responses, and such vaccines did indeed reduce the symptoms of allergic diseases [49–51]. However, given their extraordinary potency as adjuvants, they may tilt the balance towards Th1 predominance so strongly that autoimmune disorders result. Compared with these biological modifiers, we believe that γ -PGA has several therapeutic benefits. In terms of efficacy, it can inhibit the reactions mediated by multiple Th2 cytokines rather than targeting a single cytokine. In terms of safety, in addition to its edible and non-protein nature, its modest potency as an adjuvant should be adequate to re-establish an immune

balance rather than shifting the balance too far in the opposite direction.

In summary, we have shown that γ -PGA specifically activates cDCs in a TLR-4-dependent manner and alleviates pathologic symptoms in a Th2-biased asthmatic model. Our findings have implications beyond the context of asthma, because γ -PGA has been linked to the suppression of chronic allergic inflammatory diseases in which Th2 responses play an important role.

Acknowledgements

We thank Drs Shizuo Akira and Myung-Shik Lee for providing TLR-2^{-/-} mice, Ms Jiyoung Seo for technical assistance, Dr Chul-Hoon Lee for valuable discussion, and Dr Julian Gross for editorial assistance. This work was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A084660).

Competing interests: We declare that we have no competing interests.

References

- Braman SS. The global burden of asthma. *Chest* 2006; 130:4S–12S.
- Eder W, Ege MJ, von Mutius E. The asthma epidemic. *N Engl J Med* 2006; 355:2226–35.
- Zdanowicz MM. Pharmacotherapy of asthma. *Am J Pharm Educ* 2007; 71:98.
- Bloemen K, Verstraelen S, Van Den Heuvel R, Witters H, Nelissen I, Schoeters G. The allergic cascade: review of the most important molecules in the asthmatic lung. *Immunol Lett* 2007; 113:6–18.
- Wills-Karp M, Luyimbazi J, Xu X *et al.* Interleukin-13: central mediator of allergic asthma. *Science* 1998; 282: 2258–61.
- Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989; 7:145–73.
- Krieg AM, Yi AK, Matson S *et al.* CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995; 374:546–9.
- Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci USA* 1996; 93:2879–83.
- Kline JN, Waldschmidt TJ, Businga TR *et al.* Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J Immunol* 1998; 160:2555–9.
- Parronchi P, Brugnino F, Annunziato F *et al.* Phosphorothioate oligodeoxynucleotides promote the in vitro development of human allergen-specific CD4+ T cells into Th1 effectors. *J Immunol* 1999; 163:5946–53.
- Fujieda S, Iho S, Kimura Y, Yamamoto H, Igawa H, Saito H. Synthetic oligodeoxynucleotides inhibit IgE induction in human lymphocytes. *Am J Respir Crit Care Med* 2000; 162:232–9.
- Kim YK, Oh SY, Jeon SG *et al.* Airway exposure levels of lipopolysaccharide determine type 1 versus type 2 experimental asthma. *J Immunol* 2007; 178:5375–82.
- Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 2002; 196:1645–51.
- Merad M, Manz MG. Dendritic cell homeostasis. *Blood* 2009; 113:3418–27.
- Asselin-Paturel C, Boonstra A, Dalod M *et al.* Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat Immunol* 2001; 2: 1144–50.
- de Heer HJ, Hammad H, Soullie T *et al.* Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med* 2004; 200:89–98.
- Manches O, Munn D, Fallahi A *et al.* HIV-activated human plasmacytoid DCs induce Tregs through an indoleamine 2,3-dioxygenase-dependent mechanism. *J Clin Invest* 2008; 118:3431–9.
- Shao Z, Bharadwaj AS, McGee HS, Makinde TO, Agrawal DK. Fms-like tyrosine kinase 3 ligand increases a lung DC subset with regulatory properties in allergic airway inflammation. *J Allergy Clin Immunol* 2009; 123:917–24.
- Shao Z, Makinde TO, McGee HS, Wang X, Agrawal DK. Fms-like tyrosine kinase 3 ligand regulates migratory pattern and antigen uptake of lung dendritic cell subsets in a murine model of allergic airway inflammation. *J Immunol* 2009; 183:7531–8.
- Sung MH, Park C, Kim CJ, Poo H, Soda K, Ashiuchi M. Natural and edible biopolymer poly-gamma-glutamic acid: synthesis, production, and applications. *Chem Rec* 2005; 5:352–66.
- Kim S, Yang JY, Lee K *et al.* Bacillus subtilis-specific poly-gamma-glutamic acid regulates development pathways of naive CD4(+) T cells through antigen-presenting cell-dependent and -independent mechanisms. *Int Immunol* 2009; 21:977–90.
- Jang E, Cho SH, Park H, Paik DJ, Kim JM, Youn J. A positive feedback loop of IL-21

- signaling provoked by homeostatic CD4+CD25- T cell expansion is essential for the development of arthritis in autoimmune K/BxN mice. *J Immunol* 2009; **182**:4649–56.
- 23 Forsythe P, Ebeling C, Gordon JR, Befus AD, Vliagoftis H. Opposing effects of short- and long-term stress on airway inflammation. *Am J Respir Crit Care Med* 2004; **169**:220–6.
 - 24 Zhang Z, Hener P, Frossard N *et al*. Thymic stromal lymphopoietin overproduced by keratinocytes in mouse skin aggravates experimental asthma. *Proc Natl Acad Sci USA* 2009; **106**:1536–41.
 - 25 Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* 1995; **13**:251–76.
 - 26 De Smedt T, Van Mechelen M, De Becker G, Urbain J, Leo O, Moser M. Effect of interleukin-10 on dendritic cell maturation and function. *Eur J Immunol* 1997; **27**:1229–35.
 - 27 Rincon M, Anguita J, Nakamura T, Fikrig E, Flavell RA. Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells. *J Exp Med* 1997; **185**:461–9.
 - 28 Dodge IL, Carr MW, Cernadas M, Brenner MB. IL-6 production by pulmonary dendritic cells impedes Th1 immune responses. *J Immunol* 2003; **170**:4457–64.
 - 29 Brawand P, Fitzpatrick DR, Greenfield BW, Brasel K, Maliszewski CR, De Smedt T. Murine plasmacytoid pre-dendritic cells generated from Flt3 ligand-supplemented bone marrow cultures are immature APCs. *J Immunol* 2002; **169**:6711–9.
 - 30 Vermaelen KY, Carro-Muino I, Lambrecht BN, Pauwels RA. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J Exp Med* 2001; **193**:51–60.
 - 31 Asea A, Rehli M, Kabingu E *et al*. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* 2002; **277**:15028–34.
 - 32 Mu HH, Hasebe A, Van Schelt A, Cole BC. Novel interactions of a microbial superantigen with TLR2 and TLR4 differentially regulate IL-17 and Th17-associated cytokines. *Cell Microbiol* 2011; **13**:374–87.
 - 33 Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 2010; **11**:373–84.
 - 34 Wardlaw AJ. Molecular basis for selective eosinophil trafficking in asthma: a multistep paradigm. *J Allergy Clin Immunol* 1999; **104**:917–26.
 - 35 Wills-Karp M. Interleukin-13 in asthma pathogenesis. *Immunol Rev* 2004; **202**:175–90.
 - 36 To Y, Dohi M, Tanaka R, Sato A, Nakagome K, Yamamoto K. Early interleukin 4-dependent response can induce airway hyperreactivity before development of airway inflammation in a mouse model of asthma. *Lab Invest* 2001; **81**:1385–96.
 - 37 Kim TW, Lee TY, Bae HC *et al*. Oral administration of high molecular mass poly-gamma-glutamate induces NK cell-mediated antitumor immunity. *J Immunol* 2007; **179**:775–80.
 - 38 Lee TY, Kim YH, Yoon SW *et al*. Oral administration of poly-gamma-glutamate induces TLR4- and dendritic cell-dependent antitumor effect. *Cancer Immunol Immunother* 2009; **58**:1781–94.
 - 39 Nomura F, Akashi S, Sakao Y *et al*. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. *J Immunol* 2000; **164**:3476–9.
 - 40 Zacharias R, Schmidt M, Kny J *et al*. Dose-dependent effects of erythropoietin in propofol anesthetized neonatal rats. *Brain Res* 2010; **1343**:14–9.
 - 41 Crippa L, Gasparri A, Sacchi A, Ferrero E, Curnis F, Corti A. Synergistic damage of tumor vessels with ultra low-dose endothelial-monocyte activating polypeptide-II and neovasculature-targeted tumor necrosis factor- α . *Cancer Res* 2008; **68**:1154–61.
 - 42 Flood-Page P, Swenson C, Faiferman I *et al*. A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma. *Am J Respir Crit Care Med* 2007; **176**:1062–71.
 - 43 Flood-Page PT, Menzies-Gow AN, Kay AB, Robinson DS. Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. *Am J Respir Crit Care Med* 2003; **167**:199–204.
 - 44 Leckie MJ, ten Brinke A, Khan J *et al*. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 2000; **356**:2144–8.
 - 45 Haldar P, Brightling CE, Hargadon B *et al*. Mepolizumab and exacerbations of refractory eosinophilic asthma. *N Engl J Med* 2009; **360**:973–84.
 - 46 Nair P, Pizzichini MM, Kjarsgaard M *et al*. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N Engl J Med* 2009; **360**:985–93.
 - 47 Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* 2008; **118**:3546–56.
 - 48 Wenzel S, Wilbraham D, Fuller R, Getz EB, Longphre M. Effect of an interleukin-4 variant on late phase asthmatic response to allergen challenge in asthmatic patients: results of two phase 2a studies. *Lancet* 2007; **370**:1422–31.
 - 49 Tighe H, Takabayashi K, Schwartz D *et al*. Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity. *J Allergy Clin Immunol* 2000; **106**:124–34.
 - 50 Marshall JD, Abtahi S, Eiden JJ *et al*. Immunostimulatory sequence DNA linked to the Amb a 1 allergen promotes T(H)1 cytokine expression while down-regulating T(H)2 cytokine expression in PBMCs from human patients with ragweed allergy. *J Allergy Clin Immunol* 2001; **108**:191–7.
 - 51 Creticos PS, Schroeder JT, Hamilton RG *et al*. Immunotherapy with a ragweed-toll-like receptor 9 agonist vaccine for allergic rhinitis. *N Engl J Med* 2006; **355**:1445–55.