

ORIGINAL ARTICLE

Airways Disease

Allergen-specific immunotherapy enhances CD8⁺ CD25⁺ CD137⁺ regulatory T cells and decreases nasal nitric oxide

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Abstract

Background: 4-1BB (CD137), a member of the inducible tumor necrosis factor receptor (TNFR) family, is expressed on regulatory T (Treg) cells and regulates Treg cells to control allergic inflammation. Pam3CSK4, a synthetic TLR2 ligand that can expand CD8⁺ Treg function, is a promising adjuvant for allergen immunotherapy (IT). We examined whether *Dermatophagoides pteronyssinus* (Der p) IT and Pam3CSK4 could enhance CD8⁺ CD25⁺ CD137⁺ Treg suppressive function to decrease nasal nitric oxide (nNO) levels.

Methods: Nasal symptom scores, nNO levels, PBMCs, and inferior turbinate biopsies were obtained from 40 mite-sensitive perennial allergic rhinitis (PAR) patients before and after one year of Der p IT and 30 non-allergic control subjects. CD137 expression on CD8⁺ CD25⁺ T cells and suppressive function of CD8⁺ CD25⁺ CD137⁺ Tregs was measured using flow cytometry. Cytokine levels were analyzed by ELISA. Inducible nitric oxide synthase production by nasal epithelial cells after co-culturing with CD8⁺ CD25⁺ CD137⁺ T cells was analyzed by Western blotting.

Results: Der p IT improved nasal symptom scores, decreased nNO levels, and increased CD137 expression on CD8⁺ T cells in PBMCs and nasal mucosa. Pam3CSK4 expanded the CD8⁺ CD25⁺ CD137⁺ population in PBMCs. Pam3CSK4-stimulated CD8⁺ CD25⁺ CD137⁺ Tregs induced IL-10 and TGF- β and suppressed CD4⁺ CD25⁺ T-cell proliferation mainly by cell contact inhibition. CD8⁺ CD25⁺ CD137⁺ Tregs cultured with nasal epithelial cells suppressed Der p 2-induced iNOS production. Silencing CD137 in sorted CD8⁺ CD25⁺ T cells decreased Pam3CSK4-activated Foxp3 expression.

Conclusion: Der p IT expanded CD8⁺ CD25⁺ CD137⁺ Tregs and decreased nNO levels. Induced CD137 expression on CD8⁺ CD25⁺ Tregs by Pam3CSK4 stimulation may help suppress allergic inflammation during IT.

KEYWORDS

allergy, CD137, immunotherapy, regulatory T cells, Toll-like receptor 2

Abbreviations: AIT, allergen immunotherapy; Der p, *Dermatophagoides pteronyssinus*; nNO, nasal nitric oxide; PAR, perennial allergic rhinitis; PBMCs, peripheral blood mononuclear cells; TLR, Toll-like receptor; Treg, regulatory T.

Yang, Wen and Hung equally contributed to this work.

1 | INTRODUCTION

Cumulative evidence shows defective immune regulation in CD4⁺ regulatory T (Treg) cell function and diminished Foxp3 expression in nasal polyposis in allergic rhinitis (AR) patients.^{1,2} CD8⁺ Tregs express CD25 and Foxp3 and have been identified as another population of T cells with suppressive properties to maintain immune surveillance.^{3,4} CD8⁺ Tregs could suppress naive T-cell proliferation through a cell-cell contact mechanism or through soluble factors such as IL-10 and TGF- β .³⁻⁶ A reduced regulatory potential of immunosuppressive cytokine secretion, characteristic of CD4⁺ Treg-mediated regulatory function and cytotoxic ability, which is also typical of CD8⁺ Treg function, may enhance activated T-helper cells in chronic inflammation.^{6,7} Pant et al⁷ demonstrated that reduced CD8⁺ Treg population in chronic rhinosinusitis mucosa with nasal polyp (CRSwNP) indicates a crucial role for CD8⁺ Tregs in the nasal mucosa in controlling allergic inflammation.

Allergen-specific immunotherapy (AIT) induces immune tolerance to common environmental allergens by repeated subcutaneous or sublingual administration of allergen extracts.⁸ Several studies have reported that suppression of allergic responses in patients after successful AIT might be associated with the induction of Tregs.^{3,8-11} Selecting an optimal combination of TLR ligands for a given vaccine may prove to be a vital strategy in maximizing the anti-allergy immune response. TLR2 synthetic agonist Pam3CSK4 directly enhances CD4⁺ CD25⁺ Treg proliferation to modulate the Th1/Th2 balance that may benefit as an adjuvant in AIT.¹² Previously, we reported that CD8⁺ Tregs were identified during AIT, and Pam3CSK4 enhanced CD8⁺ Tregs to maintain immune tolerance in allergic asthma.³

4-1BB (CD137), a member of the tumor necrosis factor receptor (TNFR) family, is constitutively expressed on CD4⁺ Tregs.¹³ Signaling through CD137 receptor on CD8⁺ T cells plays a multifaceted role in immune regulation and contributes to T-cell activation, acquisition of effector function, and long-term memory.¹⁴ CD137 monoclonal antibodies² enhance antitumor and antiviral activity by inducing Th1 responses and promote activation and proliferation of CD8⁺ T cells and Foxp3⁺ Treg cells.^{14,15} Anti-CD137 mAb administration suppresses CD4⁺ T-cell reactivity to diminish Th2 cytokines in murine asthma models, indicating a potentially important role of CD137 for therapeutic intervention in allergic diseases.^{16,17}

CD4⁺ CD25⁺ Treg cells can be expanded by CD137 mAb stimulation and are a promising immunotherapy target.¹⁴ Myers et al¹⁸ demonstrated that the CD137 mAb, in combination with a TLR ligand, elicits CD8⁺ Treg expansion with suppressive activity. However, the role of CD8⁺ CD25⁺ CD137⁺ Tregs during AIT and the effect of Pam3CSK4 on CD8⁺ CD25⁺ Tregs are not clear. The study investigated whether *Dermatophagoides pteronyssinus* (Der p) IT could increase CD137 expression on CD8⁺ CD25⁺ Tregs in peripheral blood mononuclear cells (PBMCs) and inferior turbinate biopsies. We also examined whether Pam3CSK4-activated CD8⁺ CD25⁺ CD137⁺ Tregs could suppress mite allergen-induced airway inflammation with nasal nitric oxide (nNO)

Key Message

Functional control of CD8⁺ CD25⁺ Treg cells by TLR2 signaling via CD137 stimulation may offer new opportunities to develop a valuable potential target during allergen immunotherapy.

levels. Our findings may further provide evidence and elucidate the mechanism for novel immunotherapeutic strategies during AIT.

2 | METHODS

2.1 | Subjects

Forty moderate-to-severe perennial allergic rhinitis (PAR) subjects without asthma (aged 10-20 years), sensitive to the house dust mite (Der p), were enrolled in this study and received Der p IT. PAR was defined according to the allergic rhinitis and its impact on asthma (ARIA) guidelines.¹⁹ PAR children in the Der p IT group received subcutaneous injections of Der p extracts (10 000 AU/mL, Allermid Laboratories Inc, San Diego, CA, USA), at an initial dose of 0.5 AU/mL once a week. The dosage was increased weekly by 25%-100% to reach an optimal maintenance dose. The PAR patients received the maximum monthly tolerated dose, according to a previously described standardized protocol, and were monitored for 1 year.^{3,10} Sensitivity to Der p was demonstrated by Der p-specific IgE test using the CAP system (Pharmacia, Uppsala, Sweden). All patients rated the severity of the symptoms of rhinorrhea, sneezing, nasal obstruction, and nasal pruritus on a 4-point scale of 0-3 (0 = none, 1 = mild, 2 = moderate, and 3 = severe, up to a maximum of 12 points), which were added to obtain total nasal symptom scores (NSS; Table 1). Patients were also instructed to stop oral glucocorticoid and intranasal steroid spray at least 7 days before blood sampling. Prednisolone prescription filling records and concordance interview were used to validate adherence status with stopping treatment. All patients completed the study. Thirty healthy control subjects with normal serum IgE levels were recruited as control subjects. The samples obtained from the PAR patients who received Der p SIT were randomly subdivided into different groups for confocal immunofluorescence, flow cytometry, CD8⁺ Treg functional assay, and Western blot analysis. The study was approved by the institutional review board of Changhua Christian Hospital, and all subjects provided written informed consent.

2.2 | Nasal no measurement

nNO was measured using an online electrochemical analyzer equipped with nNO software (NIOX MINO; Aerocrine AB, Sweden) in compliance with the recommendations of the American Thoracic Society/

TABLE 1 (A) Patient characteristics and (B) clinical response with nasal symptom scores and Der p-specific IgE changes after SIT

(A)	IT group	Control group
Number of patients	40	30
Mean age (y)	14.4 ± 3.9	14.6 ± 3.6
Gender (M:F)	19:21	17:13
(B)	Baseline	Post-IT
Nasal NO (ppb)	772.6 ± 229.2	548.9 ± 145.7**
Der p-specific IgE (kU/l)	67.5 ± 24.8	52.5 ± 27.2*
Total nasal symptom cores	9.8 ± 2.1	4.5 ± 3.2**
Rhinorrhea	2.4 ± 0.8	1.4 ± 0.9**
Sneezing	2.8 ± 0.4	1.2 ± 0.9**
Nasal obstruction	2.4 ± 0.8	1.0 ± 1.2**
Nasal itching	2.2 ± 0.8	0.9 ± 1.0**

Note: Data are shown as the mean ± SD.

Significant differences were determined using Student's paired *t* test (**P* < 0.05; ***P* < 0.01).

European Respiratory Society.²⁰ The detailed methods for nNO measurement have been provided in the Supporting Information.

2.3 | Tissue samples

Inferior turbinate specimens were obtained from 10 enrolled severe PAR patients (including five PAR patients who received one year of Der p IT and another five PAR patients before administration of Der p IT) and five non-allergic controls undergoing nasal septoplasty because of anatomic variations and without other sinonasal diseases. Surgery was performed under strict clinical indications, and severe PAR patients faced difficulty in nose breathing. The detailed methods on nasal biopsy tissue culture have been provided in the Supporting Information.

2.4 | Confocal immunofluorescence

To detect CD137 expression on CD8⁺ T cells from nasal mucosa, confocal microscopic analysis on nasal tissue biopsy specimens with CD8 (FITC), CD137 (PE), and 4',6-diamidino-2-phenylindole (DAPI; nuclear stain) were performed with the Confocal Olympus FV1200 fluorescence microscope. Three randomly chosen high-power fields at 400-fold magnification per patient or controls were evaluated by two experienced researchers in a blinded manner.

2.5 | Blood CD8⁺ CD25⁺ CD137⁺ Treg cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque gradient centrifugation (Pharmacia, Uppsala, Sweden). The synthetic lipopeptide Pam3CSK4 (InvivoGen, Carlsbad, Calif) was prepared in sterile phosphate-buffered saline (PBS). A total of 1×10^6 cells were cultured with Pam3CSK4 (5 µg/mL) or the same

volume of medium as negative control for 5 days on 96-well culture plates in RPMI 1640 culture medium supplemented with L-glutamine (2 mmol/L), HEPES (20 mmol/L), sodium pyruvate (91 mmol/L), streptomycin (50 ng/mL), penicillin (100 IU/mL), and 10% fetal bovine serum (BioWhittaker, Walkersville, MD). In some experiments, CD4⁺ or CD8⁺ cells were separated directly from PBMCs using microbeads, according to the manufacturer's instructions (BD Biosciences).⁶ CD4⁺ CD25[−] T cells were isolated using a CD4⁺ T-cell enrichment kit, followed by separation with CD25 microbeads (BD Biosciences). To explore CD8⁺ Treg cell inhibition, CD8⁺ CD25⁺ CD137⁺ T cells were isolated using a CD8⁺ T-cell enrichment kit (comprising the following monoclonal antibodies: biotin mouse anti-human CD4 mAb, clone L200; anti-human CD11b mAb, clone ICRF44; anti-human CD16, clone 3G8; anti-human CD19, clone HIB19; anti-human CD41a, clone HIP8; anti-human CD45RO, clone UCHL1; anti-human γδ-TCR, clone B1; and anti-human glycoprotein A, clone GA-R2; BD Biosciences), followed by staining with anti-human CD25 FITC mAb and anti-human CD137 PE mAb (BD Biosciences). The CD8⁺ CD25⁺ CD137⁺ T-cell population was purified using the BD FACSCanto II (BD Biosciences), and the cells analyzed by flow cytometry were more than 95% pure as shown in Figure 2A.

2.6 | Flow cytometry

PBMCs were fixed with 4% paraformaldehyde and washed with PBS containing 0.2% bovine serum albumin (BSA). After washing, cells were stained for 30 minutes with fluorescein-conjugated mAbs. CD8⁺ CD25⁺ CD137⁺ T cells were stained with anti-human CD25 FITC-conjugated, CD137 PE-conjugated, CD8 PC5-conjugated, and isotype-matched control mAbs from BD Biosciences (San Diego, Calif). In order to confirm Foxp3 expression on CD8⁺ CD25⁺ CD137⁺ T cells, PBMCs were permeabilized and then stained with CD25-FITC, CD137-PE, CD8-PC5, and Foxp3-ECD mAb and isotype-matched control mAbs (BD Biosciences). Pam3CSK4-stimulated CD8⁺ CD25⁺ T cells after CD137 silencing were permeabilized and then stained with Alexa Fluor® 488 anti-human Foxp3 mAb (BD Biosciences). Cells were fixed and stained using standard procedures (eBioscience, San Diego, CA), and analyzed using a FACS Scan flow cytometer (FC500, Beckman Coulter), acquiring 10 000 events.

2.7 | Cell proliferation assay and transwell experiments

CD4⁺ CD25[−] T cells were labeled with 5 µmol/L CFSE (carboxy-fluorescein succinimidyl ester; Invitrogen, Carlsbad, CA) for 15 minutes at 37°C. For the CFSE suppression assay,³ CFSE-labeled CD4⁺ CD25[−] T cells were incubated with IL-2 (10 U/mL), anti-CD3 mAb (1 µg/mL), and Pam3CSK4 (5 µg/mL) with/without CD8⁺ CD25⁺ CD137⁺ T cells at a ratio of 10:1 for 5 days. Transwell experiments were carried out in 24-well plates (0.4 µm pore size, Nunc, Roskilde, Denmark). CD8⁺ CD25⁺ CD137⁺ T cells were added in the upper chamber, and the CFSE-labeled CD4⁺ CD25[−] T cells were placed in the lower chamber. At the end of the culture, the

proliferation of CD4⁺ T cells was analyzed by flow cytometry using CFSE fluorescence.

2.8 | Blocking experiments

CFSE-labeled, CD8-depleted PBMC was incubated with IL-2 (10 U/mL), anti-CD3 mAb (1 µg/mL), and Pam3CSK4 (5 µg/mL) with/without sorted CD8⁺ CD25⁺ CD137⁺ T cells and CD8⁺ CD25⁺ CD137⁻ T cells at a ratio of 10:1 in the presence or absence of a TNF-α inhibitor (adalimumab, 10 ng/mL; Humira, AbbVie) for 3 days. At the end of the incubation period, the proliferation of CD8-depleted PBMC cells was analyzed by flow cytometry and the supernatant was collected for subsequent cytokine determination.

2.9 | Enzyme-linked immunosorbent assay (ELISA)

Supernatants from CD8⁺ CD25⁺ CD137⁺ T-cell suppression assay were collected to measure IL-4, IFN-γ, IL-10, TGF-β, and TNF-α cytokine levels by ELISA (R&D Systems, London, UK).

2.10 | Western blot analysis

In order to examine inhibition of iNOS activity by CD8⁺ CD25⁺ CD137⁺ T cells, confluent nasal epithelial cells (1×10^6 cells) were stimulated with Der p 2 (10 µg/mL; Indoor Biotechnologies, Cardiff, UK) and cultured with or without purified CD8⁺ CD25⁺ CD137⁺ T cells for 48 hours. CD8⁺ CD25⁺ CD137⁺ T cells were separated from adherent nasal epithelial cells by washing, and the protein levels of iNOS were determined by Western blot analysis.

2.11 | CD137 gene silencing

Small interfering RNAs (siRNA) targeting human CD137 (NM_001561.5) or control siRNA were purchased from GeneDex Inc (Taiwan). Purified CD8⁺ CD25⁺ T cells were transfected with CD137 siRNA (20 µmol/L), using Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and a previously published protocol.²¹ After CD137 siRNA transfection, CD8⁺ CD25⁺ T cells were stimulated with Pam3CSK4

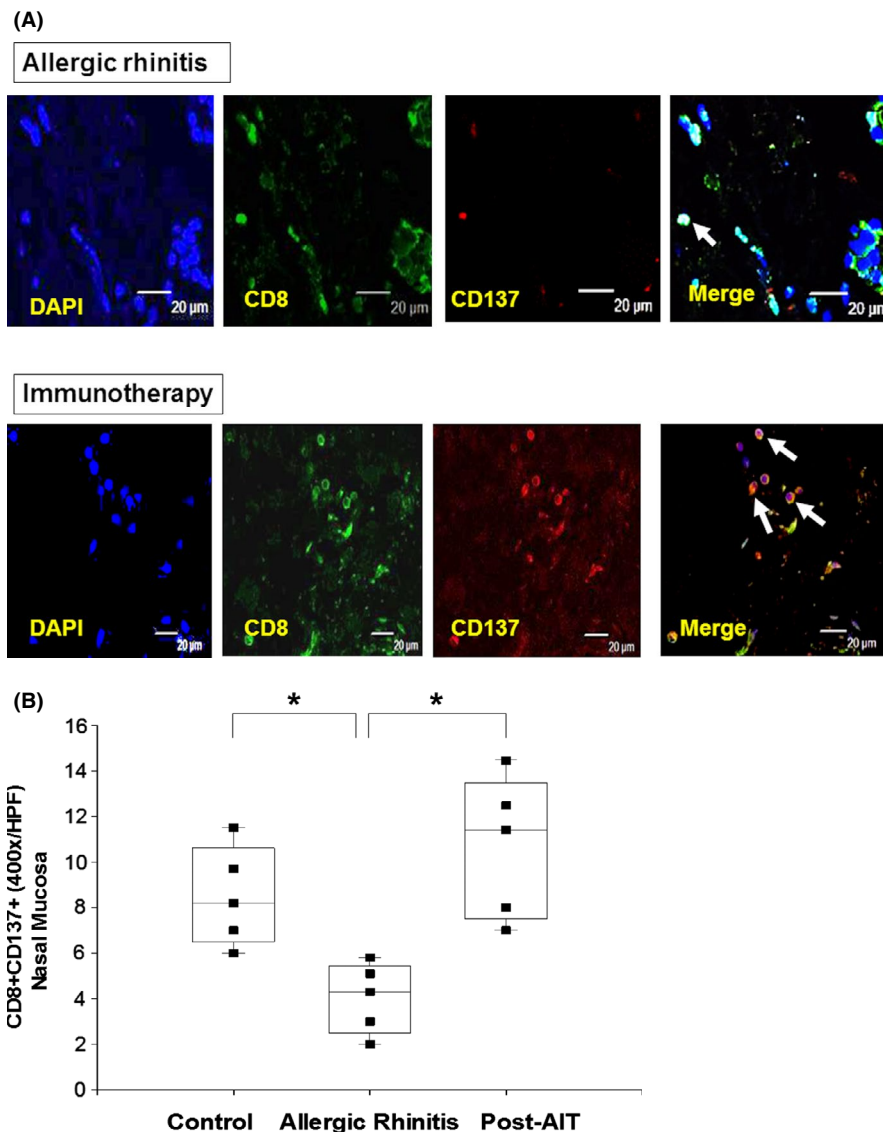
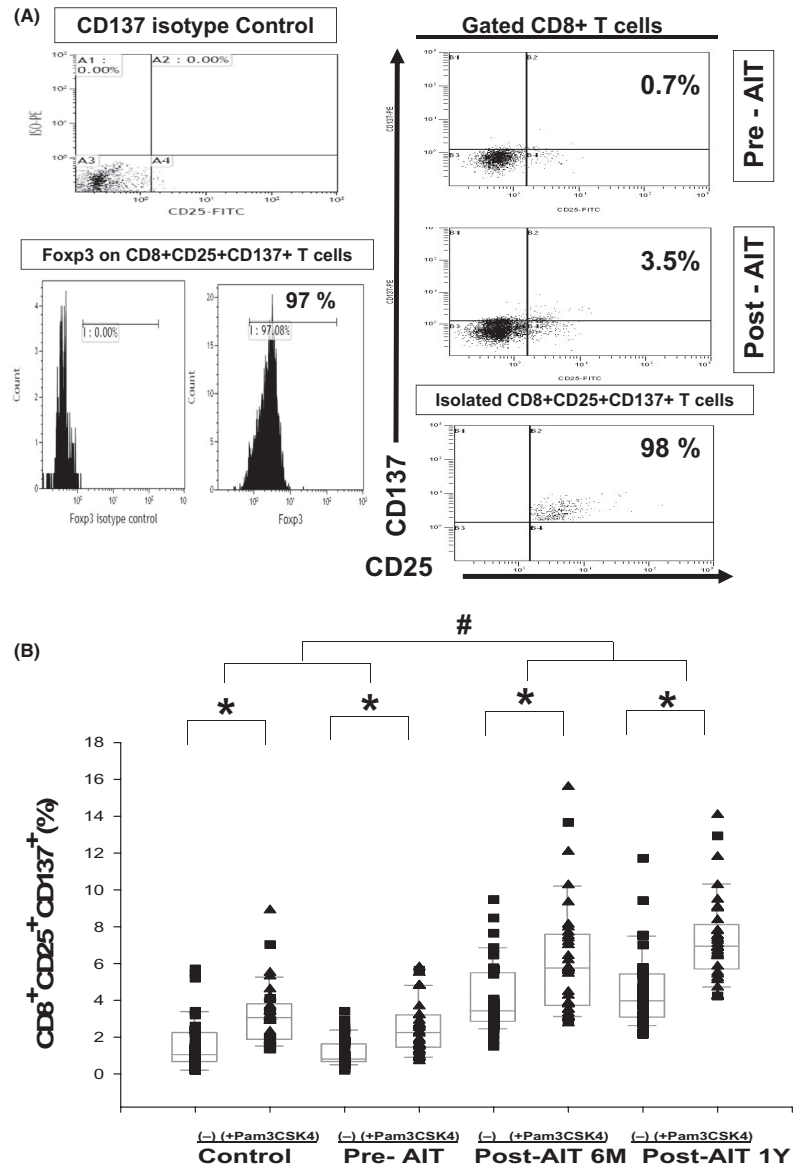


FIGURE 1 A, CD8⁺ CD137⁺ T cells (indicated by white arrows) in the nasal mucosa from perennial allergic rhinitis (PAR) patients. Primary inferior turbinate biopsies stained with CD8 (FITC), CD137 (PE), and 4',6-diamidino-2-phenylindole (DAPI; nuclear stain). Representative confocal microscopic analysis between nasal mucosa from PAR and Der p IT-treated patient. Scale bar, 20 µm. B, Quantification of CD8⁺ CD137⁺ T cells from nasal mucosal tissue biopsies between PAR patients before (n = 5) and after Der p IT (n = 5) and non-atopic control subjects (n = 5). Significant differences were determined using Kruskal–Wallis test. **P* < 0.01 [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 2 Der p IT induced the expression of CD137 on CD8⁺ CD25⁺ T cells. A, CD137 expression was assessed in CD8⁺ CD25⁺ T cells from non-atopic control subjects (*n* = 30) and PAR subjects (*n* = 30) before, and 6 and 12 months after Der p IT. PBMCs were stimulated with IL-2 (10 U/mL), anti-CD3 mAb (1 µg/mL), and Pam3CSK4 (5 µg/mL) for 5 days. Cells were fixed and stained with anti-human CD25 FITC-conjugated, CD137 PE-conjugated, CD8 PC5-conjugated, and isotype-matched control mAbs. CD8⁺ T cells were gated for surface CD137 and CD25 expression. In order to confirm Foxp3 expression on CD8⁺ CD25⁺ CD137⁺ T cells, PBMCs were permeabilized and then stained with CD25-FITC, CD137-PE, CD8-PC5, Foxp3-ECD mAb, and negative isotype control staining (BD Biosciences). Representative data before and after 12 months of Der p IT are shown. B, Summary of 30 paired experiments for CD137 expression in CD8⁺ CD25⁺ T cells. (**P* < 0.01 after Pam3CSK4 treatment by Student's paired *t* test; #*P* < 0.05 between PAR subjects before, and 6 and 12 months after Der p IT and non-atopic control group by the ANOVA test and the post hoc Dunn test)



(5 µg/mL) and IL-2 (10 U/mL) for 72 hours and nuclear proteins were extracted and examined for Foxp3 expression (Abcam, Cambridge, UK) by Western blotting.

2.12 | Reverse transcription and real-time PCR

Total RNA was extracted with Direct-zol™ RNA Kits (Zymo Research) as described by the manufacturer. For reverse transcription, cDNA was generated using SuperScript™ IV VILO™ Master Mix with the DNase™ Enzyme (Invitrogen). For real-time PCR, the primer sequences were as follows: Foxp3 sense, 5'-GGCACAATGTCTCCTCCAGAGA-3' and antisense, 5'-CAGATGAAGCCTTGGTCAGTGC-3'; CD137 sense, 5'-TCTTCCTC ACGCTCCGTTTCTC-3' and antisense, 5'-TGGAAATCGGCAGCTACA GCCA-3'; β-actin sense, 5'-CACCATTGGCAATGAGCGGTTC-3' and antisense, 5'-AGGTCTTTGCGGATGTCACGT-3'. Real-time PCR amplification of a cDNA template corresponding to 100 ng total RNA

was conducted with an ABI PRISM 7300 sequence detection system (Applied Biosystems, Carlsbad, CA, USA) using Fast SYBR™ Green Master Mix (Applied Biosystems) following the manufacturer's instructions. PCR conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, followed by a dissociation plot. For data analysis, the comparative threshold cycle³ value for β-actin was used to normalize loading variations in the real-time PCR data. The ΔΔCT value was then obtained by subtracting the control ΔCT values from the corresponding experimental ΔCT values. The ΔΔCT values were converted to fold difference over the control using the equation $2^{-\Delta\Delta CT}$.

2.13 | Statistical analysis

Demographic data from PAR patients are shown as the mean ± SD. Data for each group were found to fall under a normal distribution, prior to

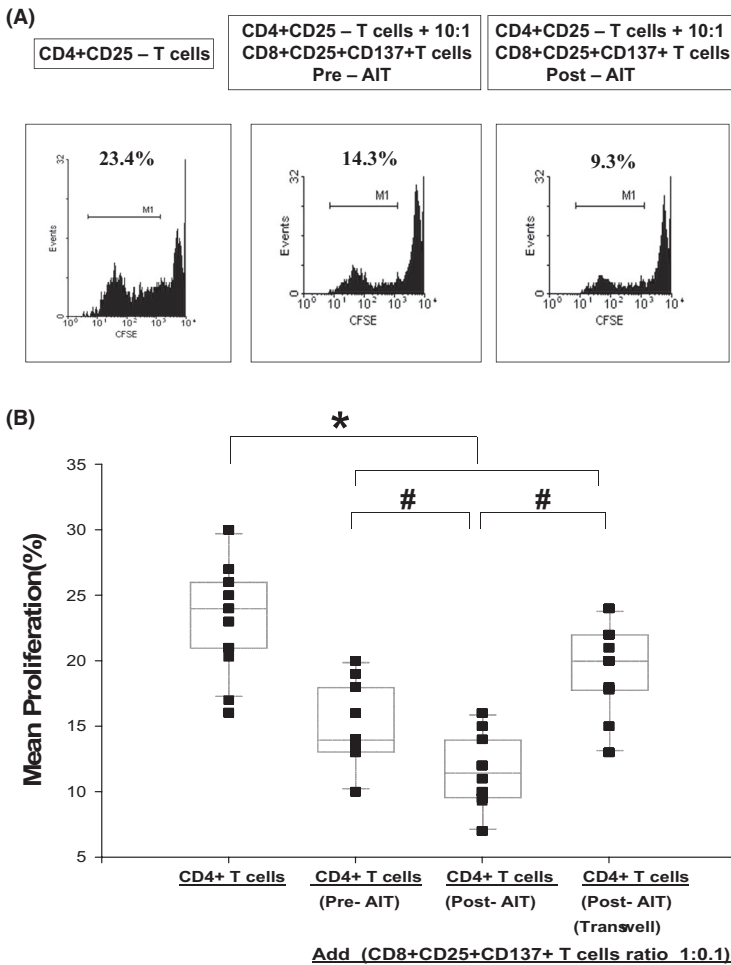


FIGURE 3 Demonstration of antiproliferative capacity in the presence of Pam3CSK4-stimulated CD8⁺ CD25⁺ CD137⁺ T cells. A, The CFSE-labeled, CD4⁺ CD25⁻ T cells were incubated with IL-2 (10 U/mL), anti-CD3 mAb (1 µg/mL), and Pam3CSK4 (5 µg/mL) with/without sorted CD8⁺ CD25⁺ CD137⁺ T cells for 5 days at a ratio of 10:1 in the same well or separated by a Transwell semipermeable membrane. Proliferation of CD4⁺ CD25⁻ T cells was analyzed by flow cytometry. B, There was a significant suppression of CD4⁺ CD25⁻ T-cell proliferation by CD8⁺ CD25⁺ CD137⁺ T cells by cell-cell contact. A summary of 20 paired samples showed a significant difference between allergic rhinitis patients after Der p IT. (**P* < 0.01 after treatment using the Wilcoxon signed-rank test; #*P* < 0.01 by the Kruskal-Wallis test on comparison between pre-IT and post-IT and Transwell treatment in the same PAR patients.)

analysis using the one-sample Kolmogorov–Smirnov test. Groups of normal distribution data were compared using the ANOVA test, followed by the Duncan test, and differences between before and after AIT among various experimental conditions were analyzed by dependent *t* test. Groups of non-normal data were compared using the Kruskal–Wallis test, and differences between before and after AIT among various experimental conditions were analyzed by Wilcoxon signed-rank test. A *P* value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Der p IT improved nasal symptoms and nasal NO levels and induced CD137 expression on CD8⁺ T cells in the nasal mucosa in PAR patients

Table 1A shows the clinical traits of 40 PAR patients and 30 matched controls. All PAR patients that underwent Der p-specific IT completed the treatment period, and no systemic side effects were reported. All PAR patients who received Der p IT had improved total nasal symptoms scores, nasal NO levels, and Der p-specific IgE (*P* < 0.05) when compared to pre-IT status (Table 1B). Confocal microscopic analysis showed increased CD137 expression on CD8⁺ T cells in nasal mucosa

biopsies among Der p IT-treated patients (*n* = 5) when compared with another group of PAR patients (*n* = 5) before Der p IT (*P* < 0.01; Figure 1).

3.2 | Der p IT induced the expression of CD137 on CD8⁺ CD25⁺ T cells

CD137 expression on CD8⁺ CD25⁺ T cells from PBMC in PAR patients had increased after 6 months ($4.1 \pm 1.8\%$) and 1 year of IT ($4.5 \pm 2.0\%$) compared to pre-IT status ($1.1 \pm 0.7\%$; *n* = 30, *P* < 0.05; Figure 2). Herein, we examined whether Pam3CSK4 stimulation could enhance CD137 expression on CD8⁺ CD25⁺ T-cell population. CD137 expression was found to have increased in CD8⁺ CD25⁺ T cells from PAR patients and control subjects after stimulation with Pam3CSK4 (*P* < 0.01; Figure 2B).

3.3 | Pam3CSK4 enhanced suppressive CD8⁺ CD25⁺ CD137⁺ Tregs via cell contact

We evaluated whether CD8⁺ CD25⁺ CD137⁺ Tregs regulate CD4⁺ CD25⁻ T-cell proliferation during Der p IT. CFSE-labeled, CD4⁺ CD25⁻ T cells from PAR patients were incubated with sorted,

anti-CD3 and Pam3CSK4-stimulated CD8⁺ CD25⁺ CD137⁺ T cells before and after Der p IT. Proliferation of CD4⁺ CD25⁺ T cells was analyzed by flow cytometry. In a representative study (Figure 3A), Pam3CSK4-stimulated CD8⁺ CD25⁺ CD137⁺ T cells suppressed the proliferation of CD4⁺ CD25⁺ T cells ($11.6 \pm 2.7\%$) after Der p IT compared to pre-IT status ($15.1 \pm 3.0\%$; $P < 0.01$, $n = 20$; Figure 3B). Transwell inset experiments (pore size $0.4 \mu\text{mol/L}$) were performed to determine whether the Pam3CSK4-mediated CD8⁺ CD25⁺ CD137⁺ T-cell suppression required cell-cell contact. Incubation of CD4⁺ CD25⁺ T cells and Pam3CSK4-stimulated-CD8⁺ CD25⁺ CD137⁺ T cells in separate chambers reversed the inhibition of CD4⁺ CD25⁺ T-cell proliferation ($19.3 \pm 3.3\%$ vs $11.6 \pm 2.7\%$; $P < 0.01$, $n = 20$; Figure 3B).

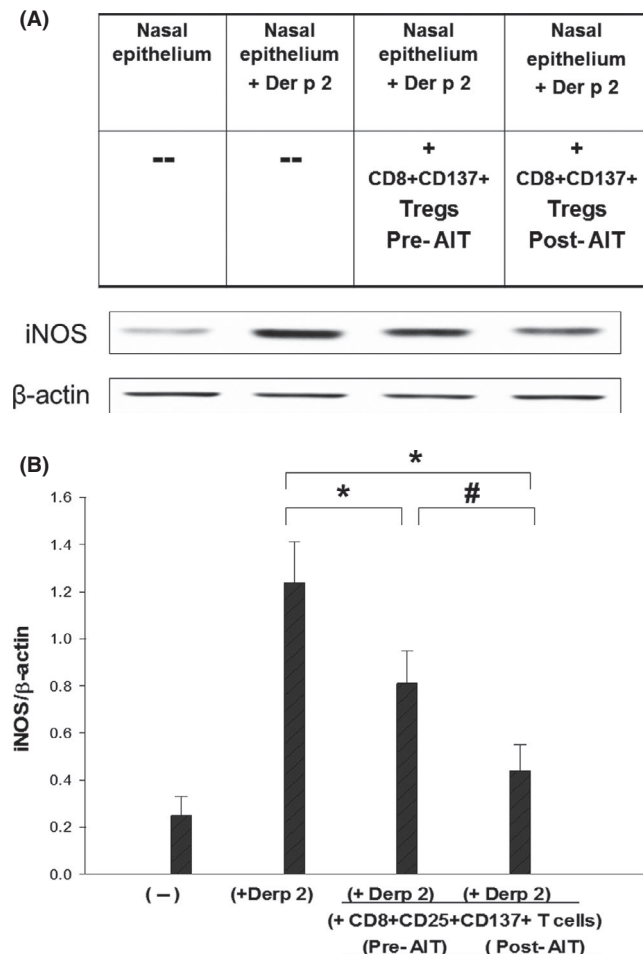


FIGURE 4 A, Inhibition of iNOS activity in Der p 2-activated primary nasal epithelial cells by Pam3CSK4-stimulated CD8⁺ CD25⁺ CD137⁺ T cells during Der p IT. Sorted CD8⁺ CD25⁺ CD137⁺ T cells were stimulated with Pam3CSK4 ($5 \mu\text{g/mL}$) and IL-2 (10 U/mL) for 72 hours and then cultured with Der p 2-activated primary nasal epithelial cells for 48 hours. iNOS proteins were extracted from confluent nasal epithelial cells and analyzed by Western blot. B, Statistical data of experiments on 10 paired samples showed a significant difference between PAR patients before and after Der p IT. (* $P < 0.01$ after treatment; # $P < 0.01$ by the Wilcoxon signed-ranks test on comparison between pre-IT and post-IT in the same PAR patients.)

3.4 | Effect of Pam3CSK4-stimulated CD8⁺ CD25⁺ CD137⁺ Treg cytokine production during Der p IT

We investigated whether Pam3CSK4-stimulated CD8⁺ CD25⁺ CD137⁺ T cells could modulate cytokine profiles in CD4⁺ CD25⁺ T cells during Der p IT. Table S1 summarizes the supernatant cytokines after Pam3CSK4-stimulated CD8⁺ CD25⁺ CD137⁺ T cells were incubated with CD4⁺ CD25⁺ T cells from 20 PAR patients during Der p IT and 20 non-atopic control subjects. Pam3CSK4-stimulated CD8⁺ CD25⁺ CD137⁺ T cells suppressed IL-4 production and increased anti-inflammatory cytokine production, such as IL-10 and TGF- β ($P < 0.05$). Der p IT elicited greater IL-10 and TGF- β production with CD8⁺ CD25⁺ CD137⁺ T cells than pre-IT status in allergic patients ($P < 0.05$; Figure S1).

4-1BB (CD137), a member of the inducible tumor necrosis factor receptor (TNFR) family, is expressed on Tregs. We further demonstrated that CD8⁺-depleted PBMC proliferation suppressed by Pam3CSK4-stimulated CD8⁺ CD25⁺ CD137⁺ T cells was greater than Pam3CSK4-stimulated CD8⁺ CD25⁺ CD137⁺ T cells and the proliferation inhibition and IL-10 production were reversed by TNF- α inhibitor ($P < 0.05$, $n = 6$; Figure S2).

3.5 | Der p 2 inhibition induced iNOS on nasal epithelial cells by CD8⁺ CD137⁺ Tregs

Recombinant house dust allergen Der p 2 was shown to activate nasal epithelial cells to induce inducible nitric oxide synthase¹ expression (Figure 4A). The effects of Pam3CSK4-stimulated CD8⁺ CD25⁺ CD137⁺ T cells in suppressing iNOS activity in Der p 2 pre-activated nasal epithelial cells were studied. CD8⁺ CD25⁺ CD137⁺ Treg cell-inhibited Der p 2-induced iNOS by nasal epithelial cells was greater in post-IT-treated allergic patients compared to pre-IT status ($P < 0.05$; $n = 10$; Figure 4B).

3.6 | Silencing CD137 decreased Pam3CSK4-activated Foxp3 in CD8⁺ CD25⁺ Tregs

Anti-CD137 mAb stimulation could promote Treg proliferation and was associated with Foxp3 expression. Herein, purified CD8⁺ CD25⁺ T cells were transfected with siRNA targeting human CD137 or control siRNA in the presence of Pam3CSK4 for 72 hours. Proteins were extracted from the CD8⁺ CD25⁺ T cells, and Foxp3 expression was analyzed by Western blotting (Figure 5A). Silencing CD137 decreased Foxp3 expression in Pam3CSK4-stimulated CD8⁺ CD137⁺ T cells as shown by three independent experiments ($P < 0.01$; Figure 5B,E).

4 | DISCUSSION

CD137 is a co-inhibitor with GATA-3 for Th2 differentiation, and CD137 engagement may thus be a potential treatment for allergic airway inflammation.¹⁷ CD137 activation might also limit

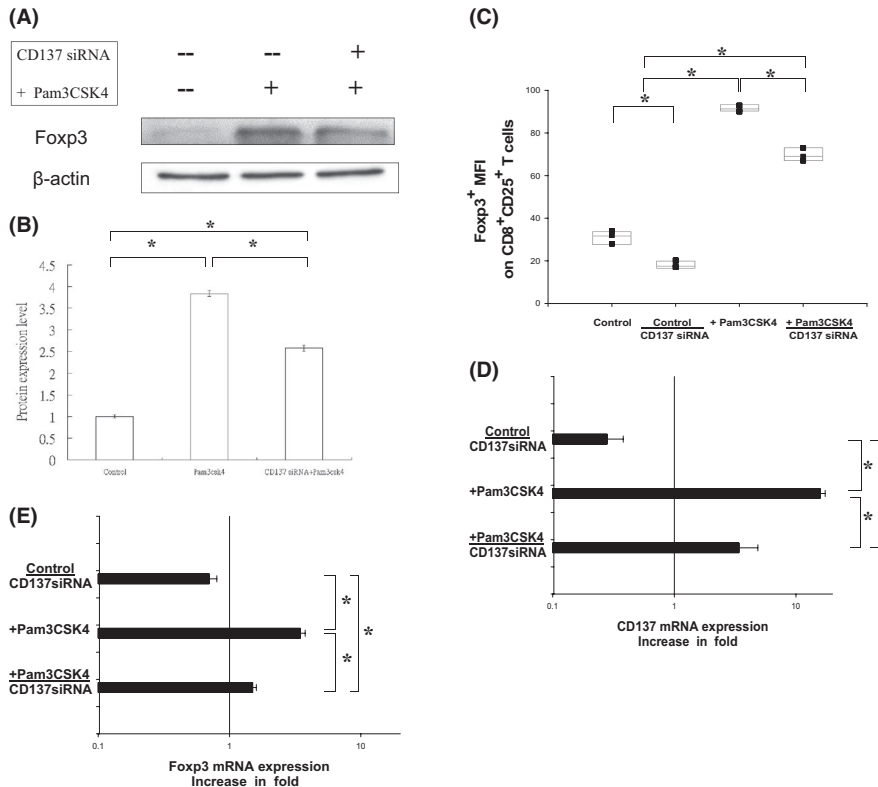


FIGURE 5 Silencing CD137 decreases Foxp3 expression in Pam3CSK4-stimulated CD8⁺ CD25⁺ T cells. A, Purified CD8⁺ CD25⁺ T cells were transfected with small interfering RNAs (siRNA) targeting human CD137 or control siRNA. After CD137 siRNA transfection, CD8⁺ CD25⁺ T cells were stimulated with Pam3CSK4 (5 μ g/mL) and IL-2 (10 U/mL) for 72 hours and nuclear proteins were extracted and examined for Foxp3 expression by Western blotting. B, Statistical data of three independent experiments are shown. C, The mean fluorescence intensity (MFI) of Foxp3 staining on CD8⁺ CD25⁺ T cells was analyzed by flow cytometry. Real-time PCR results for (D) CD137 and (E) Foxp3 genes of CD8⁺ CD25⁺ T cells after CD137 siRNA transfection are shown as the average fold induction from three independent experiments. * $P < 0.01$ after treatment. Significant differences were determined using Kruskal-Wallis test

GM-CSF-mediated and IL-5-mediated anti-apoptosis of eosinophils. A single injection of an anti-CD137 mAb has been shown to prevent eosinophilic airway inflammation and is accompanied by diminished Th2 cytokine production, decreased serum IgE levels, and reduced airway hyper-reactivity.²² Previous evidence revealed decreased percentages of CD8⁺ Foxp3⁺ Treg cells in both eosinophilic and non-eosinophilic nasal polyps.^{7,23,24} CD137 stimulation provides a potent co-stimulatory signal of T-cell responses, especially CD8⁺ Treg cell-mediated responses.²⁵ We further identified that Der p IT could increase percentage of CD8⁺ CD25⁺ CD137⁺ T cells and inhibit Der p 2-induced iNOS on nasal epithelium cells and nasal NO levels, suggesting that CD137 stimulation may benefit immune tolerance mediated by CD8⁺ Tregs during AIT.

TLR2 is related to protection against allergies and allergic asthma, playing a crucial role in balancing immune responses with tolerance.²⁶ CD4⁺ Tregs by TLR2 stimulation could affect CD4⁺ Treg cell suppressive capacity and prevent excessive allergen-induced inflammation.¹² We demonstrated the role of Pam3CSK4 in regulating CD8⁺ Treg cell-mediated suppression during mite allergy.³ Enhanced CD137 signal expression by TLR ligand stimulation has also been reported. TLR2 can interact with CD137 on the macrophage surface to participate in CD137 ligand-mediated TNF production.²⁷ Immunization combined with anti-CD137 mAb and TLR ligand and poly I:C induced CD8⁺ Treg generation to suppress antigen-specific proliferative responses of CD4⁺ T cells.¹⁸ After "knocking down" CD137 with small interfering RNA, we first confirmed that Pam3CSK4 could cross-link CD137 in sustaining Foxp3 expression in the CD8⁺ CD25⁺ Tregs. These data suggest that modulation of

CD137 signaling pathway by Pam3CSK4 plays an important role in CD8⁺ CD25⁺ Treg cells to control allergic inflammatory diseases.

Several mechanisms have been proposed for CD8⁺ Treg cell-mediated suppression including cell contact inhibition and secretion of immunosuppressive cytokines.⁴⁻⁶ In addition to cell-cell contact inhibition, Pam3CSK4-stimulated CD8⁺ CD25⁺ CD137⁺ Treg cells can secrete these suppressive cytokines with IL-10 and TGF- β , which may be beneficial in controlling allergic inflammation. Co-expression of TGF- β 1 and IL-10 by regulatory T cells is required for complete suppression of airway hyper-reactivity in murine asthma.²⁸ In experimental models, co-stimulation by TGF- β induced CD8⁺ Treg proliferation from naïve CD8⁺ T lymphocytes and neutralization by anti-TGF- β mAb reversed the suppressive effects of CD8⁺ Tregs.²⁹ CD137 is required for induction of CD8⁺ Treg cellular activity, which can enhance the function of suppressive molecules such as TGF- β and IL-10.²⁷ Further studies are required to determine whether CD8⁺ CD137⁺ Treg cells can resolve airway inflammation during allergen immunotherapy.

Direct and indirect effects of CD137 agonists on regulatory T cells and adoptive T-cell therapy have been reported for cancer immunotherapy.^{14,15} However, the significance of such mechanisms being proposed in CD137-based immunotherapy remains to be completely elucidated. Immunomagnetic sorting of CD137-positive T cells among tumor-infiltrating lymphocytes offers a great opportunity to raise the efficacy of current cancer immunotherapies.³⁰ In summary, functional control of CD8⁺ Tregs by TLR2 signaling via CD137 stimulation may offer new avenues toward developing a valuable potential target during AIT.

CONFLICTS OF INTEREST

The authors have no conflicts of interest relevant to this article to disclose.

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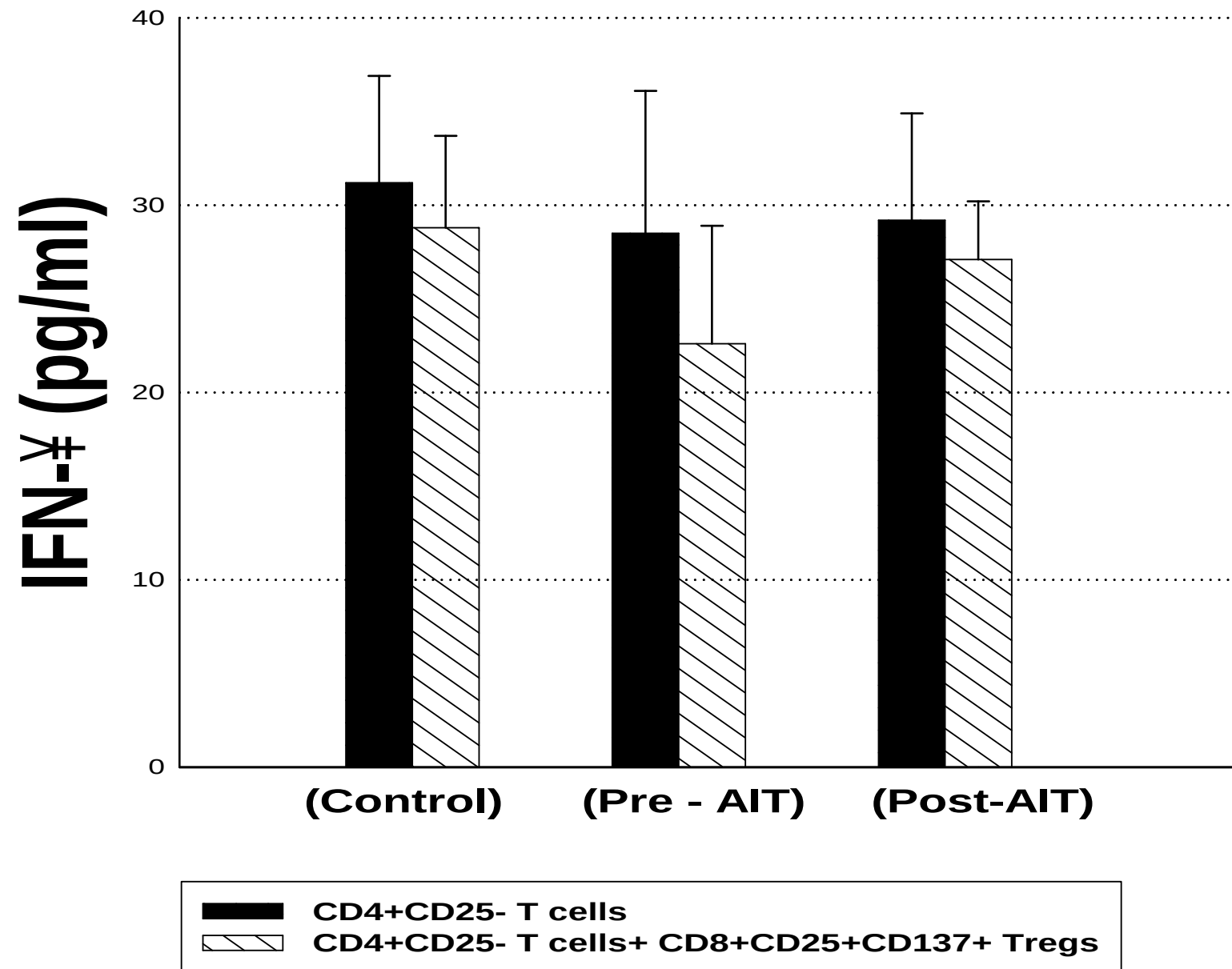
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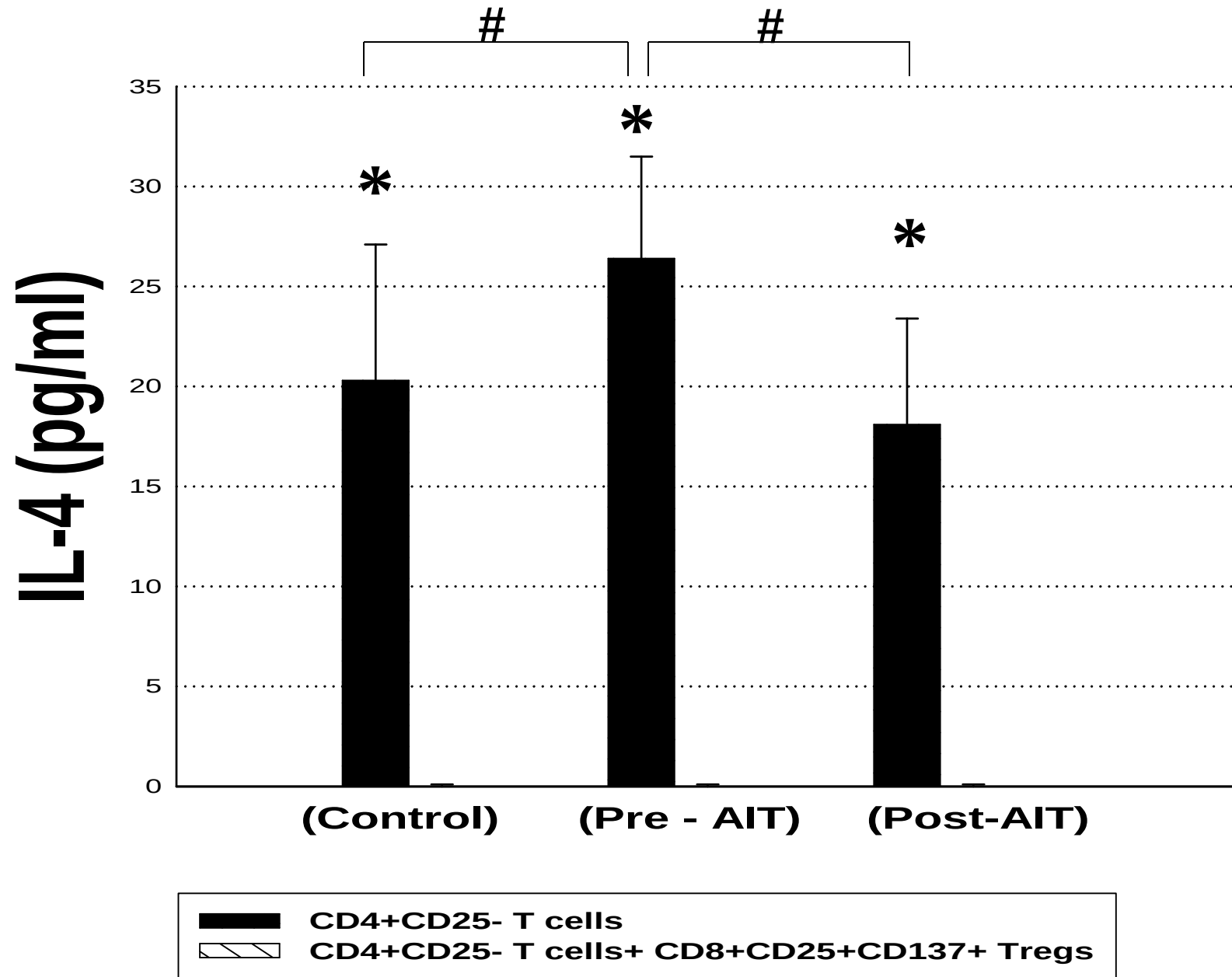
SUPPORTING INFORMATION

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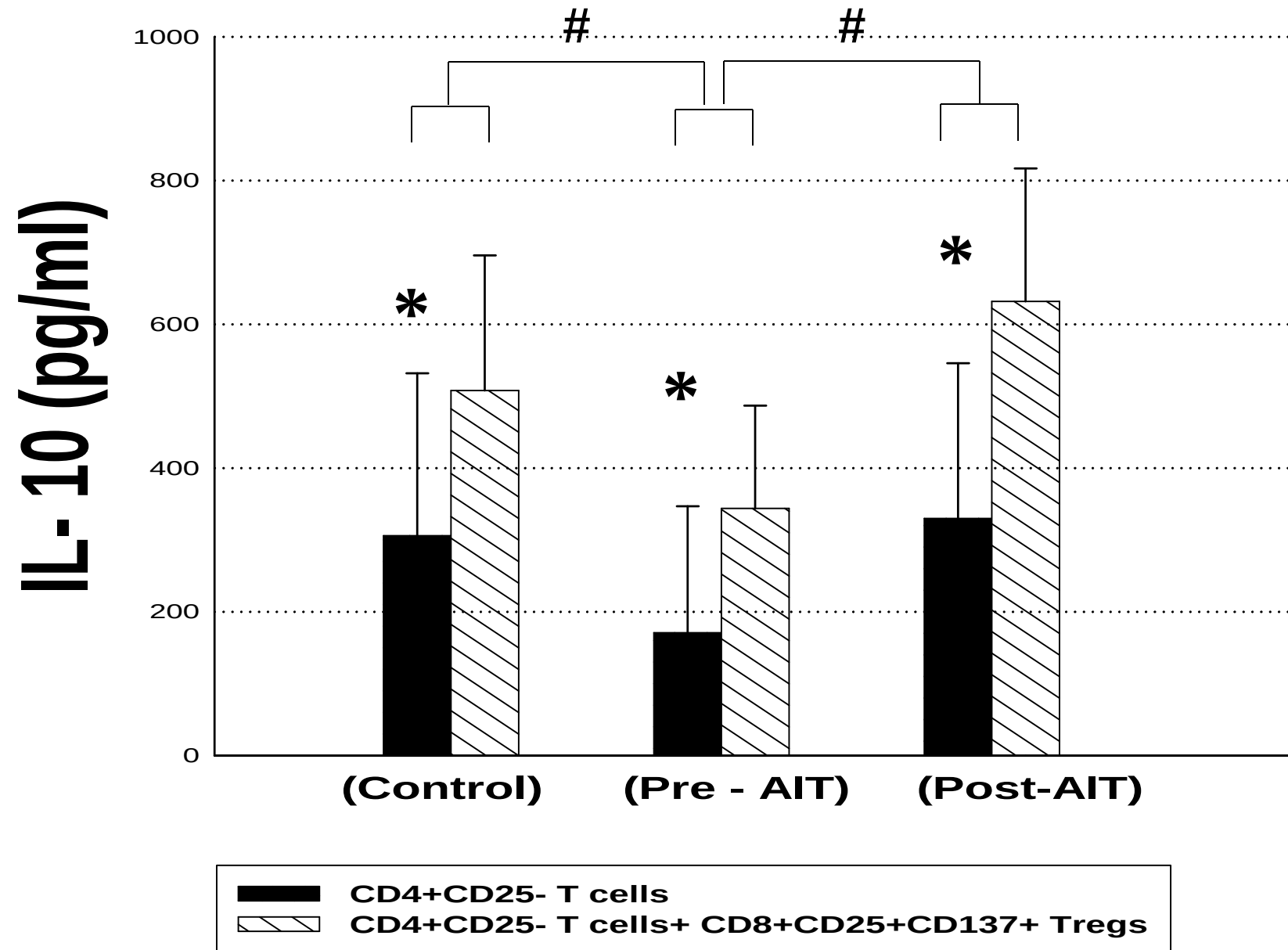
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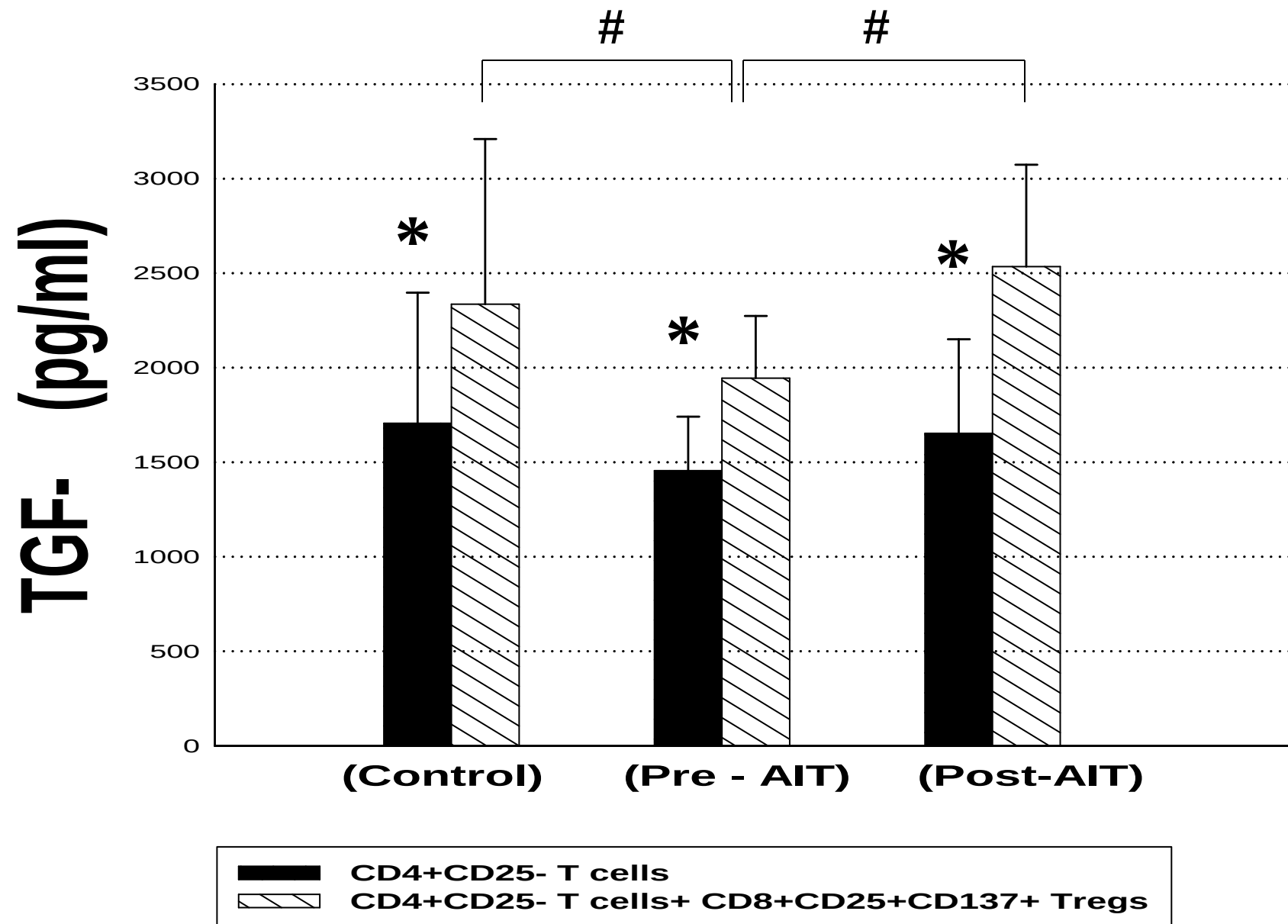
Supplement Figure 1A



Supplement Figure 1B

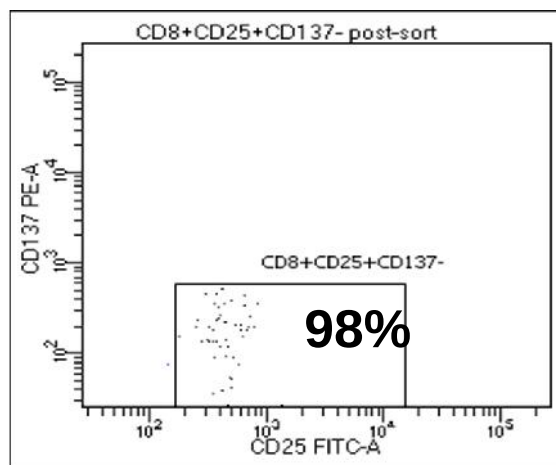


Supplement Figure 1C

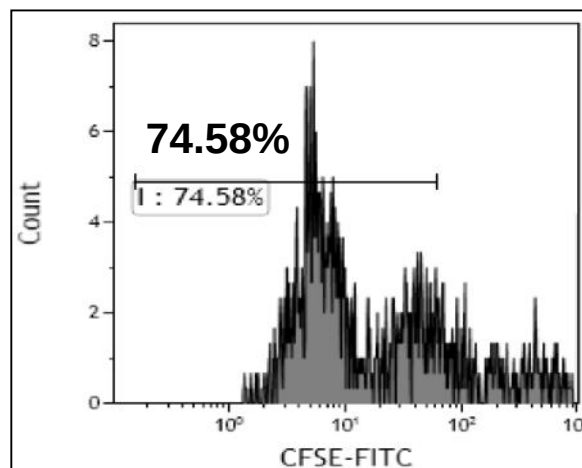


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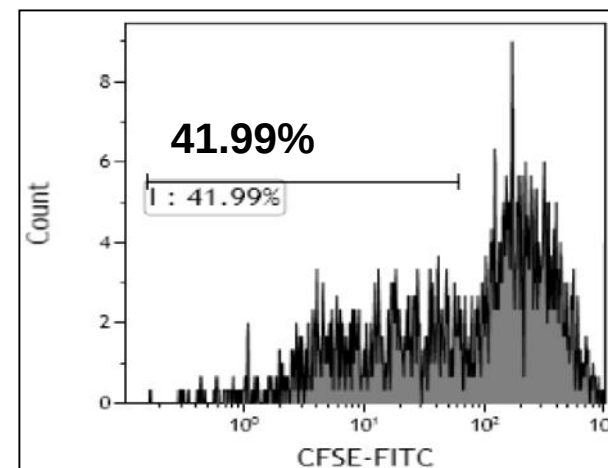
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CD8+CD25+CD137- T cells**



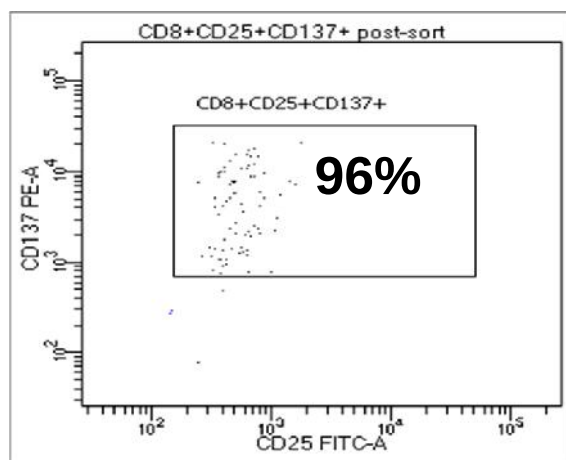
CD8- PBMCs



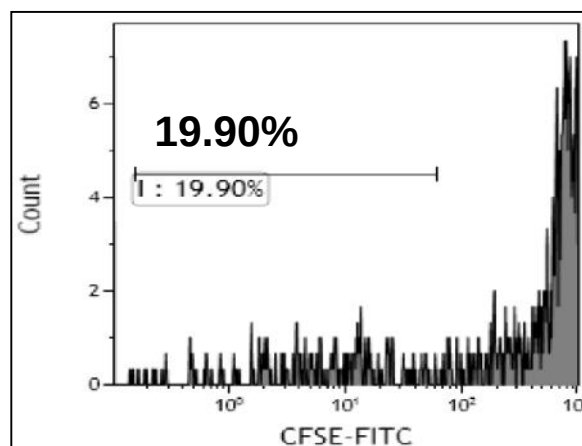
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+ CD8+CD25+CD137- T cells**



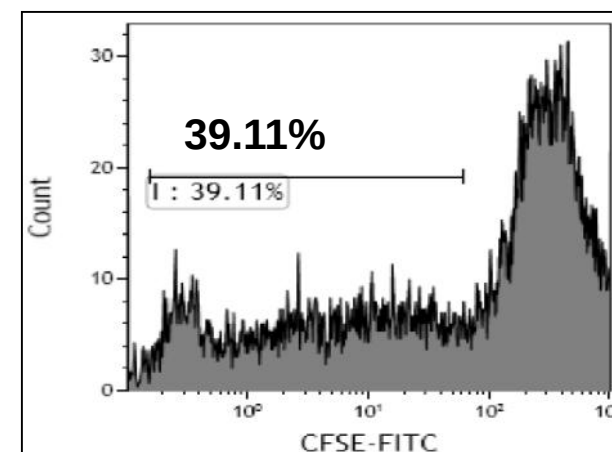
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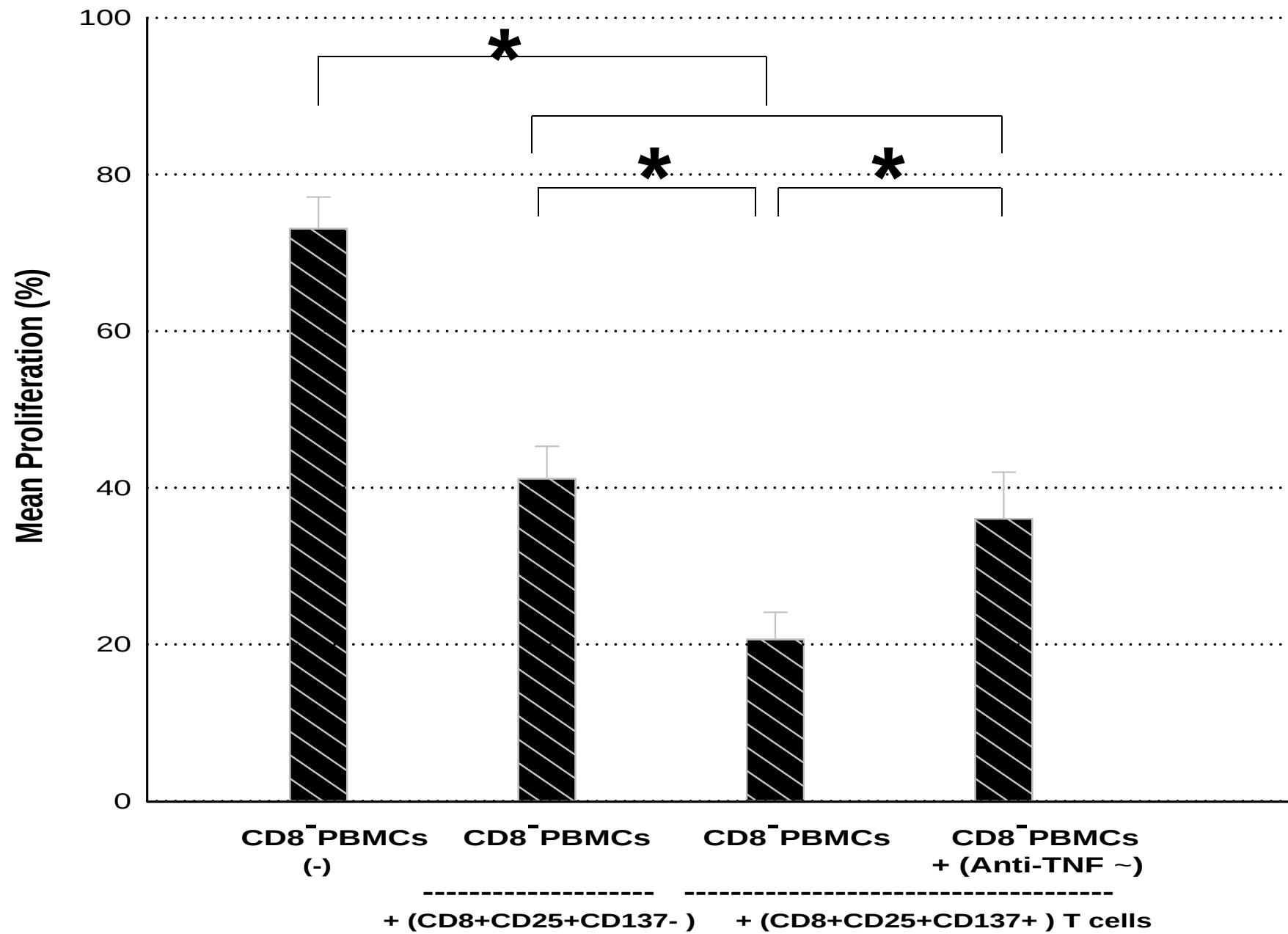
**CD8- PBMCs
+ CD8+CD25+CD137+ T cells**



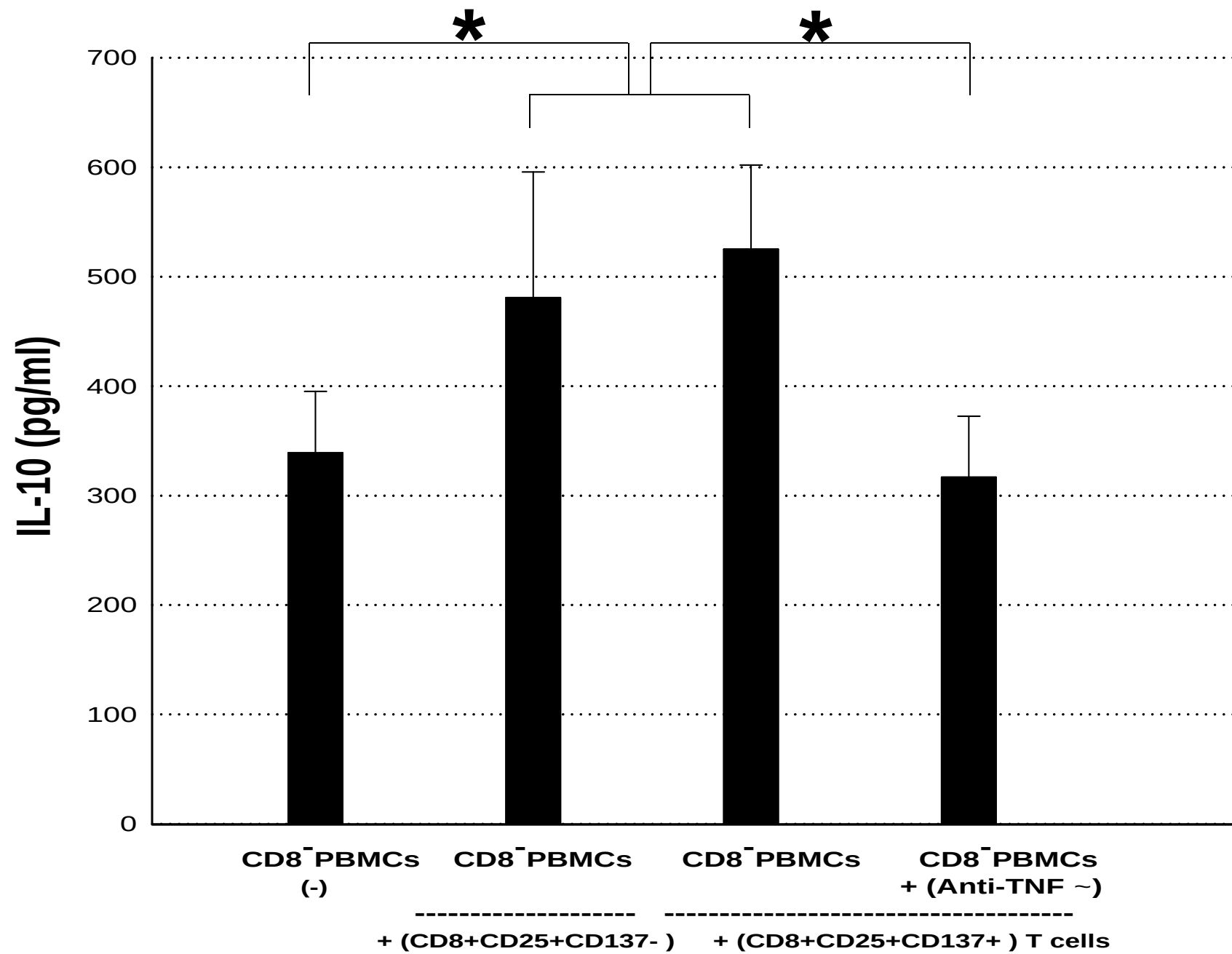
**CD8- PBMCs
+ CD8+CD25+CD137+ T cells
+ Anti-TNF α mAb**



Supplement Figure 2A



Supplement Figure 2B



Supplement Figure 2C

1

2 **Supplement Table 1.** Cytokine levels in culture supernatant before and after addition
 3 of CD8⁺CD25⁺CD137⁺ T cells during Der p SIT

Cytokine (pg/ml)	IFN- γ	IL-4	IL-10	TGF- β
Normal subjects				
CD4⁺CD25⁻ T cells	31.2 $\pm$ 5.7	20.3 $\pm$ 6.8	306 $\pm$ 226	1706 $\pm$ 691
CD25⁻ & CD8⁺Tregs	28.8 $\pm$ 4.9	U.D.*	508 $\pm$ 188*	2336 $\pm$ 874*
Pre – IT subjects				
CD4⁺CD25⁻ T cells	28.5 $\pm$ 7.6	26.4 $\pm$ 5.1 ⁺	171 $\pm$ 176 ⁺	1456 $\pm$ 285
CD25⁻ & CD8⁺Tregs	22.6 $\pm$ 6.3	U.D.*	344 $\pm$ 143 ⁺⁺	1945 $\pm$ 329 ⁺⁺
Post – IT subjects				
CD4⁺CD25⁻ T cells	29.2 $\pm$ 5.7	18.1 $\pm$ 5.3	330 $\pm$ 216	1653 $\pm$ 498
CD25⁻ & CD8⁺Tregs	27.1 $\pm$ 3.1	U.D.*	632 $\pm$ 185*	2535 $\pm$ 539*

4

5 CD4⁺CD25⁻ T cells were stimulated with IL-2 (10 U/ml) in the presence or absence
 6 of CD8⁺CD25⁺CD137⁺ T cells for 5 d, and their supernatants were collected to
 7 measure IL-4, IFN- γ , IL-10, TGF- β cytokines content by ELISA.

8 Data are shown as the mean $\pm$ SD. U.D. = Undetectable * $p < 0.05$ compared after
 9 addition of CD8⁺CD25⁺CD137⁺ T cells. ⁺ $p < 0.05$ compared between nonatopic
 10 control subjects (n=20) and PAR subjects (n=20) during Der p IT.

11

12

13 **Supplement Material and Methods.**

14 ***Nasal NO Measurement***

15 nNO were measured using an online electrochemical analyzer equipped with
16 nNO software (NIOX MINO; Aerocrine AB, Sweden) in compliance with American
17 Thoracic Society/European Respiratory Society recommendations [20]. Briefly, nNO
18 levels were measured from the nostril while breath was held for 45s with an aspiration
19 flow of 5 mL/s. The NO levels derived from the nose were recorded by introducing a
20 nasal olive connected to the analyzer into one nostril. The nNO concentration was
21 automatically calculated by the NIOX MINO system. The nasal olive was then placed
22 in the other nostril and the test was repeated. Measurements were made in triplicate
23 for both nostrils and the mean nNO level was used for the analysis. All children
24 avoided physical exercise and consumption of foods rich in nitrogen such as sausages,
25 various animal offal, lettuce, and spinach within two hours of NO measurement.
26 Finally, all enrolled children successfully completed the nNO measurements.

27

28 ***Cells and Tissue Sample***

29 Inferior turbinate specimens were obtained from 10 enrolled severe PAR patients
30 (including 5 PAR patients who received one year of Der p IT) and 5 non-allergic
31 controls undergoing nasal septoplasty because of anatomical variations and without
32 other sinonasal diseases. Surgery was done under strict clinical indications, and severe
33 PAR had a difficulty in nose breathing. Nasal biopsy specimens were cut into small
34 pieces and placed in trypsin (0.025%)/EDTA (0.01%; Gibco, Grand Island, NY) for 3
35 hours at 37°C and 5% CO₂. Cells were strained through 70-mm nylon mesh (Becton
36 Dickinson Labware, Franklin Lakes, NJ), washed, seeded at a density of 4×10^3
37 cells/cm² in T-75 cell culture flasks (Hyclone, GE Healthcare, USA), and cultured in
38 medium (Hyclone), supplemented with 100 U/mL penicillin, 100 mg/mL

streptomycin, and 0.25 mg/mL amphotericin B (Gibco) in a humidified atmosphere containing 5% CO₂ at 37°C.

CD8⁺CD25⁺CD137⁺ Treg cells isolation and cell culture

Peripheral mononuclear cells (PBMCs) were isolated by Ficoll-paque gradient centrifugation (Pharmacia, Uppsala, Sweden). The synthetic lipopeptide Pam3CSK4 (InvivoGen, Carlsbad, Calif) was prepared in sterile phosphate-buffered saline (PBS). A total of 1×10^6 cells were cultured with Pam3CSK4 (5 µg/ml) or the same volume of medium as negative control for 5 days on 96-well culture plates in RPMI-1640 culture medium supplemented with L-glutamine (2 mmol/L), HEPES (20 mmol/L), sodium pyruvate (91 mmol/L), streptomycin (50 ng/ml), penicillin (100 IU/ml) and 10% fetal bovine serum (Bio-Whittaker, Walkersville, Md). In some experiments, CD4⁺ or CD8⁺ cells were separated directly from PBMCs using microbeads, according to manufacturer's instructions (BD Biosciences) [6]. CD4⁺CD25⁻ T cells were isolated using a CD4⁺ T cells enrichments kit, followed by separation with CD25 microbeads (BD Biosciences). To explore CD8⁺ Treg cells inhibition, CD8⁺CD25⁺CD137⁺ T cells were isolated using a CD8⁺ T cells enrichment kit (BD Biosciences) followed by stained with anti-human CD25 FITC mAb and anti-human CD137 PE mAb (BD Biosciences). The CD8⁺CD25⁺CD137⁺ T cells population were purified using the BD FACSCanto II (BD Biosciences) and cells analyzed by flow cytometry was greater than 95% as shown in Figure 2A.