

Oral administration of poly- γ -glutamic acid prevents the development of atopic dermatitis in NC/Nga mice

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Abstract: *Bacillus subtilis*-derived poly- γ -glutamic acid (γ PGA) has demonstrated adjuvant activity in promoting Th1/Th17 cell differentiation. Here, the NC/Nga (NC) mouse model was used to determine whether γ PGA modulates the outcome of atopic dermatitis (AD), which is known to be a Th2-biased immune disease. We found that oral administration of γ PGA dramatically reduced the development of AD in NC mice. Antigen-presenting cells activated with γ PGA produced pro-inflammatory cytokines, such as IL12/23 and IFN γ , which, in turn, induced the differentiation of Th1 and Th17 cells. Concomitantly, Th2 responses, such as high levels of serum IgE, were dramatically

decreased. Furthermore, *in vivo* γ PGA treatment altered several cellular components of allergic reactions, such as mast cells and eosinophils. Taken together, our results strongly demonstrate that *in vivo* treatment with γ PGA at early time points can prevent the development of AD in NC mice and suggest that γ PGA may have therapeutic applications for human AD.

Key words: atopic dermatitis – cytokine – mast cell – poly- γ -glutamic acid – T helper differentiation

Accepted for publication 19 June 2013

Background

Atopic dermatitis (AD) is known to be caused by inappropriate Th2-initiated responses to environmental allergens in genetically predisposed individuals (1). Th2-type cells play a key role in AD development, as shown by the increased levels of serum IgE and the IgE-mediated activation of mast cells in affected individuals (2–4). Defects in the Th1 response have also been associated with the severity of AD (5). Given the functional antagonism between Th1 and Th2 cells, controlling the Th1/Th2 imbalance has been proposed as a strategy for the prevention of AD (2). Pathogen-associated molecular patterns, among others, regulate T helper cell differentiation. For example, the bacterial CpG motif, a toll-like receptor (TLR) 9 ligand, promotes Th1 responses but inhibits Th2 responses (6). The therapeutic effects of CpG motifs have been well established in mouse models of asthma (7). However, as CpG induces strong Th1 responses (8), it may produce side effects, such as the aggravation of the chronic stage of AD, which is characterized by abnormal Th1-type responses (9). Therefore, milder and safer adjuvants without side effects would be ideal for the treatment of human AD.

Questions addressed

Poly- γ -glutamic acid (γ PGA) is a natural product synthesized by *Bacillus subtilis* found in chungkookjang or natto, traditional foods in East Asian countries, such as Korea and Japan (10). Previous studies have revealed that γ PGA exerts antitumor effects via the activation of both NK cells and TLR4-expressing dendritic cells (DCs) (11,12). Furthermore, γ PGA activates DCs to promote CD4⁺ T cell differentiation towards the Th1, rather than the Th2, cell type (13). γ PGA has also been demonstrated to affect T helper polarization in a murine asthma model (14). Therefore, we investigated the potential immunomodulatory effects of γ PGA on

inflammatory skin diseases, such as AD, which are known to be initiated by Th2 responses.

Experimental design

See supporting information for details (Appendix S1).

Results

To determine whether γ PGA can modulate AD, we used NC/Nga (NC) mice, an established animal model of human AD (15). NC mice were treated orally with either γ PGA or PBS under conventional conditions for the spontaneous development of AD. The severity of skin damage was scored according to the criteria described in Appendix S1. Mice were analysed for up to 12 weeks following transfer to conventional conditions, as NC mice usually show maximal AD-like symptoms at 18–20 weeks of age. Over time, the severity of dermatitis gradually increased in the PBS-treated control group, whereas γ PGA-treated mice exhibited significantly lower levels of skin inflammation (Fig. 1a). To evaluate histological changes in the skin lesions of the γ PGA-treated mice, the thickness of the skin epidermis was measured by haematoxylin and eosin (H&E) staining. The epidermal thickness was found to be significantly reduced in the γ PGA-treated group compared with the control group (Fig. 1b). In addition, γ PGA administration dramatically reduced the serum IgE levels from approximately 8 weeks postinjection (Fig. 1c). NC mice maintained under specific pathogen-free (SPF) conditions were used as negative controls. We also studied the effects of γ PGA stimulation on innate immune cells, such as mast cells, basophils and eosinophils, which have been associated with the allergic response. In particular, mast cells and eosinophils are critical effectors of various allergic diseases, such as AD (3,16). Compared with the control group, γ PGA-treated mice exhibited reductions in both the frequencies and absolute numbers of splenic mast

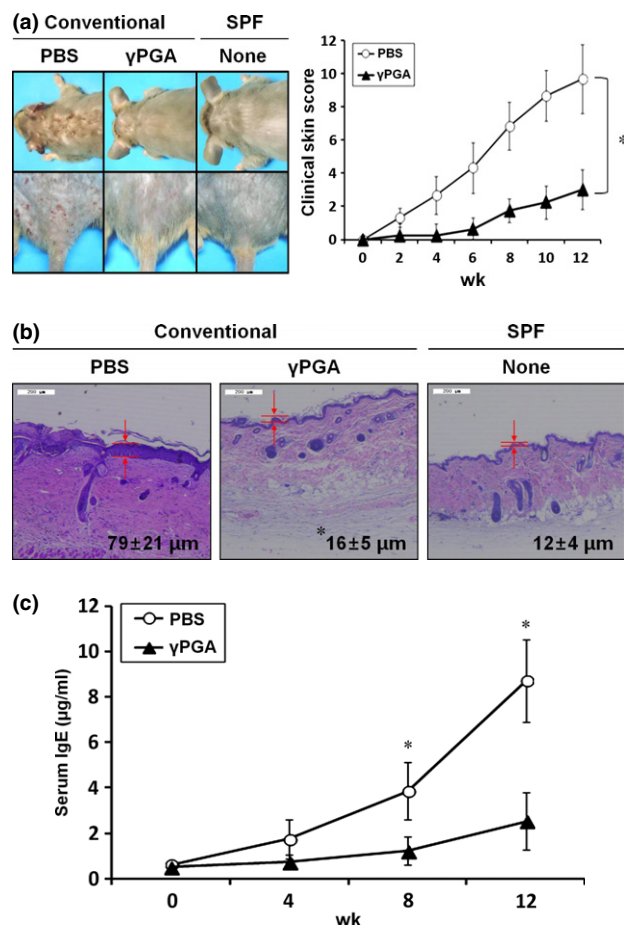


Figure 1. Oral administration of poly- γ -glutamic acid (γ PGA) prevents atopic dermatitis (AD) development in NC mice. (a) NC mice were treated orally with either γ PGA (2 mg/injection) or PBS three times a week for 12 weeks, and the clinical symptoms were measured once a week. One representative set of clinical scoring data is from three independent experiments with similar results ($n = 5$ –6 for each group, mean values $\pm$ SD, $*P < 0.05$). (b) Skin lesions were sectioned and stained with H&E. The thickness of the epidermis was measured in 10 random high-power fields (400 $\times$) per sampled lesion. Values indicate the mean $\pm$ SD (lower right, $n = 5$, $*P < 0.05$). (c) Both groups were maintained in conventional conditions for spontaneous AD development. Serum was collected from each mouse every 4 weeks, beginning at the time of the first injection. Serum IgE levels were measured by ELISA. Values indicate as the mean $\pm$ SD ($n = 5$, $*P < 0.05$).

cells, basophils and eosinophils (Fig. S1a). The skin distributions of mast cells and eosinophils were examined by staining with toluidine blue and Congo red, respectively. We observed that *in vivo* γ PGA treatment significantly decreased the skin infiltration of mast cells and eosinophils (Fig. S1b). In addition, the levels of IgE bound on mast cells, an additional indicator of the allergic response, were also dramatically reduced in the γ PGA-treated mice (Fig. S1c). Based on these evidences, we concluded that *in vivo* injection of γ PGA can modulate the outcome of AD in NC mice. To elucidate the mechanisms by which γ PGA mediates its inhibitory effect on the development of AD in NC mice, we examined the effects of γ PGA on DCs. Oral injection of γ PGA was found to up-regulate the surface expression of CD86 (Fig. S2a) and significantly increase the gene expression of pro-inflammatory cytokines, such as IL12p40, IL12p35, IL23p19, IL6, TNF α and IFN γ , in the DCs (Fig. 2a). In addition, γ PGA stimulation induced

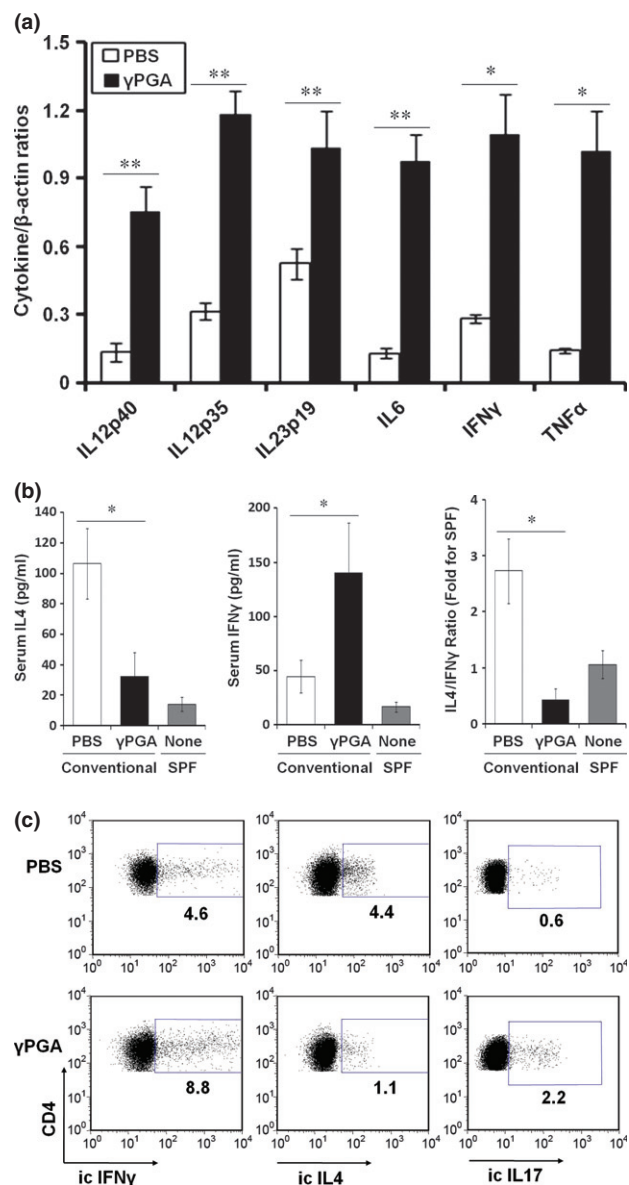


Figure 2. Oral administration of poly- γ -glutamic acid (γ PGA) to NC mice induces Th1 and Th17 responses but inhibits Th2 responses. NC mice were treated orally with either γ PGA (2 mg/injection) or PBS three times per week for 12 weeks. (a) Splenic CD11c $^{+}$ dendritic cells (DCs) were isolated from either γ PGA- or PBS-injected NC mice, and transcriptional levels of IL12p19, IL12p35, IL12p40, IL6, TNF α and IFN γ were quantified in purified DCs using semi-quantitative RT-PCR. β -actin was used for standardization. Values indicate as the mean $\pm$ SD ($n = 5$, $*P < 0.05$, $**P < 0.01$). (b) Sera were collected from either γ PGA- or PBS-injected NC mice 24 h after the final injection, and serum levels of IFN γ and IL4 were measured by ELISA (mean values $\pm$ SD, $n = 5$, $*P < 0.05$). (c) Purified splenic CD4 $^{+}$ T cells from either γ PGA- or PBS-injected NC mice were activated with plate-bound anti-CD3 (10 μ g/ml) and anti-CD28 (1 μ g/ml) mAbs for 16 h and were further restimulated with PMA/ionomycin for 2 h in the presence of Brefeldin A (10 μ g/ml). The intracellular expression of IFN γ , IL4 and IL17 was then analysed by flow cytometry.

the secretion of IL12 and IL23 from splenic DCs (Fig. S2b). We subsequently evaluated the effects of *in vivo* γ PGA injection on T helper cell differentiation. First, the serum levels of IFN γ and IL4 were measured by ELISA after 12 weeks of oral γ PGA treatment. Compared with the PBS-treated controls, γ PGA-treated mice

exhibited lower levels of serum IL4 but higher levels of serum IFN γ (Fig. 2b). These increased levels of serum IFN γ were associated with increased IFN γ production by NK and $\gamma\delta$ T cells (Fig. S3). In addition, flow cytometric analysis of intracellular cytokine production by T helper cells demonstrated that the *in vivo* injection of γ PGA enhanced Th1 and Th17 differentiation but inhibited Th2 differentiation in an antigen-specific manner (Figs 2c and S4). This result was further supported by the finding that γ PGA treatment up-regulated the expression of T-bet and ROR γ t but down-regulated the expression of GATA-3 (Fig. S5). Th1 and Th17 induction in γ PGA-treated NC mice was correlated with a decrease in regulatory T (Treg) cells (Fig. S6), in agreement with previous reports demonstrating that Th1 and Th17 immune responses suppress Treg responses (17,18). As Th2 responses play critical roles in the pathogenesis of AD (19), our data indicate that the reduced Th2, but enhanced Th1 and Th17, responses that resulted from γ PGA treatment contributed to the prevention of AD development in NC mice by modulating the Th1/Th2 balance.

Conclusions

Here, we report for the first time that the oral administration of γ PGA prevents the development of AD-like symptoms in NC

mice. Our data suggest that γ PGA is capable of enhancing Th1 and Th17 responses and inhibiting Th2 responses, such as IL4-mediated IgE production. In addition, we found that *in vivo* γ PGA treatment has therapeutic effects on fully developed AD in NC mice (unpublished preliminary data). Therefore, despite its lack of potent adjuvant activity, γ PGA could be widely applied for the modulation of allergic immune diseases due to its edible and non-toxic properties and its lack of side effects (20).

Acknowledgements

This study was supported by a grant from the Korean Health Technology R&D Project, Ministry of Health and Welfare, Republic of Korea (A090645).

Author contribution

S.W. Lee and H.J. Park contributed equally to all of the experiments and data analysis of this study as well as to the writing of the manuscript. S.-H. Park and S. Hong contributed not only to the experimental design and data interpretation of this study but also the writing and editing of the manuscript. All of the authors read and approved the final manuscript.

Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effects of oral γ PGA treatment on allergic effector cells.

Figure S2. Effects of oral γ PGA treatment on dendritic cells.

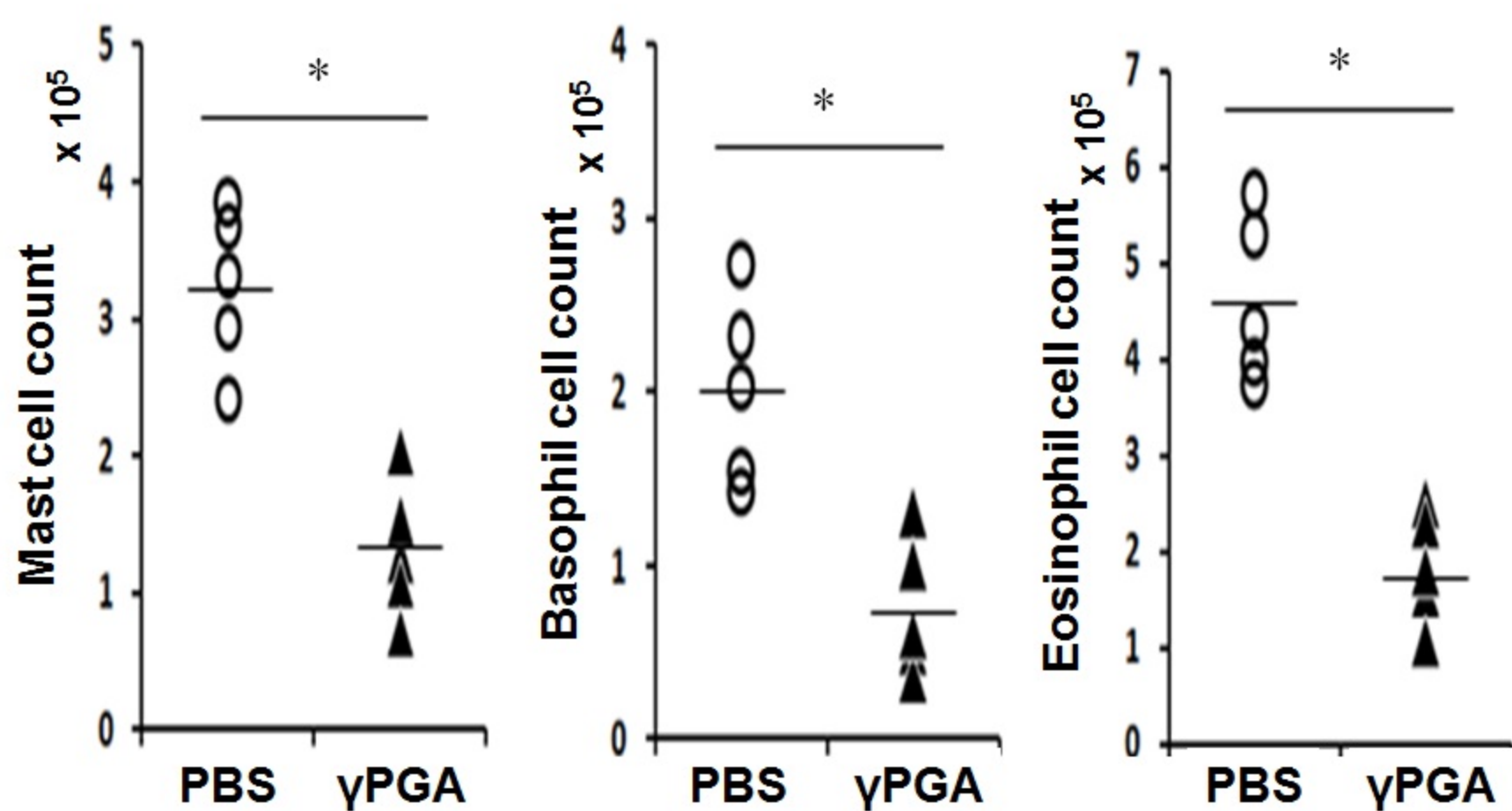
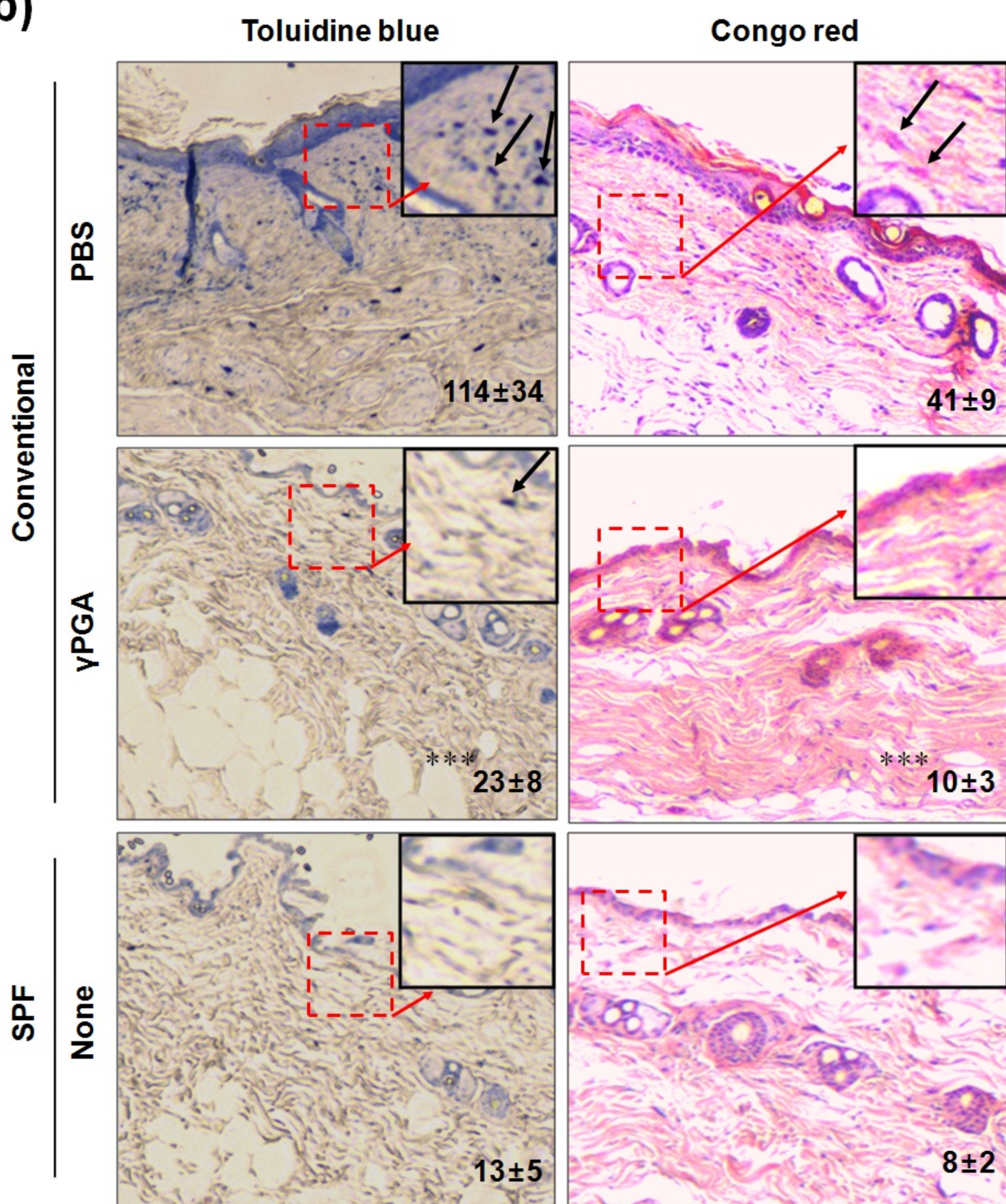
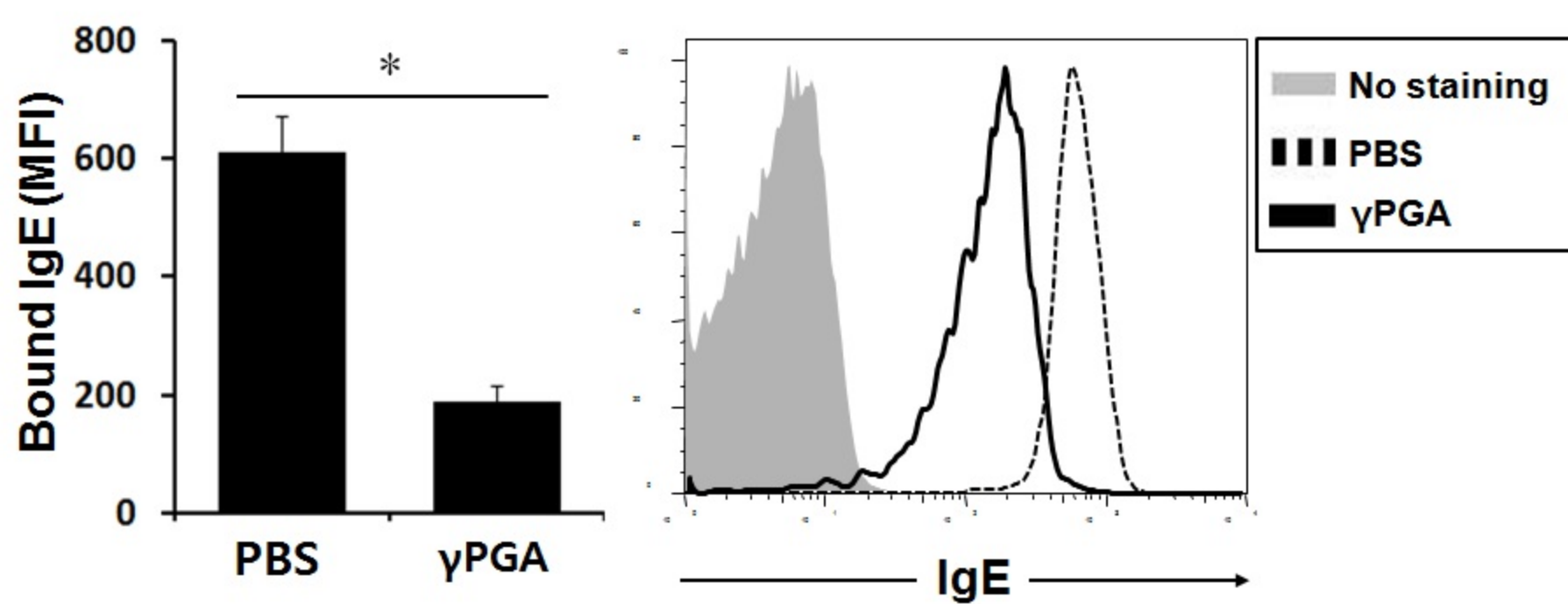
Figure S3. Oral administration of γ PGA increases IFN γ production by NK and $\gamma\delta$ T cells.

Figure S4. *In vivo* injection of γ PGA induces the polarization of Th1 and Th17 cells but inhibits the polarization of Th2 cells in an antigen-specific manner.

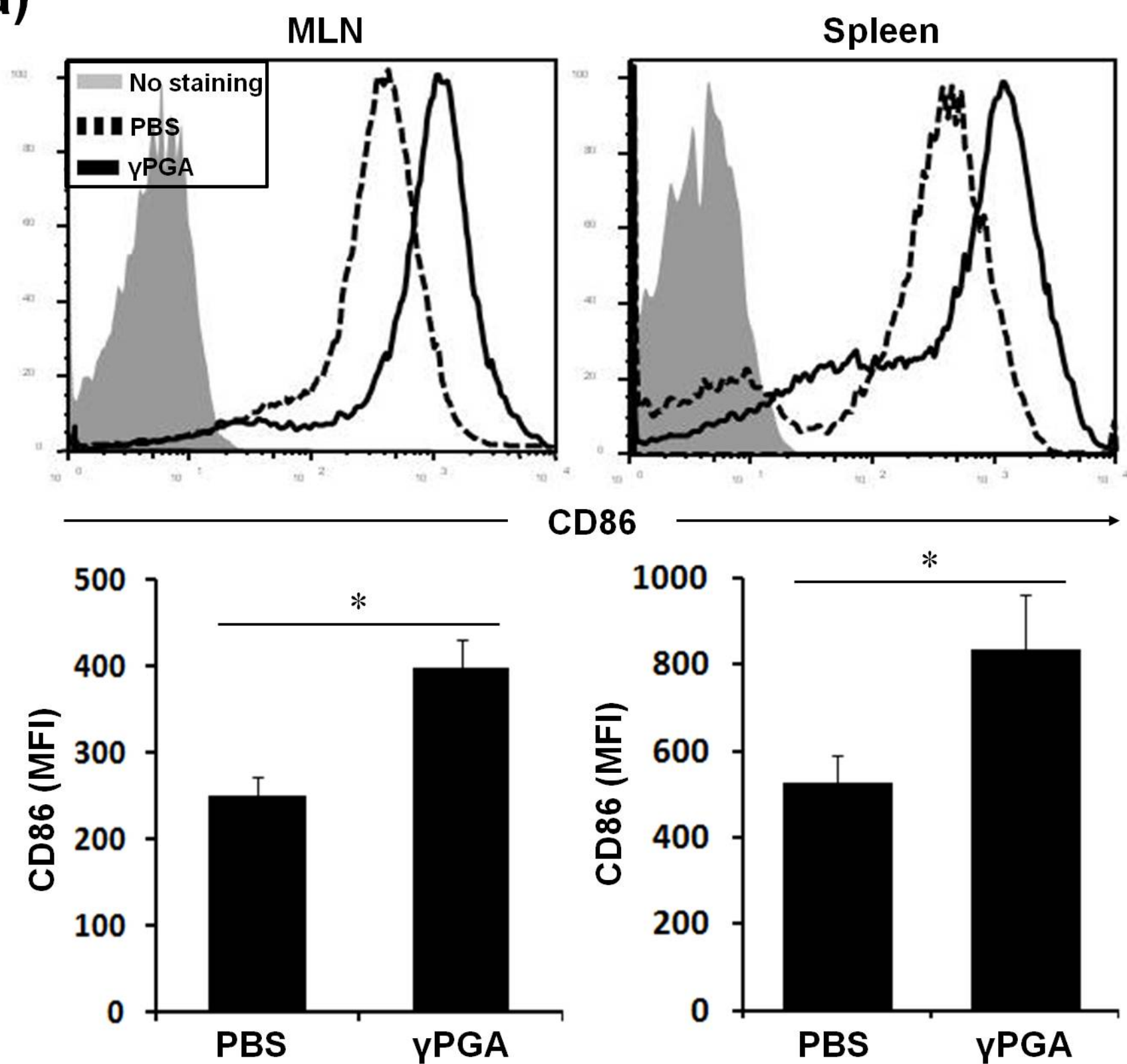
Figure S5. Oral administration of γ PGA up-regulates T-bet and ROR γ t expression but down-regulates GATA-3 expression.

Figure S6. Oral administration of γ PGA down-regulates the *in vivo* generation of Treg cells.

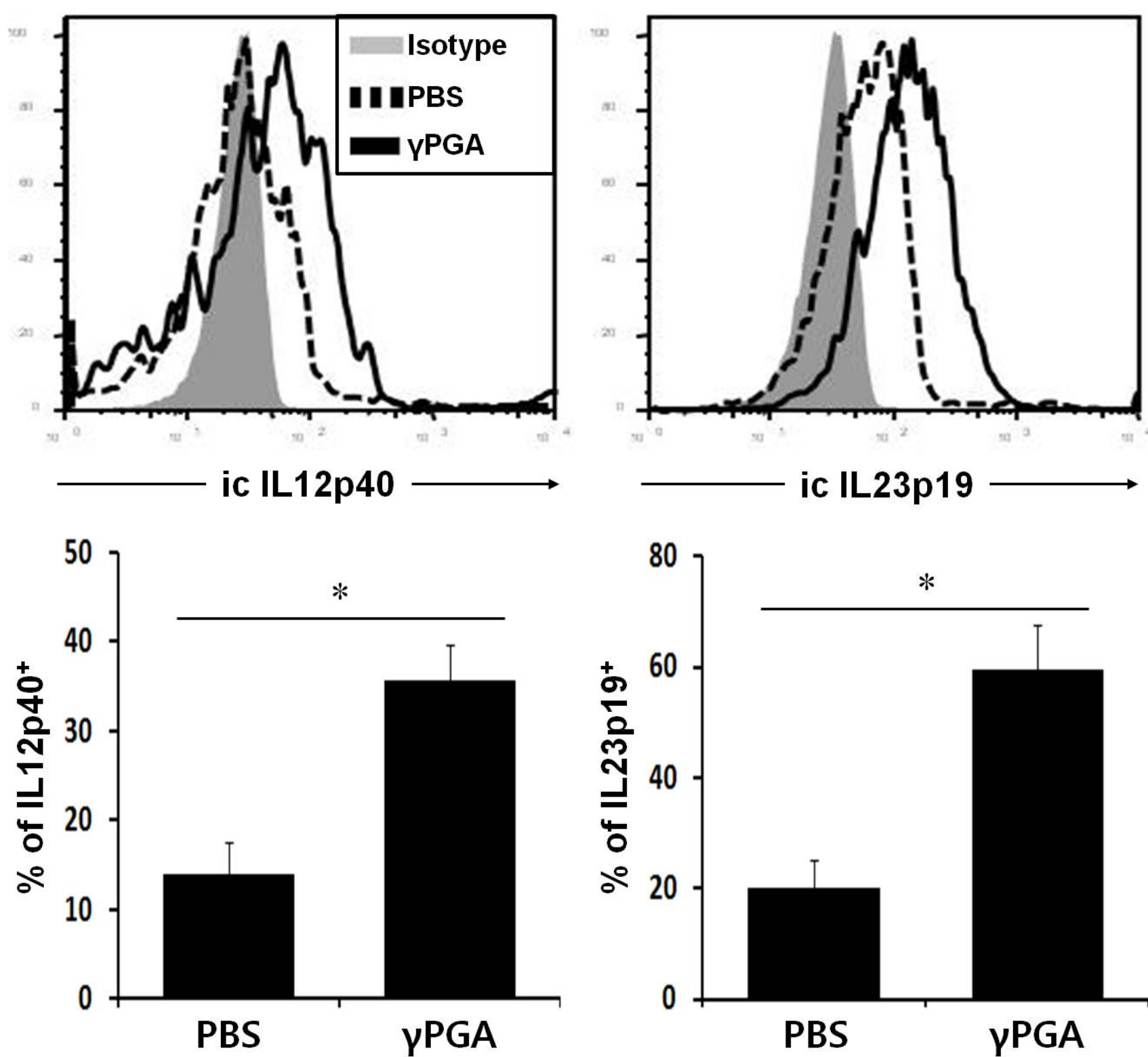
Appendix S1. Materials and methods.

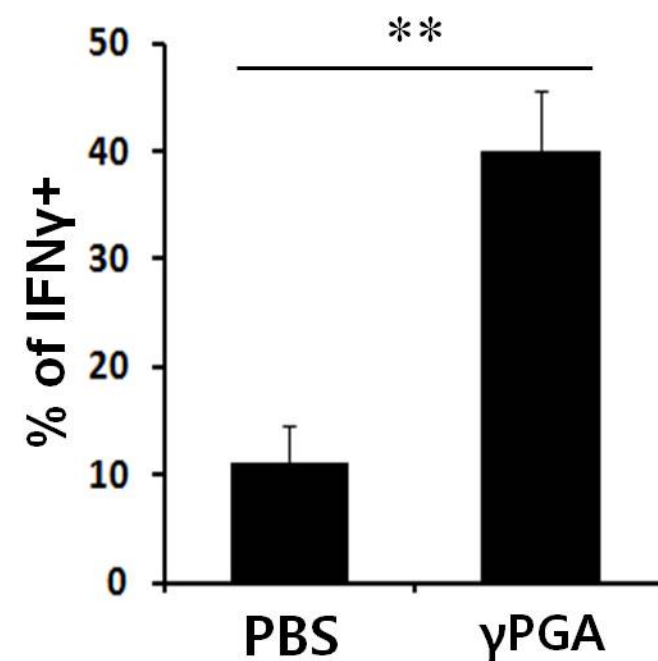
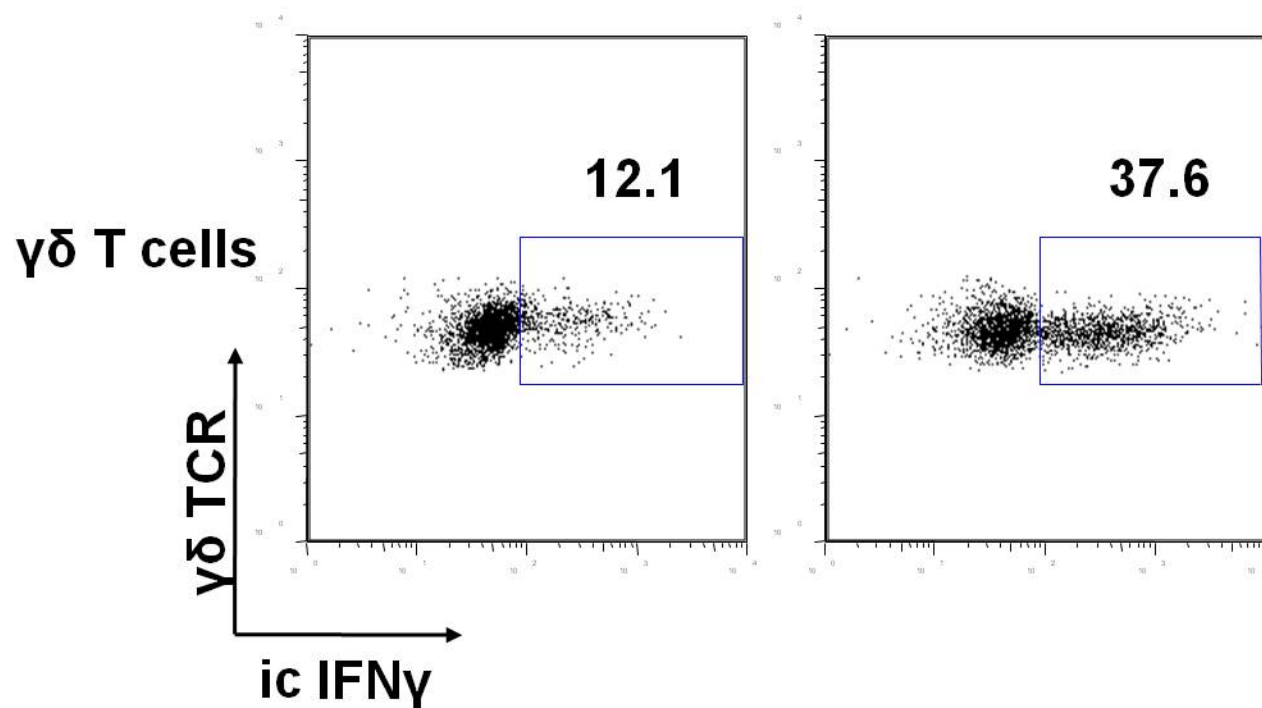
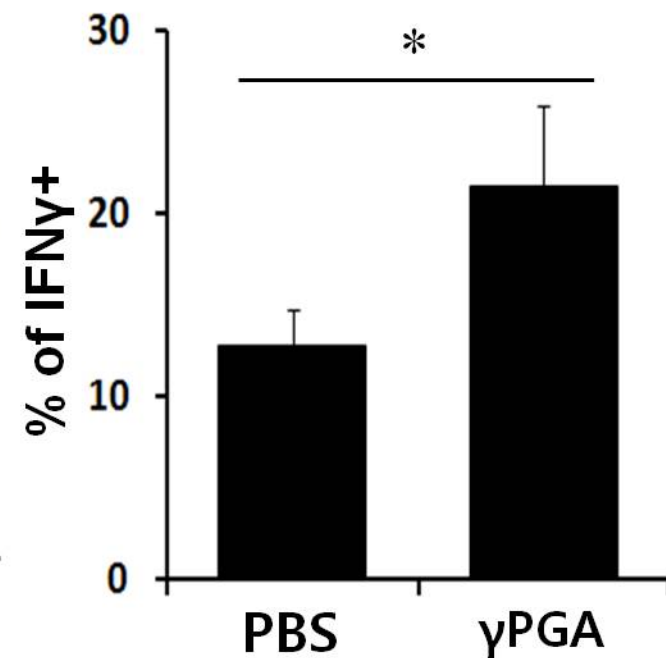
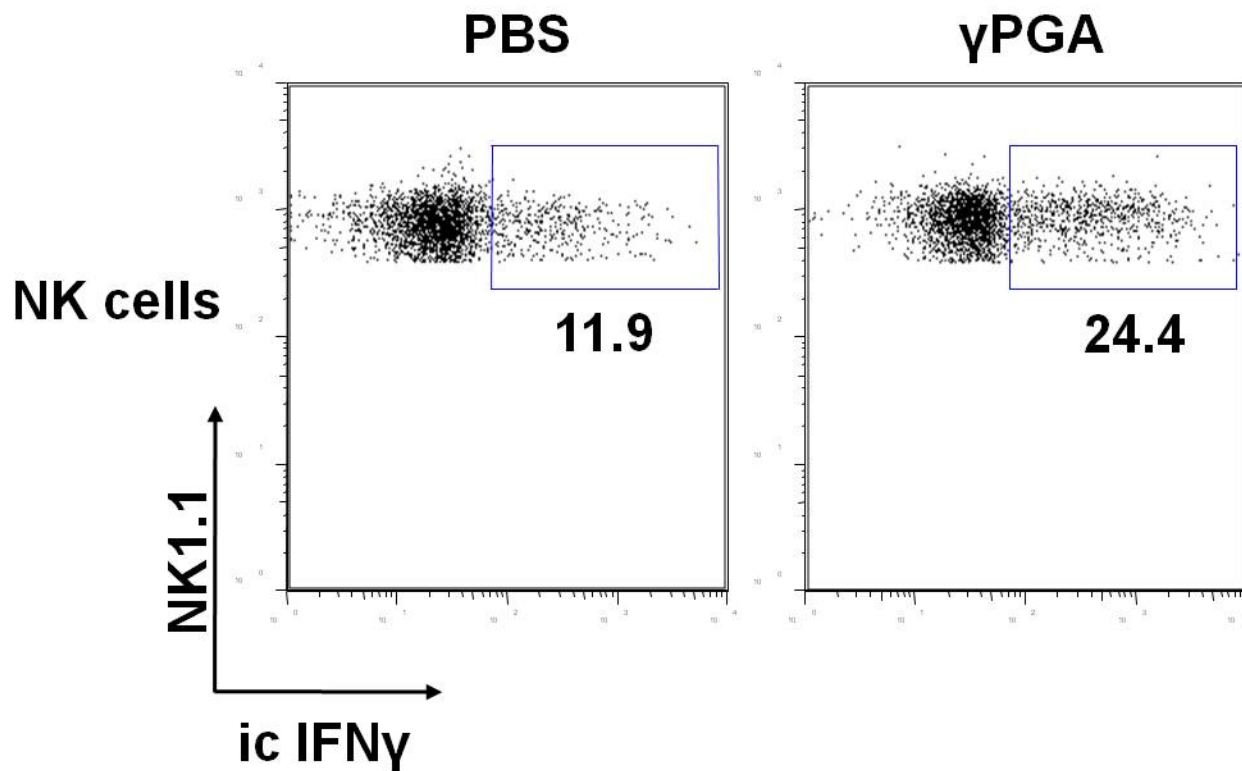
(a)**(b)****(c)**

(a)



(b)





Unpulsed DC

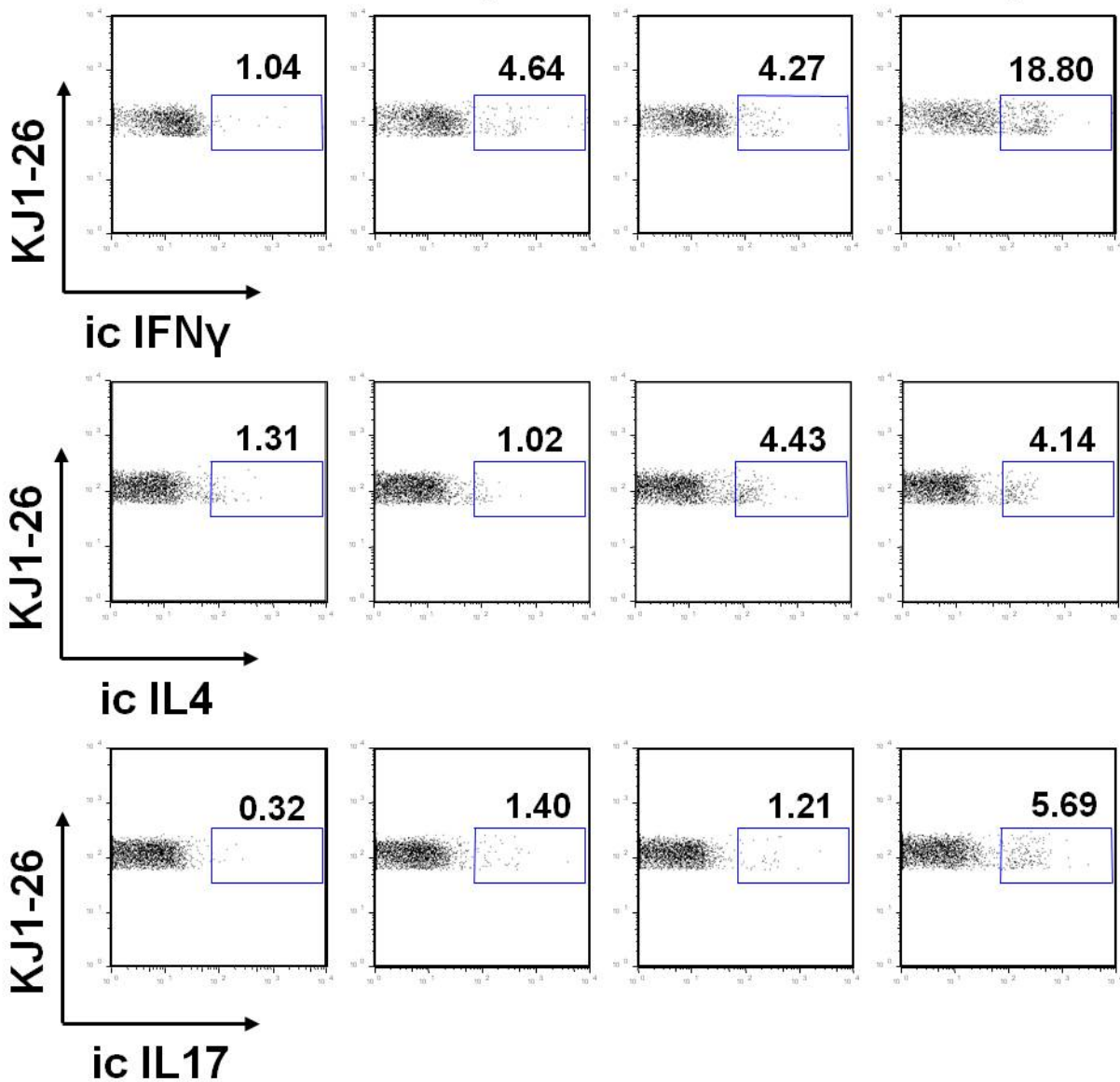
Pulsed DC

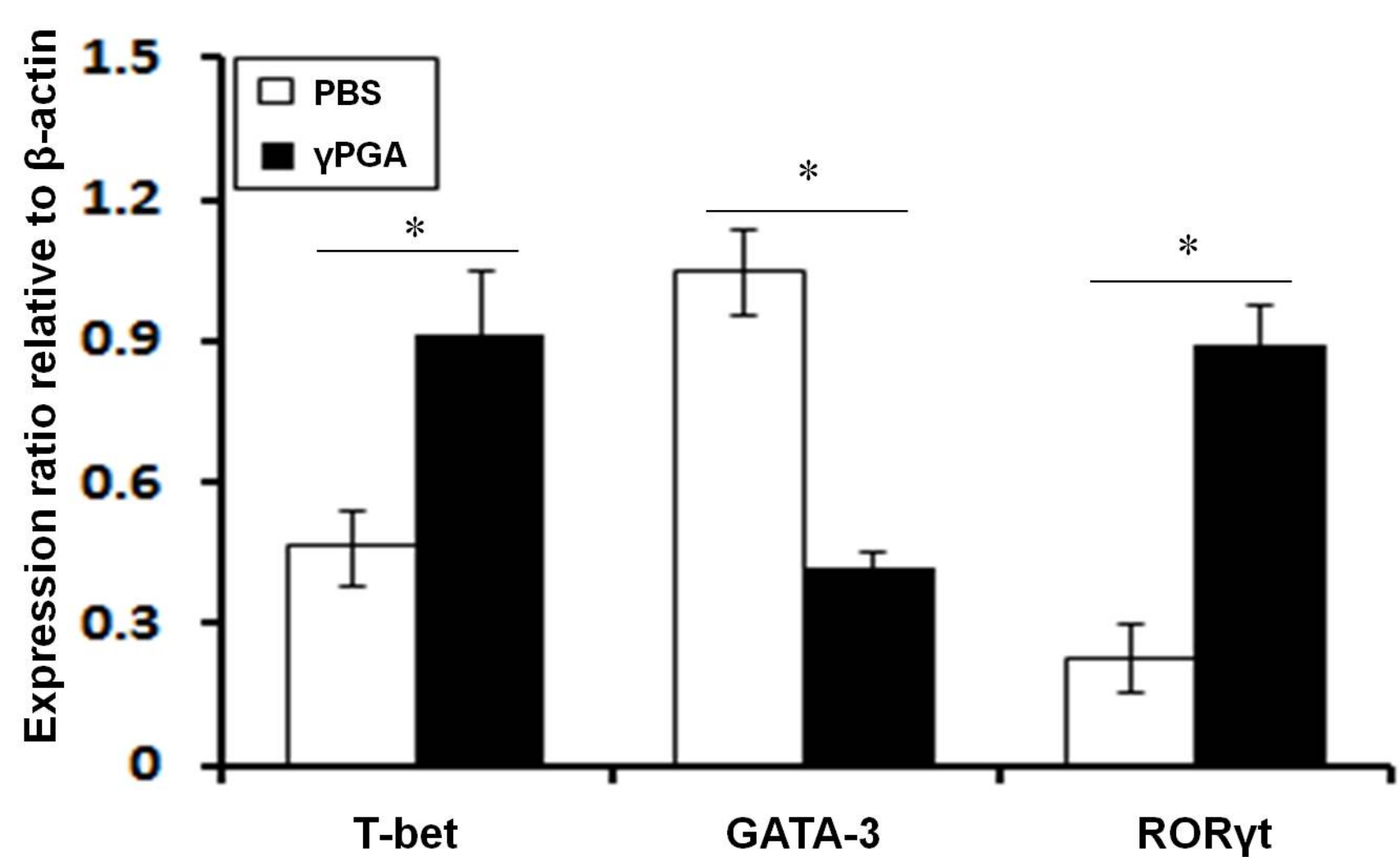
OVA

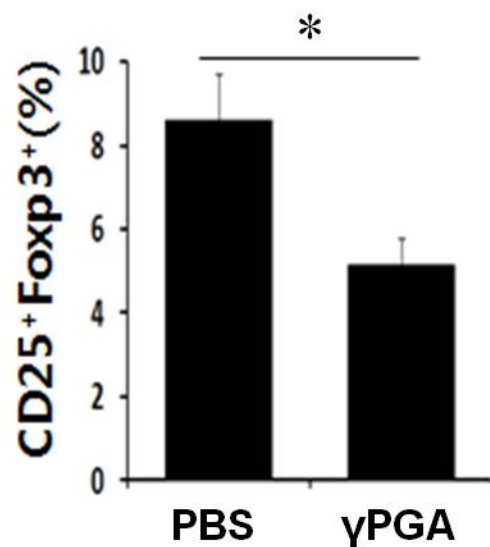
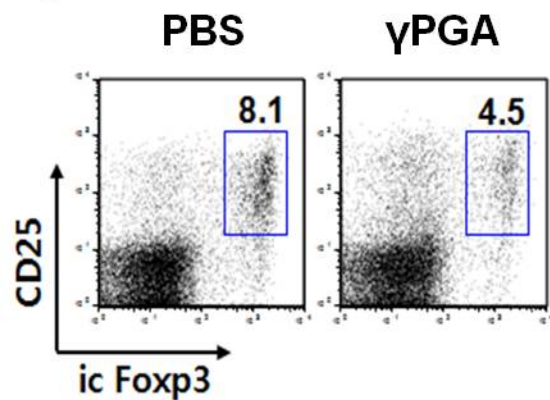
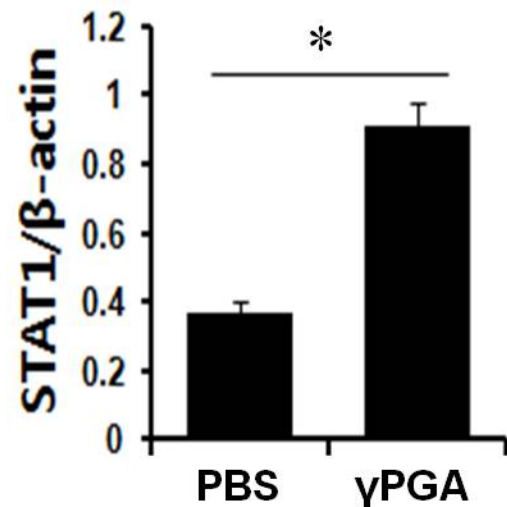
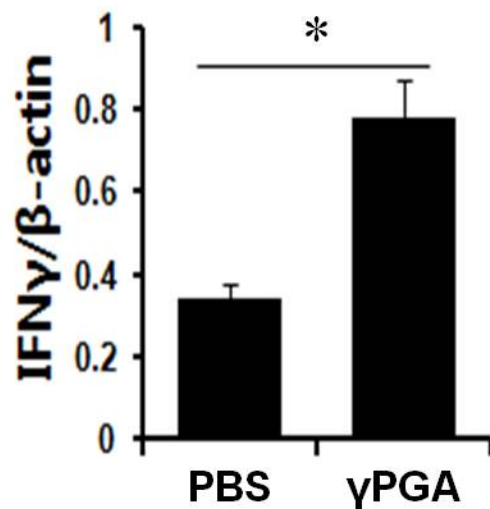
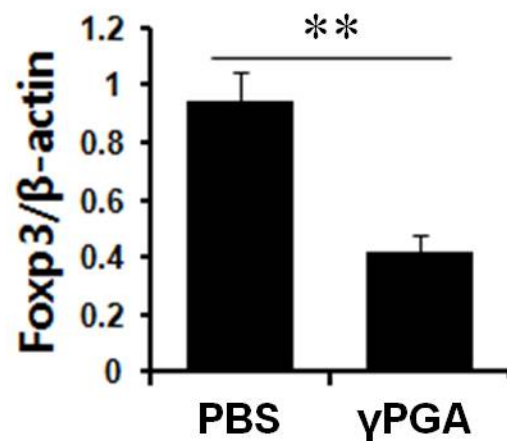
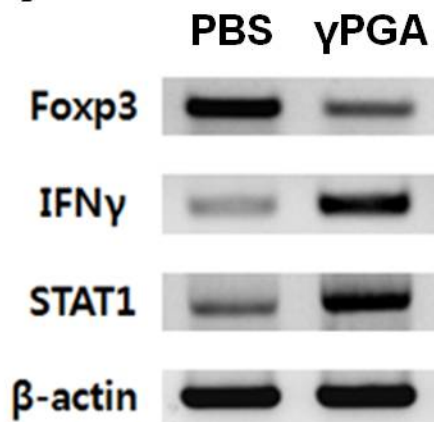
OVA+ γ PGA

OVA

OVA+ γ PGA





(a)**(b)**

Appendix S1. Materials and methods

Mice and reagents

NC/Nga (NC) wild type (WT) mice were purchased from Jung Ang Lab Animal Inc. (Seoul, Korea). DO11.10 OVA-specific TCR Tg Balb/c mice were backcrossed to NC mice for more than eleven generations. The mice used in this study were all obtained from an NC genetic background. These mice were bred and maintained at Sejong University and were 6-12 weeks old in all of the experiments that were performed (unless otherwise specified). The mice were fed a γ -irradiated sterile diet and autoclaved distilled water. They were injected orally with either γ PGA (2 mg/mouse) or PBS alone starting within one week after mice were transferred to conventional condition. The animal experiments were approved by the Institutional Animal Care and Use Committee of Sejong University (SJ-20100401009). The OVA₃₂₃₋₃₃₉ peptide (ISQAVHAAHAEINEAGR) was synthesized by Peptron (Daejeon, Korea), and γ PGA was purchased from Bioleaders (Daejeon, Korea). γ PGA was dissolved in a neutral pH buffer, diluted in PBS, and utilized at a final concentration of 10 mg/ml.

Oral administration of γ PGA to NC mice

Male NC mice were transferred to conventional conditions at the age of 6–8 weeks and were treated orally by using oral gavage with either γ PGA (2 mg/injection) or PBS three times per week for 12 weeks. Three independent experiments were performed, with each group containing 5-6 mice in each experiment. Six- to eight-week-old male NC mice maintained under SPF conditions were used as negative controls for AD development.

Cell isolation and culture

Splenic CD4 T cells were isolated from either γ PGA- or PBS-treated NC mice using a magnetic activated cell sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's instructions. The CD4 T cells were >97% after MACS. In addition, for the preparation of CD11c⁺ total DCs, whole splenocytes from either γ PGA-treated or PBS-treated NC mice were stained with anti-CD11c mAb for MACS and enriched for CD11c⁺ DCs by positive selection. The DC population was >95% after MACS. Primary cells were cultured in RPMI 1640 (Gibco BRL, USA) culture media supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 100 units/mL penicillin-streptomycin, and 5 mM 2-mercaptoethanol.

Flow cytometric analysis

The following mAbs were obtained from BD Biosciences (San Jose, USA): fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or allophycocyanin (APC)-conjugated anti-CD25 (clone PC61); FITC-, PE-, or APC-conjugated anti-CD4 (clone RM4-5); PE- or APC-conjugated anti-NK1.1 (clone PK-B6); FITC-, PE-Cy7-, or APC-conjugated anti-CD3 ϵ (clone 145-2C11); biotin-conjugated anti-CD86 (clone GL1); FITC-, PE-, PE-Cy7-, or APC-conjugated anti-CD11c (clone HL3); biotin-conjugated anti-TCR $\gamma\delta$ (clone GL3); PE-conjugated anti-Siglec-F (clone E50-2440); PE-Cy5-conjugated anti-clonotypic TCR (clone KJ1-26); biotin-conjugated anti-IgE (clone R35-118); PE-conjugated anti-IL12p40 (clone C15.6); FITC-conjugated anti-IgG2b (κ isotype control) (A95-1); and PE-conjugated anti-IgG1 (κ isotype control) (clone R3-34). The following mAbs were obtained from eBioscience (San Diego, USA): FITC- or APC-conjugated anti-CD19 (clone ID3); PE-Cy7-conjugated anti-CD117 (c-kit) (clone 2B8); FITC- or PE-conjugated anti-Fc ϵ RI (clone MAR-1); PE-conjugated anti-IFN γ (clone XMG1.2); FITC- or PE-conjugated anti-IL4 (clone BVD6-24G2); FITC- or PE-conjugated anti-IL17A (clone eBio17B7); and FITC- or PE-conjugated anti-Foxp3 (clone NRRF-30). The PE-conjugated anti-IL23p19 (clone 320244) mAb was obtained from R&D Systems. The flow cytometric data were acquired with a FACSCalibur system (Becton Dickinson, USA) and analyzed with FlowJo software (Tree Star, USA).

For surface staining, cells were harvested and washed twice with cold 0.5% BSA-PBS (FACS buffer). To block the Fc receptors, the cells were incubated with anti-CD16/CD32 mAbs on ice for 10 min and were subsequently stained with fluorescence-labeled mAbs. For intracellular staining, splenocytes were incubated in RPMI medium containing the intracellular protein transport inhibitor brefeldin A (10 μ g/ml) for 4 hr at 37°C. The cells were stained with the indicated mAbs (FITC- or APC-conjugated anti-CD3; PE-Cy7- or APC-conjugated anti-CD11c; APC-conjugated anti-NK1.1; FITC- or PE-Cy7-conjugated anti-CD4; or APC-conjugated anti-CD25) for 30 min at 4°C, fixed with 1% formaldehyde in PBS, and subsequently treated with a permeabilizing solution containing 1% BSA and 0.1% saponin for 10 min at room temperature. The permeabilized cells were then stained for an additional 30 min at room temperature with the indicated mAbs (PE-conjugated anti-IFN γ , anti-IL12, anti-IL23, or anti-Foxp3; FITC-conjugated anti-IL17 or anti-Foxp3; or PE-conjugated isotype control rat IgG mAbs). More than 5,000 cells per sample were acquired using a FACSCalibur system and analyzed with the FlowJo software package.

***In vivo* T helper cell differentiation assay**

DO11.10 TCR Tg NC mice were immunized i.p. with 100 µg of the OVA₃₂₃₋₃₃₉ peptide in the presence of either 2 mg of γPGA or PBS. Five days later, CD4⁺ T cells isolated from the immunized mice were co-cultured with DCs pulsed with OVA₃₂₃₋₃₃₉ (10 µg/ml) at a ratio of 10 to 1 for 48 hr. CD4⁺ T cells were stimulated with OVA *in vitro*, and cytokine production was determined by intracellular cytokine staining.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from CD4⁺ cells using the Easy Red reagent (Intron, Korea) and was reverse-transcribed into cDNA with oligo(dT) primers and M-MLV RT (Invitrogen Life Technologies, USA), according to the manufacturers' instructions. PCR amplification was performed according to the recommended protocols for the PCR Amplification Kit; for each PCR amplification, 1 µg of cDNA, 5 pmol of each primer, and HiPi PCR Premix (ELPiS Biotech, Korea) were included in a 20 µl reaction volume. PCR was conducted in an XP Thermal Cycler (Bioer Technology, Hangzhou, China) with the following thermal cycling parameters: 35 cycles of 94°C for 5 min, 94°C for 45 sec, 57°C for 45 sec, 72°C for 1 min, and 72°C for 5 min for IL12p40, IL12p19, IL12p35, TNFα, IL6, and IFNγ; 35 cycles of 94°C for 5 min, 94°C for 45 sec, 61°C for 45 sec, 72°C for 1 min, and 72°C for 5 min for T-bet, STAT1, GATA-3, RORγt, and Foxp3; and 30 cycles of 94°C for 5 min, 94°C for 30 sec, 54°C for 30 sec, 72°C for 35 sec, and 72°C for 5 min for β-actin. Equal amounts of RT-PCR product were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The intensity of the resulting bands was measured with a Gel Logic 100 Imaging System (Kodak, NY, USA) and analyzed using the Image J software package (National Institutes of Health). The sequences of the PCR primers were as follows: β-actin forward, 5'-GTATGGAATCCTGTGGCATC-3' and β-actin reverse, 5'-AAGCACTTGC GGTCACGAT-3'; IL12p40 forward, 5'-CAGAAGCTAACCATCTCCTGGTTTG-3' and IL12p40 reverse, 5'-TCCGGAGTAATTTGGTGCTTCACAC-3'; IL12p19 forward, 5'-CTGTGCCTAGGAGTAGCAGT-3' and IL12p19 reverse, 5'-TGCTTATAAAAAGCCAGACC-3'; IL12p35 forward, 5'-TCAGCGTTCCAACAGCCTC-3' and IL12p35 reverse, 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'; IFNγ forward, 5'-AGCTCTTCCTCATGGCTGTT-3' and IFNγ reverse, 5'-TTTGCCAGTTCCTCCAGATA-3'; TNFα forward, 5'-GGCAGGTCTACTTTGGAGTCATTG-3' and TNFα reverse, 5'-ACATTCGAGGCTCCAGTGAATTCGG-3'; IL6 forward, 5'-GTGGCAGGTAGAGCAGGAAG-3' and IL6 reverse, 5'-CCACCTGAAAGGCACTCTGT-3'; STAT1 forward 5'-CTTCTTCCTGAACCCCCCG-3', and STAT1 reverse, 5'-CCCATCATTCCAGAGGCACAG-3'; T-bet forward 5'-

AACCAGTATCCTGTTCCCAGC-3', and T-bet reverse, 5'-TGTCGCCACTGGAAGGATAG-3'; GATA-3 forward, 5'- CTCCTTTTGTCTCTCCTTTTC-3' and GATA-3 reverse, 5'- AAGAGATGAGGACTGGAGTG-3'; RORyt forward, 5'-ACTTTTCCCACTTCCTCAGCG-3' and RORyt reverse, 5'-AGAGTTCCTTATAGAGTGGCGGG-3'; Foxp3 forward, 5'-GAAACAGCACATTCCCAGAGTTC-3' and Foxp3 reverse, 5'-ATGGCCCAGCGGATGAG-3'.

ELISA

Serum IgE levels were measured with a sandwich ELISA (clone R35-72 for capturing IgE and R35-118 for detecting IgE; BD PharMingen, USA). The quantities of IFN γ and IL4 in the culture supernatants were determined using ELISA kits, according to the manufacturer's instructions (BD PharMingen, USA). The optical density was measured at 450 nm with an immunoreader (Bio-Tek ELX-800, USA).

Analyses of skin sections

Dorsal skin samples were obtained 4 days after the final injection, fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 6- μ m sections using a microtome (RM 2235, Leica, Germany). The sections were then stained with H&E for the analysis of histological changes and additionally stained with toluidine blue and Congo red for the detection of mast cells and eosinophils, respectively. The cells were counted with a microscope at a magnification of 400 times. The cell density was expressed as the total number of cells in ten high-power fields (400x) for each section.

Scoring the severity of skin lesions

Skin lesions were scored at the indicated time points after oral injection with γ PGA. The scoring was based on the severity of lichenification, edema, erosion/excoriation, scarring/dryness, and erythema/hemorrhage. The total clinical skin severity score was defined as the sum of the five signs (none=0; mild=1; moderate=2; and severe=3).

Statistical analysis

Statistical significance was determined using Excel software (Microsoft, USA). To compare two groups, Student's t-test was performed. *P<0.05, **P<0.01, and ***P<0.001 were considered significant.

Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1. Effects of oral γ PGA treatment on allergic effector cells.

(a) Decrease in splenic mast cells, eosinophils, and basophils following oral administration of γ PGA. The absolute numbers of mast cells (c-kit⁺Fc ϵ RI⁺), basophils (c-kit⁺Fc ϵ RI⁺), and eosinophils (siglec-F⁺) in the spleen were determined by flow cytometry. The mean values $\pm$ SD are provided (n=5, *P<0.05). (b) Oral administration of γ PGA results in the reduction of mast cells. Skin lesions were sectioned and stained for mast cells and eosinophils with toluidine blue and Congo red, respectively. Mast cells and eosinophils were counted in 10 random high-power fields (400x) for each section. The mean values $\pm$ SD are provided (lower right panel, n=5, ***P<0.001). (c) Reduced levels of IgE on the surface of splenic mast cells following oral administration of γ PGA. Upon allergen binding, the cross-linking of IgE-bound Fc ϵ RI on activated mast cells initiates the allergic response (1). The extent to which mast cells express IgE-bound Fc ϵ RI indicates the status of an allergic response, and we therefore measured IgE levels on mast cells by flow cytometry. γ PGA treatment resulted in a dramatic reduction of IgE bound on mast cells, suggesting that γ PGA stimulation can alter mast cell function. The expression levels of membrane-bound IgE in mast cells (c-kit⁺Fc ϵ RI⁺) is represented as mean fluorescence intensities (MFI). The mean values $\pm$ SD are provided (n=5, *P<0.05).

Figure S2. Effects of oral gPGA treatment on dendritic cells.

(a) Oral administration of γ PGA induces maturation of dendritic cells. Cells from the mesenteric lymph node (MLN) and spleen were prepared from either γ PGA- or PBS-injected NC mice. These cells were subsequently stained for the co-stimulatory molecule CD86, and the expression levels of CD86 in the CD11c⁺-gated cell population were measured by flow cytometry. The mean values $\pm$ SD are provided (n=5, *P<0.05). (b) Oral administration of γ PGA induces pro-inflammatory cytokine production by dendritic cells. Flow cytometry was utilized to measure the intracellular (ic) expression levels of the IL12p40 and IL23p19 proteins in purified splenic DCs after stimulation with PMA (50 ng/ml) and ionomycin (1 μ g/ml) for 2 hr. The mean values $\pm$ SD are provided (n=5, *P<0.05).

Figure S3. Oral administration of γ PGA increases IFN γ production by NK and $\gamma\delta$ T cells.

Although it has been reported that IFN γ secretion by splenic NK cells is greatly increased by *in vivo* treatment with high-molecular-mass γ PGA (2), here we investigated the effect of low-molecular-mass γ PGA and found that γ PGA stimulation could induce IFN γ production from $\gamma\delta$ T cells in addition to NK cells. The frequencies of IFN- γ -producing populations of splenic NK cell and from $\gamma\delta$ T cells from either γ PGA- or PBS-treated NC mice were assessed by flow cytometry. The mean values $\pm$ SD are provided (n=5, *P<0.05, **P<0.01).

Figure S4. *In vivo* injection of γ PGA induces the polarization of Th1 and Th17 cells but inhibits the polarization of Th2 cells in an antigen-specific manner.

To verify the adjuvant capability of γ PGA in an antigen-specific manner, we generated an OVA-specific TCR transgenic NC mouse model by backcrossing DO11.10 TCR Tg Balb/c mice more than 10 times onto an NC genetic background. To measure antigen-specific T helper cell differentiation, DO11.10 NC mice were immunized i.p. with OVA peptide in combination with either γ PGA or PBS. Five days later, CD4⁺ T cells were isolated from immunized mice and restimulated *in vitro* for 2 days with OVA-pulsed DCs. The cytokine production profiles of the CD4⁺ T cells were then evaluated by an intracellular cytokine staining assay. Our results demonstrated that *in vivo* γ PGA stimulation increased antigen-specific Th1 and Th17 responses but not Th2 responses.

Figure S5. Oral administration of γ PGA up-regulates T-bet and ROR γ t expression but down-regulates GATA-3 expression.

We utilized RT-PCR to evaluate the mRNA levels of the following specific transcription factors that orchestrate the differentiation of T helper subsets in both the γ PGA- and PBS-treated groups: T-bet (Th1 cells), GATA-3 (Th2 cells), and ROR γ t (Th17 cells) (3). The gene expression levels of T-bet, GATA-3, and ROR γ t in splenic CD4⁺ T cells purified from γ PGA- or PBS-injected NC mice were determined by RT-PCR. γ PGA treatment led to an up-regulation of T-bet and ROR γ t but a down-regulation of GATA-3, consistent with the cytokine profile of T helper cells. Mean values $\pm$ SD are shown (n=5, *P<0.05).

Figure S6. Oral administration of γ PGA down-regulates the *in vivo* generation of Treg cells.

Previous studies have demonstrated the importance of the Th1 cytokine IFN γ and the transcription factor STAT1 in controlling the generation of peripheral Treg cells (4, 5). Because γ PGA injection induces the up-regulation of IFN γ by innate immune cells such as NK cells and $\gamma\delta$ T cells, the Th1 immune responses elicited

by γ PGA might result in a decrease of CD25⁺ Foxp3⁺ Treg cells. To assess this possibility, we measured the transcriptional levels of the STAT1, IFN γ , and Foxp3 genes in splenic CD4⁺ T cells from either γ PGA- or PBS-treated NC mice using semi-quantitative RT-PCR. The results demonstrated that the CD4⁺ T cells from γ PGA-treated mice exhibited markedly reduced levels of Foxp3 mRNA but increased levels of STAT1 and IFN γ , compared to the CD4⁺ T cells from PBS-treated mice. The mean values $\pm$ SD are provided (n=5, *P<0.05, **P<0.01) (b). In addition, the percentage of CD25⁺Foxp3⁺ Treg cells from γ PGA- or PBS-treated NC mice was evaluated by flow cytometry. The mean values $\pm$ SD are provided (n=5, *P<0.05) (a). Progressive Th1 immune responses following γ PGA stimulation produce negative effects on the generation of peripheral Foxp3⁺ Treg cells, suggesting that Treg cells have little impact on the regulation of Th2 allergic responses in the context of Th1-dominant immune responses.

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