

Nasal peanut+ CpG immunotherapy enhances peanut-specific IFN- γ in Th2 cells and IL-10 in non-Th2 cells in mice

To the Editor,

Peanut-specific allergen immunotherapy reduces peanut-specific serum IgE and T helper 2 (Th2) cell cytokines, IL-5 and IL-13, and increases peanut-specific serum IgG, IgG4, mucosal IgA, and T regulatory (Treg) cells^{1,2} in hypersensitive individuals. However, it is not well understood how peanut-specific Th2 cells change after immunotherapy. This study aimed to evaluate potential mechanisms allergen-specific immunotherapy may use to reduce Th2 responses in peanut-hypersensitive mice.

Utilizing a mouse nasal allergen-specific immunotherapy (NIT) model, we confirmed the beneficial effects of including the Toll-like receptor 9 ligand, CpG, as a Th1-enhancing adjuvant in peanut NIT^{3,4} to reduce allergic disease severity. The comparison of CpG-adjuvanted to unadjuvanted peanut-specific NIT was performed in IL-13-lineage-tracing mice to evaluate the contribution of CpG combined with peanut on peanut-specific Th2 cells in hypersensitive mice. This study tested the hypothesis that CpG-adjuvanted peanut NIT transitioned peanut-specific Th2 cells into peanut-specific Th1 and/or Treg cells.

Peanut hypersensitivity was induced in C57BL/6J mice by gastric gavage with peanut extract (PN) and cholera toxin (CT) (Figure S1). PN-specific immunity induced by sensitization was similar among all peanut-hypersensitive mice since there were no differences in serum and fecal antibodies measured prior to immunotherapy (Figure S2). After a 4-week rest, peanut-hypersensitive mice were nasally treated with saline (placebo), peanut alone (PN), CpG alone (CpG), or peanut+ CpG (PN+ CpG), three times a week for 4 weeks. Postimmunotherapy, PN and PN+ CpG enhanced PN-specific serum and mucosal antibodies compared to placebo and CpG groups (Figure 1A,B). PN+ CpG also increased PN-specific serum IgG2c and fecal IgA compared to PN. NIT was specific to PN responses since CT-specific antibodies were similar among all treatment groups after immunotherapy (Figure S3).

One-week postimmunotherapy, PN-induced anaphylaxis determined whether NIT reduced allergy severity. Body temperature and allergy clinical symptoms were monitored for one hour postchallenge (Figure 1C,D). Unsensitized mice maintained stable body temperature after challenge; however, PN-hypersensitive mice rapidly displayed hypothermia post-PN exposure. Hypersensitive mice treated with PN+ CpG began to recover from hypothermia and demonstrate improved allergic symptoms 60 minutes postchallenge compared to placebo-treated mice while neither PN nor CpG treatments were effective. Our model suggests both PN and CpG are required for NIT to reduce PN-induced anaphylaxis severity.

PN-specific T-cell cytokines that may suggest disease severity after NIT (Figure 2) were evaluated. PN-induced IL-13, IFN- γ , and IL-10 were measured to monitor Th2, Th1, and Treg responses, respectively. Cells from PN-sensitized mice secreted more IL-13 after PN-stimulation compared to unsensitized mice (Figure 2A), but IL-13 secretion was significantly reduced by PN+ CpG compared to placebo, PN, and CpG. PN-induced IL-10 (Figure 2B) and IFN- γ (Figure 2C) secretion was increased in PN-sensitized mice compared to unsensitized mice, but PN+ CpG further enhanced IL-10 and IFN- γ compared to the other immunotherapy groups. Thus, PN+ CpG therapy decreases IL-13 and increases IFN- γ and IL-10.

To test the hypothesis that CpG-adjuvanted peanut NIT induced the transition of peanut-specific Th2 cells into Th1 and/or Treg cells, CD4+ cells from PN-hypersensitive IL-13-lineage-tracing mice treated with PN or PN+ CpG NIT were assessed for peanut-induced IL-10 and IFN- γ . PN+ CpG increased PN-induced IL-10+ cells from non-Th2 cells (Tom-) (Figure 2D) and IFN- γ + from Th2 cells (Tom+) (Figure 2E) compared to PN NIT. There was no difference in IL-13+ cells after PN+ CpG and PN NIT. Our data suggest that PN+ CpG enhances the number of IL-10+ and IFN- γ + cells without effecting the number of IL-13+ cells compared to NIT with PN alone.

This study demonstrated enhanced PN-specific serum IgG2c, mucosal IgA, IL-10, and IFN- γ and decreased IL-13 in mice with improved allergy severity after PN+ CpG NIT. PN+ CpG increased IFN- γ in CD4+ T cells that once produced IL-13 (ie, Th2 cells) and IL-10 in non-Th2 cells (never produced IL-13) in IL-13-lineage-tracing mice. These results suggest, for the first time, that CpG-adjuvanted peanut NIT can modulate IL-13-producing Th2-cell cytokine profile to enhance IFN- γ and generate additional IL-10-producing cells, which may reduce anaphylaxis severity.

PN+ CpG induced peanut-specific immune responses in PN-hypersensitive mice similar to peanut-allergic individuals who have achieved immunological tolerance, which reduces allergen-specific Th2 responses, after PN immunotherapy.^{1,2} PN NIT enhanced sensitization-induced antibody responses compared to placebo or CpG. However, PN+ CpG further boosted PN-specific serum IgG2c and mucosal IgA responses compared to NIT with PN alone and increased peanut-induced IFN- γ and IL-10 while decreasing peanut-induced IL-13 secretion, suggesting a shift from Th2 immunity toward Th1 and Treg immunity. Our results support clinical observations that also report decreased Th2-associated immunity and increased Th1 and/or Treg immunity.²

Changes in secreted IL-13, IL-10, and IFN- γ after PN+ CpG immunotherapy led us to investigate the cellular source of these

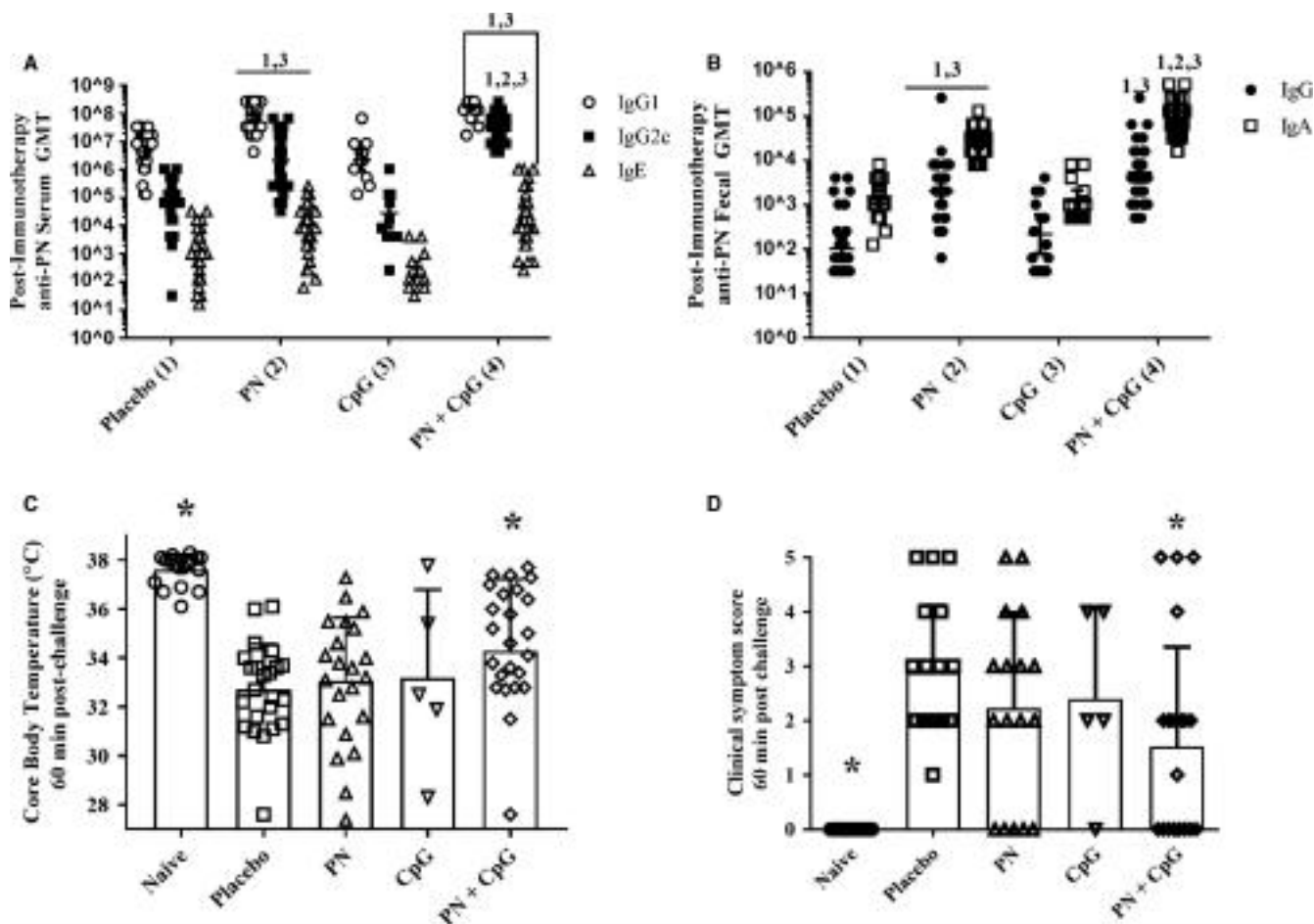


FIGURE 1 CpG enhances host responses influenced by peanut-specific immunotherapy and reduces allergen severity. Peanut-hypersensitive C57BL/6 mice received saline (placebo), PN, CpG, or PN+ CpG NIT. PN-specific (A) serum and (B) fecal antibodies reported as geometric mean titers (GMT) were measured after therapy (N = 14-41). Systemic anaphylaxis was induced with PN. C, Core body temperature and (D) allergy clinical symptom scores were measured for one hour after peanut exposure (N = 5-25) and reported as the mean + standard deviation. Statistical differences in antibodies were determined by one-way ANOVA with Tukey's multiple comparison ($P < 0.05$). Numbers denote a significant increase compared to corresponding therapy group. Two-way ANOVA determines a significant reduction in disease severity compared to placebo indicated by * ($P < 0.05$)

cytokines. We hypothesized that adjuvanted immunotherapy may change PN-specific T-cell cytokines through at least two mechanisms, including (a) enhanced production of Th1 and Treg cytokines in Th2 cells and/or (b) generation of peanut-specific Th1 or Treg cells from naïve precursor T cells that reduce Th2 responses. Th2 cells produce several cytokines, including IL-4, IL-5, and IL-13; however, IL-13 is involved in tissue Th2 responses⁵ and may contribute to allergic symptoms. Our study demonstrates reduced IL-13 secretion with PN+ CpG NIT compared to PN NIT but similar numbers of IL-13+ CD4+ cells, which suggests that PN+ CpG therapy decreases the amount of IL-13 produced without effecting the number of IL-13+ cells. One mechanism that may reduce IL-13 production is enhanced plasticity in Th2 cells to acquire cytokines that suppress Th2 cytokine responses. IL-13 may be produced by Th1 and Th17 cells⁶; however, low Th1 transcription factor levels are observed in IL-13+/IFN- γ cells,⁶ suggesting that the IL-13+ IFN- γ cells are plastic Th2 cells evolving to acquire a Th1 phenotype. We observed increased IFN- γ in CD4+ IL-13+ T cells after IT, which

may support Th2 cell plasticity. Th2 to Th2+ Th1 cell plasticity is reported in mouse effector and memory Th2 cells that produce IFN- γ after in vivo activation with Th1-inducing stimuli.⁷ Therefore, it is possible that the addition of CpG to PN immunotherapy formulations increases IFN- γ in Th2 cells to reduce pro-allergy effector cytokines.

PN+ CpG may also enhance cytokine responses from non-Th2 cells to suppress IL-13 secretion. Enhanced IL-10 in CD4+ IL-13- T cells supports an important role for IL-10 in reducing allergic disease severity, since immunological tolerance to peanut is not achieved in the absence of IL-10.⁸ Naïve T cells exposed to PN+ CpG may possibly differentiate into IL-10+ effector T cells to suppress allergic Th2 responses. Peanut oral immunotherapy (OIT) reduces IL-4+ cells and increases IFN- γ + cells in peanut-hypersensitive subjects.⁹ Since similar cytokine profiles in specific T-cell clones were observed in subjects receiving OIT as placebo therapy, it is unlikely that OIT enhanced Th2-cell plasticity but instead increased non-Th2 cells that minimize the effects of the Th2 cells.⁹ While our data also agree

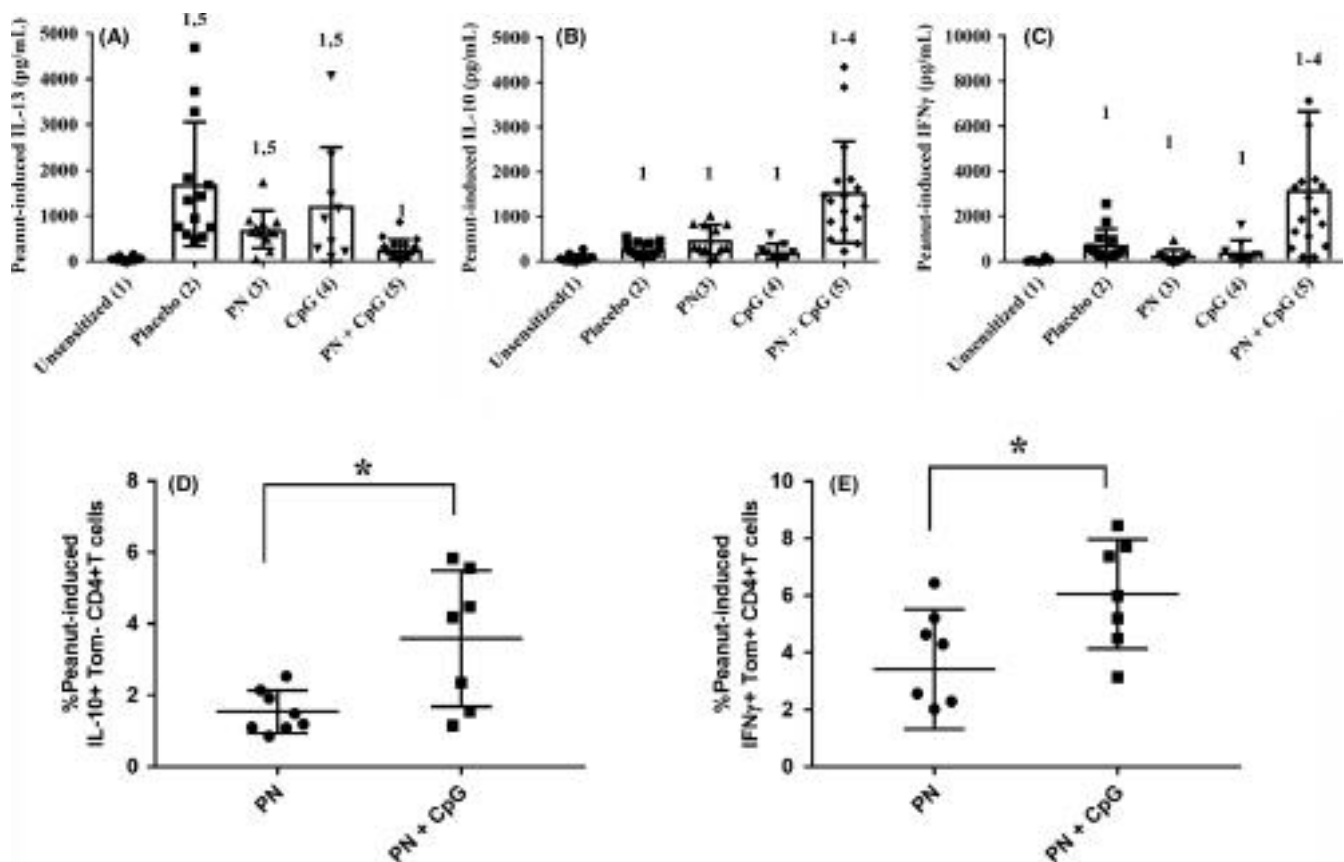


FIGURE 2 Allergen-specific immunotherapy modifies PN-induced cytokine responses. Peanut-induced responses were determined by subtracting values in unstimulated cells from peanut-stimulated cells. Significant differences in peanut-induced secreted (A) IL-13, (B) IL-10, and (C) IFN- γ , measured postimmunotherapy, were determined by one-way ANOVA with Tukey's multiple comparisons ($P < 0.05$). Numbers above denote increased cytokine responses compared to corresponding treatment group below ($n = 9-17$). IL-13-lineage-tracing mice revealed changes in peanut-induced cytokine-positive cells (D-E) after PN or PN+ CpG therapy ($n = 7-8$). CD4+ splenocytes were monitored for IL-13 lineage by TdTomato (Tom) expression, (D) IL-10, and (E) IFN- γ . Mann-Whitney determined significant differences in peanut-induced cytokine-positive cells between PN- and PN+ CpG-treated mice based on IL-13 expression. *indicates significant increase in cytokines compared to PN alone ($P < 0.05$)

with peanut immunotherapy generating non-Th2 cells, such as IL-10+ cells, to reduce allergic Th2-cell effects and desensitize or tolerate the host, it is possible that including potent Th1-enhancing adjuvants, such as CpG, to clinical peanut immunotherapy formulations will both generate new T cells and induce Th2-cell plasticity to, more effectively, reduce allergic disease severity compared to therapy with peanut allergens alone.

Collectively, our study suggests CpG-adjuvanted PN NIT may generate new IL-10+ T cells and induce Th2-cell plasticity to enhance IFN- γ to reduce allergy severity. This information may provide insight toward developing improved peanut allergy immunotherapy formulations.

CONFLICTS OF INTEREST

The authors do not have any conflicts of interest to report.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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IL-10 mRNA levels in whole blood cells correlate with house dust mite allergen immunotherapy efficacy

To the Editor,

Allergen immunotherapy (AIT) is recognized as a safe and efficacious treatment for IgE-mediated respiratory allergies to pollen and house dust mite (HDM) allergens.¹ Whereas patients' eligibility to AIT relies on symptoms severity, reactivity to skin prick tests, and/or high allergen-specific IgEs titers,¹ and patients would greatly benefit from the identification of biomarkers capable to predict the likelihood of clinical improvement during AIT. With this need in mind, we undertook a gene expression analysis on whole blood cells obtained from a sub-group of 152 Japanese patients included in a double-blind placebo-controlled clinical HDM AIT study (S-524101)² to search for predictive biomarker candidates.

Specifically, in this study, a total of 152 HDM allergic patients receiving daily either a 300 IR HDM ($n = 75$) or placebo ($n = 77$) tablet sublingually for 12 months were stratified into four subgroups based upon the median percentage (51.18%) of AASS improvement (cf. Methods S1), namely high active responders (ARs^{high} ; $n = 38$), low active responders (ARs^{low} ; $n = 37$), high placebo responders (PRs^{high} ; $n = 18$), and low placebo responders (PRs^{low} ; $n = 59$). The demographics and baseline characteristics of patients were similar in all groups (Tables S1 and S2). We evaluated polarization markers (ie, *Tbet*, *MX1*, *IFN- γ* , *GATA3*, *OX40L*, *RIPK4*, *IL-13*, *ROR γ -t*, *IL-23*, *IL-17*, *Foxp3*, *C1q*, *STAB1*, and *IL-10*) described to be involved in the allergic reaction or in its resolution³ as predictive markers of clinical responses to AIT. We thus monitored by qPCR the expression of the aforementioned markers and endogenous control β -actin gene in

whole blood cells collected before AIT, and we calculated the copy numbers of each gene per million copies of β -actin mRNA. Among these 14 gene expression analyses, we highlighted that *IL-10* mRNA levels are predictive of clinical responses to AIT.

As shown in Figure 1A, *IL-10* mRNA levels were significantly ($P < 0.01$) higher in whole blood cells from ARs^{high} than in ARs^{low} , whereas no such difference was observed in the placebo group between PRs^{high} and PRs^{low} . In addition, there was no potential bias due to differences in *IL-10* mRNA levels between the active and the placebo groups (Figure 1A). No statistical differences could be observed in the gene expression of other markers between ARs and ANRs (Figure S1). When plotted against percentages of AASS improvement (V17/V2), mRNA levels of *IL-10* were significantly correlated (with a Spearman correlation $R = 0.3509$, $P = 0.002$) with clinical benefit within patients from the active group (Figure 1B, upper panel), whereas no such a correlation was observed in the placebo group (Figure 1B, lower panel). We also measured *IL-10* expression by qPCR in whole blood collected after 2-month immunotherapy (V6) from 152 HDM allergic patients receiving either 300 IR HDM ($n = 75$) or placebo ($n = 77$) tablet sublingually, once a day, for 12 months. When plotted against percentages of AASS improvement (V17/V2) for each individual patient, levels of *IL-10* mRNA were significantly correlated (with a Spearman correlation $R = 0.3258$, $P = 0.0043$) with clinical benefit within patients from the active group (Figure S2, upper left panel), whereas no such a correlation was observed in placebo-treated patients (Figure S2, upper right panel). No such relationship could be

Supporting Information

Supporting Methods:

Mice: Female C57BL/6J and B6.129S6-Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze/J} mice [(ROSA)26/TdTomato; 6-8 weeks old] were purchased from Jackson Laboratories (Bar Harbor, ME). Male IL-13 YFP-Cre (YetCre-13)¹ mice were generously provided by Dr. Lee Reinhardt. ROSA26/TdTomato and YetCre-13 mice were bred to produce IL-13 lineage-tracing mice. All experimental procedures were conducted with the approval of the Duke University Institutional Animal Care and Use Committee.

Peanut Sensitization and Immunotherapy: Crude peanut extract (PN) was prepared, as previously described² from 12% fat lightly roasted peanut flour. PN (5 mg) and cholera toxin (CT (15 µg), List Biologicals; Campbell, CA) was gastrically delivered once a week for 4 weeks to induce sensitization. After a 4-week rest, peanut-hypersensitive mice received placebo therapy with saline (APP Pharmaceuticals; Schaumburg, IL), 40 µg PN alone (PN), 20 µg CpG-1826 alone (CpG) (Invivogen; San Diego, CA) or 40 µg PN + 20 µg CpG (PN+CpG) given in 15 µL three times a week for 4 weeks. We compared immunotherapy groups that have been similarly reported others³⁻⁶.

Allergen Provocation Challenge: While evaluation of allergen-specific immunotherapy in subjects with mild to moderate allergen-hypersensitivity may be safer than evaluation in individuals with severe allergen-hypersensitivity, subjects with severe allergic disease may be more at-risk of life-threatening accidental exposures. Recent studies demonstrated the feasibility of peanut-specific oral immunotherapy in severely allergic individuals⁷ and we have utilized a mouse model of severe systemic peanut-induced anaphylaxis to evaluate CpG-adjuvanted peanut-specific nasal immunotherapy. Mice were challenged with 1.25 mg PN injected intraperitoneally to induce severe anaphylaxis one week after therapy. A second challenge dose was administered 20-minutes post-challenge if placebo-treated mice did not display allergy symptoms. Allergic symptoms and body temperature, using implanted temperature transponders (BioMedic Data Systems; Seaford, DE), were monitored for 60 minutes after challenge. Clinical symptom scores, ranging from 0-5, were assigned to each animal as previously described⁸ based on the following symptoms: 0, no symptoms; 1, repetitive scratching; 2, decreased activity, self-isolation, swelling around the eyes and snout; 3, motionless for greater than 1 minute, lying prone on the stomach; 4, no response to whisker stimulation or prodding and 5, convulsion, death or temperature decrease greater than 5°C.

Sample Collection: Serum and fecal samples were collected from each mouse at the conclusion of peanut sensitization and immunotherapy regimens as previously described⁹. Samples were stored at -20°C until ELISA analysis.

Peanut-Specific ELISA: PN-specific serum IgG1 and IgG2c and fecal IgG and IgA antibodies were measured as previously described¹⁰ except 2 µg/mL of PN was used to coat plates. PN-specific serum IgE was measured by a capture ELISA with biotinylated PN as previously described¹¹. Geometric Mean Titers (GMT) were reported as the last log₂ dilution with a positive signal three-fold greater than a naïve reference sample at the same dilution.

Cytokine Analysis: PN-induced cytokine responses were measured by Luminex assay (Biorad; Hercules, CA). One week after immunotherapy 2.5 x 10⁶ splenocytes were incubated in medium or PN (200 µg/mL) in 48 well plates. After 96 hours of culture at 37°C with 5% CO₂, supernatants were collected and stored at -80°C until multiplex analysis to determine pg/mL of IL-10, IL-13 and IFN-γ. PN-induced cytokine responses were calculated by subtracting cytokine pg/mL of unstimulated from PN-stimulated cells.

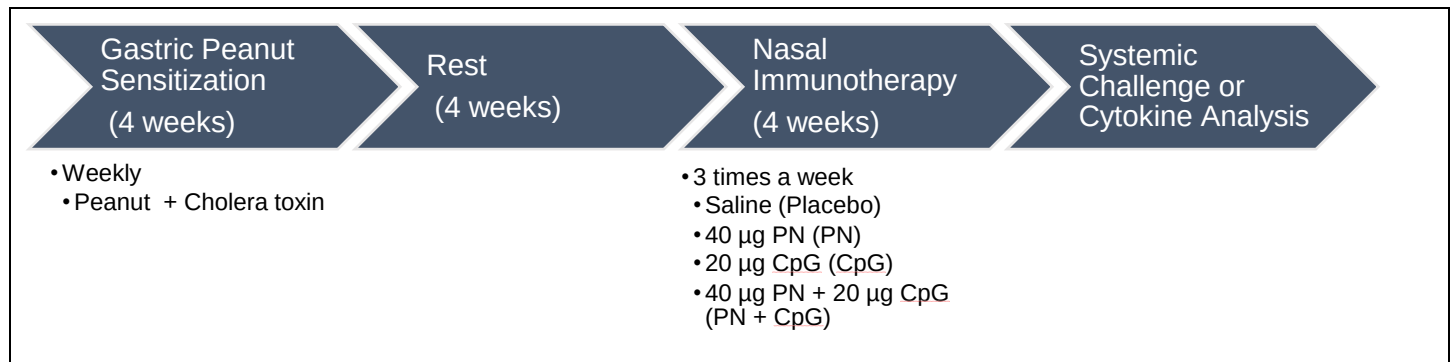
Cytokine Lineage-Tracing: IL-13-lineage-tracing mice produce red fluorescence IL-13+ cells if IL-13 has ever been produced in that cell lineage^{12 13}. CD4+IL-13+ (Tom+) cells (*Supporting Figure 4*) are considered Th2 cells and CD4+IL-13- (Tom-) cells are defined as non-Th2 cells in this study. The influence of immunotherapy on CD4+ T cell cytokines was evaluated by re-stimulation with PN (200 µg/mL) for 7 days¹⁴ to expand the population of PN-responsive cells or left unstimulated for 7 days. Both PN-expanded and unstimulated cells were activated

with PMA (50 ng/mL)/Ionomycin (1 µg/mL) in the presence of monensin for 4 hours¹⁵. IFN-γ and IL-10 were then measured by intracellular cytokine staining and flow cytometric analysis. PN-induced responses were determined by subtracting the percent of cytokine positive cells not expanded by PN-stimulation (unstimulated) from cells expanded in the presence of PN as similarly described by others¹⁶. The number of peanut-induced IL-10 or IFN-γ+ CD4+ T cells were compared between PN+CpG-treated mice and mice treated with peanut alone in both the Tom+ and Tom- populations.

Flow Cytometry Analysis: IL-13 lineage tracing splenocytes were washed (PBS/1% BSA) before staining with Live/Dead Fixable dye (Thermo Fisher; Waltham, MA) for 30 minutes, then stained with PE-Cy7-rat anti-mouse CD4 (BD; San Jose, CA). Cells were fixed and permeabilized with a Cytofix/Cytoperm solution (BD) then labeled with BV421 IFN-γ (Biolegend; San Diego, CA) and IL-10-APC (BD) antibodies. Cells were suspended in PBS/1%BSA and analyzed using a BD LSRII flow cytometer in the Duke Human Vaccine Institute Flow Cytometry Facility.

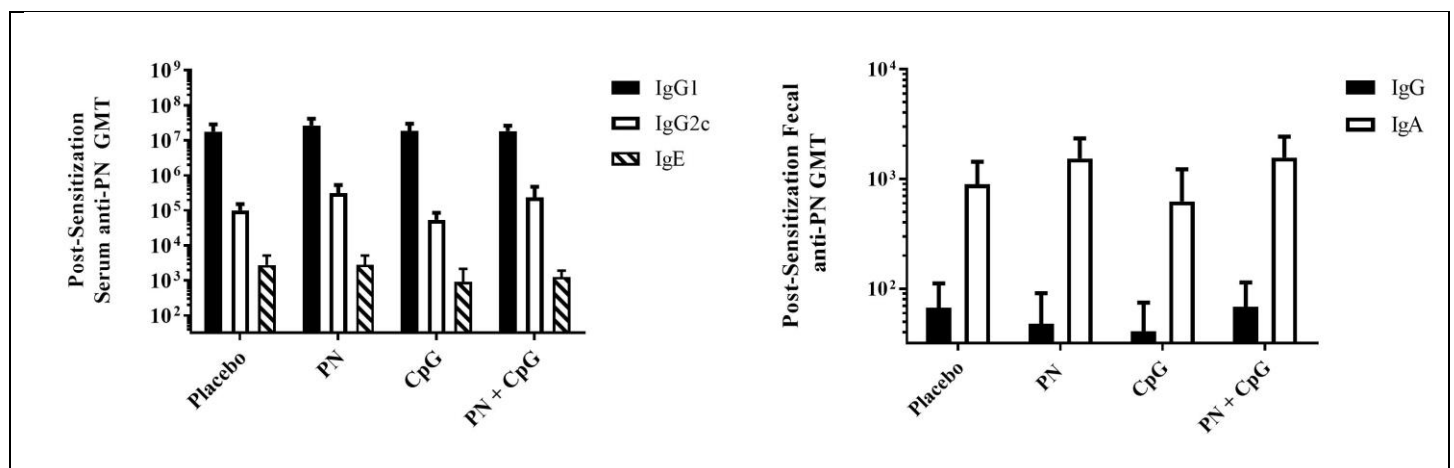
Statistical Analysis: GraphPad Prism 7 (San Diego, CA) was used for statistical analysis. Two-way analysis of variance (ANOVA) with repeated measures was used for PN-challenge analyses. One-way ANOVA with Tukey's multiple comparison was used to measure ELISA and luminex data. Mann-Whitney test analyzed intracellular-cytokine responses between PN and PN+CpG immunotherapy-treated mice.

Supporting Figures:

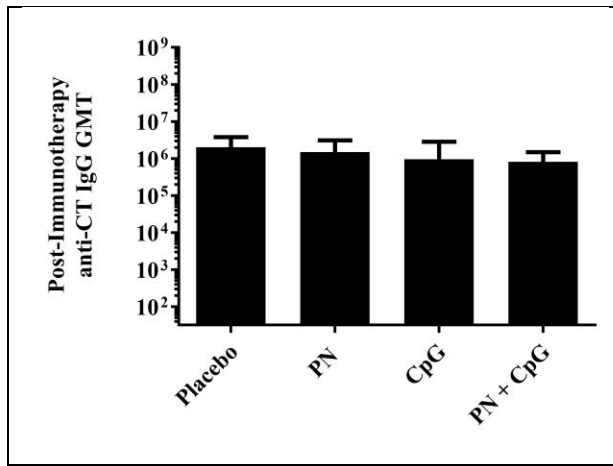


Supporting Figure 1. Experimental Timeline.

Peanut hypersensitization was induced by gastric gavage with PN combined with CT once a week for four weeks. Mice were allowed to rest after sensitization for four weeks before immunotherapy. Immunotherapy formulations that contained saline as placebo, peanut extract alone (PN), CpG alone (CpG) or peanut combined with CpG (PN + CpG) were administered by the nasal route, three times a week for four weeks. One week after immunotherapy mice were either challenged by systemic exposure to peanut to induce severe anaphylactic reactions or euthanized and harvested for splenocytes to monitor peanut-induced cytokine responses.



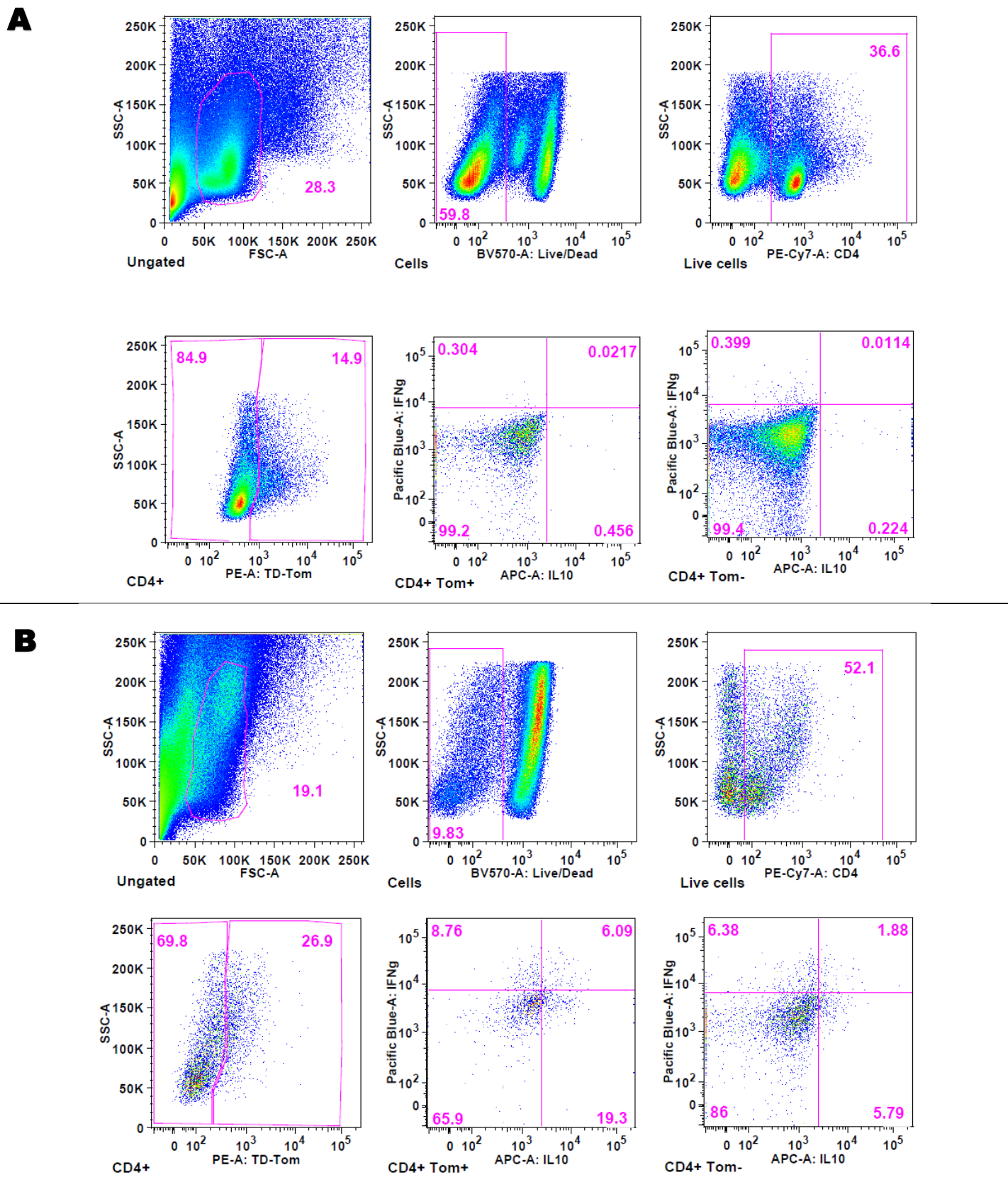
Supporting Figure 2: Peanut-sensitization is comparable in all immunotherapy groups before treatment. C57BL/6 mice were sensitized to peanut. Serum and fecal extracts were analyzed for peanut-specific antibodies before immunotherapy with saline (placebo), PN, CpG or PN + CpG. PN-hypersensitive mice displayed similar serum and fecal PN-specific antibody responses before beginning their respective immunotherapy regimen.



Supporting Figure 3: Nasal peanut immunotherapy specifically modifies peanut-specific immune responses. PN-sensitization was induced by gastric gavage with PN and CT. PN-hypersensitive mice received NIT with saline (placebo), PN, CpG or PN + CpG. CT-specific serum IgG was measured at the conclusion of immunotherapy. Similar levels of serum CT-specific IgG was observed therapy in all groups.

PN-Serum					PN-Fecal		60 minutes post-challenge	
Treatment	IgG1	IgG2c	IgE	G2c:E Ratio	IgG	IgA	Symptom Score	Body Temp
Naïve (1)	N/A	N/A	N/A	N/A	N/A	N/A	0 ± 0*	37.6 ± 0.634*
Placebo (2)	4.11E+06 ± 4.26	5.62E+04 ± 8.46	658 ± 7.51	85.4	102 ± 5.13	1531 ± 2.70	2.89 ± 1.23	32.7 ± 1.86
PN (3)	8.04E+07 ± 2.93 ^(2,4)	2.10E+06 ± 7.92 ^(2,4)	8190 ± 8.20 ^(2,4)	256	2048 ± 5.30 ^(2,4)	3.05E+04 ± 3.39 ^(2,4)	2.24 ± 1.75	33.1 ± 2.59
CpG (4)	2.20E+05 ± 4.71	2.82E+04 ± 8.53	232 ± 4.53	122	221 ± 5.85	1188 ± 2.78	2.40 ± 1.67	33.2 ± 3.61
PN + CpG (5)	1.59E+08 ± 1.82 ^(2,4)	2.93E+07 ± 2.71 ^(2,4)	1.69E+04 ± 8.09 ^(2,4)	1729	5513 ± 4.51 ^(2,4)	9.18E+04 ± 2.95 ^(2,4)	1.52 ± 1.83*	34.3 ± 2.93*

Peanut-hypersensitive C57BL/6 mice received saline (placebo), PN, CpG or PN+CpG NIT. Immunotherapy-influenced PN-specific serum and fecal antibodies are reported as GMT ± Geometric SD. PN-induced anaphylaxis was monitored for 60 minutes. Core body temperature and allergy clinical symptom scores at the end of the 60-minute monitoring period is reported for each group. One-way ANOVA with Tukey's multiple comparison determined significant differences between antibody responses and two-way ANOVA with Dunnet's comparison to the placebo group determined significant differences between allergy symptoms ($p < 0.05$). The superscript numbers after each GMT indicate a significant increase compared to the corresponding therapy group. * indicates a significant reduction in allergic disease severity compared to Placebo.



Supporting Figure 4: Intracellular cytokine staining flow cytometry gating strategy. Splenocytes were isolated from IL-13-lineage tracing mice at the conclusion of immunotherapy. Cells were not expanded (**A**) or expanded with PN (**B**) for 7 days. All cells were stimulated with PMA/ionomycin for 4 hours before adding monensin and stained for intracellular IL-10 and IFN- γ expression. Live cells were selected based on low BV570-expression using fixable live/dead stain. CD4 $^{+}$ cells were then analyzed for TdTomato (Tom) expression. Populations of IL-10 and IFN- γ were quantified in both CD4 $^{+}$ Tom $^{+}$ and CD4 $^{+}$ Tom $^{-}$ cellular populations.

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