

Letter to the Editor

Comparison of the allergic and nonallergic CD4⁺ T-cell responses to the major dog allergen Can f 1

To the Editor:

Although it is well established that pathogenic T_H2-deviated CD4⁺ T-cell responses dominate in individuals with allergy, the nature of allergen-specific CD4⁺ T-cell responses in individuals without allergy exposed to the same allergens is still largely unclear. A number of nonexclusive mechanisms, such as immune ignorance of nonpathogenic environmental antigens, anergy, or deletion of allergen-specific T cells, and immune deviation to T_H0/T_H1 phenotype, have previously been proposed to explain the tolerance to allergens in these individuals. In recent years, however, the role of regulatory T (Treg) cells in the induction and maintenance of peripheral tolerance to allergens has been emphasized.

At least 2 distinct subsets of Treg cells exist in human beings: CD4⁺CD25⁺forkhead box protein 3 (Foxp3)⁺ Treg cells and IL-10–producing type 1 Treg (Tr1) cells. Several recent studies have convincingly demonstrated that immune tolerance induced by specific immunotherapy is mainly mediated by Tr1 cells.^{1–3} However, whether Treg cells play a dominant role in maintaining tolerance to common respiratory allergens in healthy subjects without allergy is less clear. The seminal studies of Ling et al⁴ and Akdis et al⁵ suggested that immune tolerance to respiratory allergens is maintained by CD4⁺CD25⁺Foxp3⁺ Treg cells or Tr1 cells, respectively. However, more recent studies have not been able to corroborate these findings convincingly, challenging the dominant role of Treg cells in the healthy immune response against respiratory allergens.^{3,6–8}

In the current study, we aimed to dissect the factors associated with an allergic or nonallergic CD4⁺ T-cell response to the major dog allergen, Can f 1. To this end, we analyzed Can f 1–specific proliferative responses of peripheral blood CD4⁺ T cells of 15 subjects with dog allergy and 11 healthy nonallergic dog owners by using the carboxyfluorescein succinimidyl ester (CFSE) dilution assay (Fig 1, A; see this article's Methods in the Online Repository at www.jacionline.org).

The proliferative responses in the Can f 1–stimulated cultures from subjects with allergy were weak but statistically significantly stronger than those in unstimulated cultures (Fig 1, B). No proliferative responses were observed in Can f 1–stimulated cultures from subjects without allergy. Robust proliferative responses to the control antigens streptokinase and purified protein derivative (PPD) were detected in the cultures from both the subjects with and without allergy (see this article's Fig E1 in the Online Repository at www.jacionline.org; data not shown).

Next, we investigated whether suppression by CD4⁺CD25⁺Foxp3⁺ Treg cells explains the absence of proliferative responses to Can f 1 in subjects without allergy. No differences in the frequencies of CD4⁺CD25⁺Foxp3⁺ or CD4⁺CD25^{hi}Foxp3⁺ cells were detected in the PBMCs of subjects with and without allergy (see this article's Fig E2 in the Online Repository at www.jacionline.org). To evaluate the role of CD4⁺CD25⁺Foxp3⁺ Treg cells at the functional level, we used anti-CD25–magnetic beads to specifically deplete CD25^{hi} cells from PBMCs (Fig E2). After the CD25 depletion, there was a general increase in proliferative responses in the cultures. However,

the proliferative responses in Can f 1–stimulated cultures from the subjects with allergy remained statistically significantly stronger than those in unstimulated cultures (Fig 1, C). In contrast, the specific proliferative responses to Can f 1 in the cultures from the subjects without allergy were not enhanced by the CD25 depletion. The specific proliferative responses to streptokinase were not affected by the CD25 depletion (Fig E1).

To evaluate whether suppression by IL-10–producing Tr1 cells contributes to the absence of proliferative responses to Can f 1 in subjects without allergy, we stimulated PBMCs with Can f 1 or streptokinase in the presence or absence of neutralizing levels of anti-IL-10 antibodies. No effect on the proliferative responses to Can f 1 (Fig 1, D) or streptokinase (Fig E1) was observed in the subject groups either with or without allergy. Moreover, only low levels of soluble IL-10 could be detected in the supernatants of either undepleted or CD25-depleted Can f 1–stimulated cultures, and these levels did not differ between subjects with and without allergy (data not shown). Collectively, our current findings do not support the notion that suppression by either CD4⁺CD25⁺Foxp3⁺ Treg cells or IL-10–producing Tr1 cells explains the absence of proliferative responses to Can f 1 in subjects without allergy.

Next, we wanted to investigate whether the frequency of Can f 1–specific memory CD4⁺ T cells in peripheral blood or the functional characteristics of the cells at the single cell level explain the observed differences in proliferative responses between subjects with and without allergy. To enable the detection of rare Can f 1–specific memory CD4⁺ T cells, a complementary approach, the split well method (see Methods in the Online Repository) was used to generate oligoclonal long-term T-cell lines (TCLs) from peripheral blood CD4⁺CD45RA[–]CD45RO⁺ T cells.

With this technique, we were able to obtain at least 1 Can f 1–specific TCL from 14 (93%) of 15 subjects with allergy but only from 6 (55%) of 11 subjects without allergy (Fig 2, A). The mean frequency of specific TCLs obtained from 30 replicate cultures was 8.2% for subjects with allergy and 4.2% for subjects without allergy ($P = .03$; Fisher exact test). On the basis of these results, it seems that Can f 1–specific memory CD4⁺ T cells are either extremely rare or absent in most individuals without allergy. Our current data corroborate the findings of recent HLA class II tetramer studies, which consistently demonstrate a lower frequency of allergen-specific CD4⁺ T cells in subjects without allergy.^{6,7,9}

Importantly, the functional T cell receptor (TCR) avidity, estimated by effective concentration 50 (EC₅₀) values (ie, the concentration of Can f 1 needed to induce a half-maximal proliferative response), appeared to be higher in the Can f 1–specific TCLs from subjects with allergy than in those from subjects without allergy (Fig 2, B). Although most of the induced TCLs from both subjects with and without allergy were of low functional avidity, higher-avidity TCLs—that is, TCLs showing positive proliferative responses at lower concentrations of Can f 1—were induced only from subjects with allergy. Using an arbitrary cutoff of EC₅₀ ≤ 2 μg/mL, 12 (33%) of 36 TCLs from subjects with allergy but none of the TCLs from subjects without allergy could be classified as being of higher functional TCR avidity ($P = .01$; Fisher exact test).

Finally, the cytokine production of the established TCLs was analyzed by measuring IL-4, IL-5, IL-10, IFN-γ, and IL-17 in the

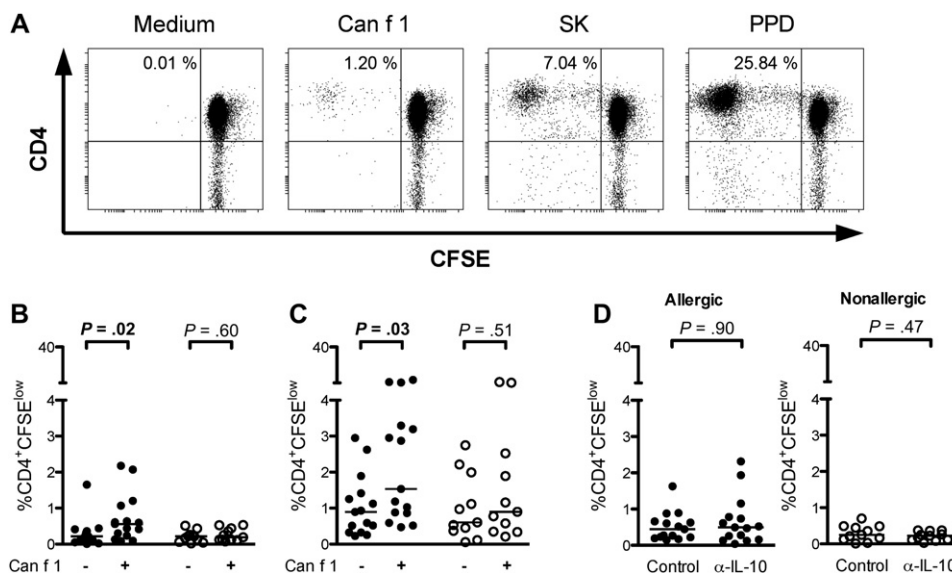


FIG 1. Detection of antigen-specific proliferation of peripheral blood CD4⁺ T cells by the CFSE dilution assay after a 7-day culture. A representative example of antigen-specific responses from a subject with allergy (A). Proliferative responses of peripheral blood CD4⁺ T cells from subjects with (●) and without allergy (○) on stimulation with 100 µg/mL Can f 1 before (B) and after depletion of CD25^{hi} cells (C), and in the presence of an isotype control or anti-IL-10 antibody (10 µg/mL; D). Results are expressed as the percentage of CD4⁺CFSE^{low} cells. SK, Streptokinase.

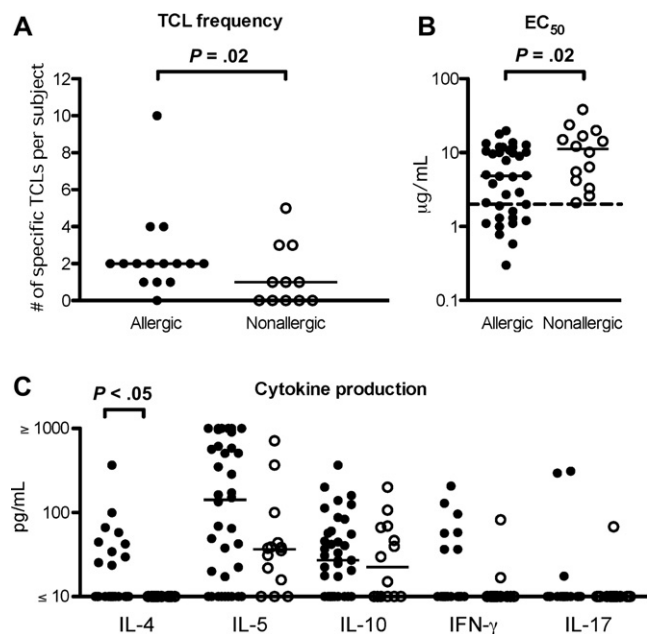


FIG 2. The frequencies and functional characteristics of long-term Can f 1-specific memory T cells. A, The number of Can f 1-specific T cells generated from subjects with (●) and without allergy (○; per 30 replicate wells seeded). The functional TCR avidity (B) and cytokine production (C) of the T cells. Functional avidity was evaluated by assessing EC₅₀ values from the titration curves of the T cells. Cytokine production was measured from the supernatants of cultures stimulated with 100 µg/mL Can f 1.

culture supernatants (Fig 2, C). In general, the levels of cytokines in the supernatants were low. However, IL-4 production was observed only in the T cells from subjects with allergy, not in those from subjects without allergy ($P < .05$; Fisher exact test). Moreover, the T cells from subjects with allergy tended to produce

higher levels of IL-5 than those from subjects without allergy, although this did not reach a statistical significance. Interestingly, the highest levels of IL-4 and IL-5 production were observed in the higher-avidity T cells (EC₅₀ ≤ 2 µg/mL) from subjects with allergy (see this article's Fig E3 in the Online Repository at www.jacionline.org). No differences in the production of IL-10 could be observed between the T cells from subjects with and without allergy, and only a few T cells produced IFN-γ or IL-17. Collectively, these results demonstrate that some of the T cells from subjects with allergy, in particular those with a higher functional TCR avidity, are clearly T_H2-biased, whereas the T cells from subjects without allergy exhibit mostly an undifferentiated T_H0 phenotype.

Taken together, we have demonstrated in this study that subtle differences in the frequency, functional TCR avidity, and phenotype of Can f 1-specific memory CD4⁺ T cells exist between subjects with dog allergy and subjects without allergy. However, the mechanisms leading to these differences remain to be elucidated. Importantly, we found no evidence for differences in either the frequency or function of CD4⁺CD25⁺Foxp3⁺ Treg cells or IL-10-producing Tr1 cells between the subject groups with and without allergy. This finding is in line with the emerging evidence that immune tolerance to allergens in healthy individuals without allergy cannot be explained solely by Treg cell function.¹⁰ One intriguing possibility that has not been examined so far in detail is that the nature of the nonallergic response may depend on the type of allergen: for some allergens, active suppression by regulatory T cells may be important for maintaining tolerance, whereas for other allergens, T_H1 deviation or immune ignorance may be the prevalent mechanism. Moreover, it is possible that the mechanisms conferring protection against a particular allergen may be different in truly nonatopic individuals than in those sensitized to other allergens. Obviously, studies comparing CD4⁺ T-cell responses to different allergens in different subject groups are needed to investigate these hypotheses further.

The skillful technical assistance of Virpi Fisk and Raija Tukiainen is gratefully acknowledged.

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Supported by Kuopio University Hospital (project no. 5021605) and the Academy of Finland (contract no. 115160).

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

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doi:10.1016/j.jaci.2010.06.005

METHODS

Subjects and antigens

PBMCs from 15 subjects with dog allergy and positive skin prick tests to Can f 1^{E1} and from 11 nonatopic dog owners with negative skin prick tests to the allergen were separated by Ficoll-Paque Plus density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden). The study was approved by the Ethics Committee of the Kuopio University Hospital, and written informed consent was given by all subjects. The antigens used in this study were recombinant Can f 1 produced in *Pichia pastoris*,^{E1} streptokinase (Sigma-Aldrich, St Louis, Mo), and PPD (Statens Serum Institut, Copenhagen, Denmark). Recombinant Can f 1 was determined to be endotoxin-free (<0.1 EU/mL) by limulus assay (Lonza, Walkersville, Md).

Depletion of CD4⁺CD25^{hi} cells

To deplete CD25^{hi} cells, 20 μ L anti-CD25-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) was suspended together with 8×10^6 PBMCs in a total volume of 100 μ L and incubated for 15 minutes at +4°C. After washing, the cells were passed through a MS column (Miltenyi Biotec). Undepleted and CD25^{hi}-depleted PBMCs were stained with anti-CD4-fluorescein isothiocyanate and anti-CD25-phycoerythrin (BD Biosciences, San Jose, Calif) for 20 minutes at +4°C. After washing, the cells were fixed and permeabilized according to the manufacturer's instructions (Human FoxP3 Buffer Set; BD Biosciences) and stained with anti-Foxp3-Alexa Fluor 647 (BD Biosciences) for 30 minutes at room temperature. Finally, the cells were washed twice and analyzed with a BD FACSCanto II flow cytometer (BD Biosciences).

CFSE dilution assay

Cells were cultured in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 20 μ mol/L 2-mercaptoethanol, 1 mmol/L sodium pyruvate, non-essential amino acids, 100 IU/mL penicillin, 100 μ g/mL streptomycin, 10 mmol/L HEPES (all from Lonza, Verviers, Belgium), and 5% inactivated human AB serum (Sigma-Aldrich). Proliferation of peripheral blood CD4⁺ cells was determined by using the CFSE dilution assay as described elsewhere.^{E2,E3} Briefly, undepleted or CD25^{hi}-depleted PBMCs were incubated in PBS at 37°C for 10 minutes with 1 μ mol/L CFSE (Molecular Probes, Eugene, Ore). Staining was terminated by adding culture medium and subsequent washing. Stained cells were cultured in quadruplicate at 2×10^5 cells per well in 96-well round-bottom plates (Corning Inc, Corning, NY). The cultures were stimulated with 1 to 100 μ g/mL Can f 1, 200 U/mL streptokinase, and 1 μ g/mL PPD. In some experiments, 10 μ g/mL neutralizing anti-IL-10 or an isotype-matched control antibody (BD Biosciences) was added to the cultures. After 7 days of culture, the cells were stained with anti-CD3 peridinin chlorophyll protein-cyanine5.5, anti-CD4-allophycocyanin, and anti-CD8-phycoerythrin (BD Biosciences) before flow-cytometric analyses.

Generation and functional analyses of long-term memory CD4⁺ T-cell lines

Memory (CD45RA⁻CD45RO⁺) CD4⁺ T cells were isolated from freshly purified PBMCs with the "no touch" memory CD4⁺ T-cell isolation kit

(Miltenyi Biotec). The mean purity of the memory cells was 95% (range 90% to 99%) as assessed by staining with anti-CD4-fluorescein isothiocyanate, anti-CD45RA-allophycocyanin, and anti-CD45RO-phycoerythrin-Cy7 (BD Biosciences).

Long-term TCLs were generated by the split-well method, as previously described.^{E4,E5} Briefly, 2.5×10^4 memory CD4⁺ T cells were seeded in 96-well round-bottom plates (30 replicate wells) together with autologous γ -irradiated (3000 rad) PBMCs and 10 μ g/mL Can f 1. After 5 days, fresh medium and 10 IU/mL IL-2 were added to the cultures. After 10 days, the cultures were restimulated with autologous irradiated PBMCs, Can f 1, and 10 IU/mL IL-2. On day 20, half of the T cells in each well were split into 2 new daughter plates and stimulated with autologous irradiated PBMCs (5×10^4 per well) with or without Can f 1. After 3 days, [³H]thymidine was added (1 μ Ci per well; GE Healthcare, Little Chalfont, United Kingdom), and after an additional 16 hours, the cells were harvested onto glass fiber filters (Wallac, Turku, Finland). Radioactivity was measured by scintillation counting (Wallac Micro Beta 1450) and the results expressed as counts per minute. The stimulation index (counts per minute in the presence of Can f 1 divided by counts per minute in the absence of Can f 1) was determined. Positive cultures (stimulation index > 2) were transferred to a 48-well plate and expanded with 1×10^6 autologous irradiated PBMCs, Can f 1, and 25 IU/mL IL-2 for 2 weeks before analyses.

T-cell proliferation assays were set up in triplicate in 96-well round-bottom plates with 2.5×10^4 T cells together with 5×10^4 autologous irradiated PBMCs as APCs, and increasing concentrations of Can f 1 (0.01 to 100 μ g/mL). After 3 days, proliferation was measured as described. Supernatants (100 μ L/well) were collected from the cultures stimulated with 100 μ g/mL Can f 1 after 3 days. The production of IL-4, IL-5, IL-10, IFN- γ , and IL-17 was measured by using commercial ELISA kits (DuoSet; R&D Systems, Minneapolis, Minn).

Statistical analyses

Statistical analyses were conducted by using GraphPad Prism (Graphpad Software, San Diego, Calif). The Mann-Whitney *U* test and Fisher exact test were used as indicated. *P* values of .05 or less were considered significant.

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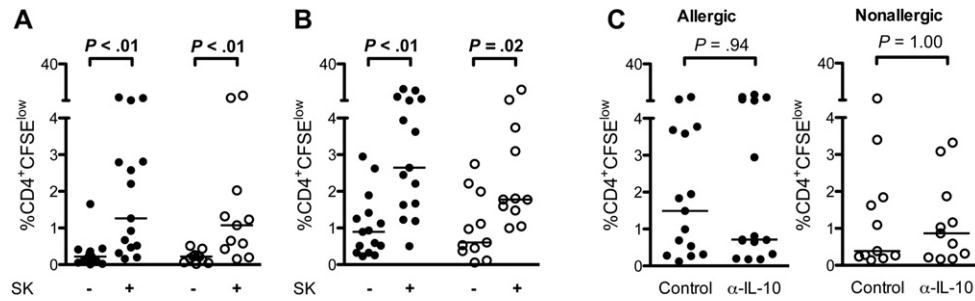


FIG E1. Proliferative responses of peripheral blood CD4⁺ T cells from subjects with (●) and without allergy (○) on stimulation with 200 U/mL streptokinase (SK) before (A) and after depletion of CD25^{hi} cells (B) and in the presence of an isotype control or anti-IL-10 antibody (10 μ g/mL; C). Results are expressed as the percentage of CD4⁺CFSE^{low} cells.

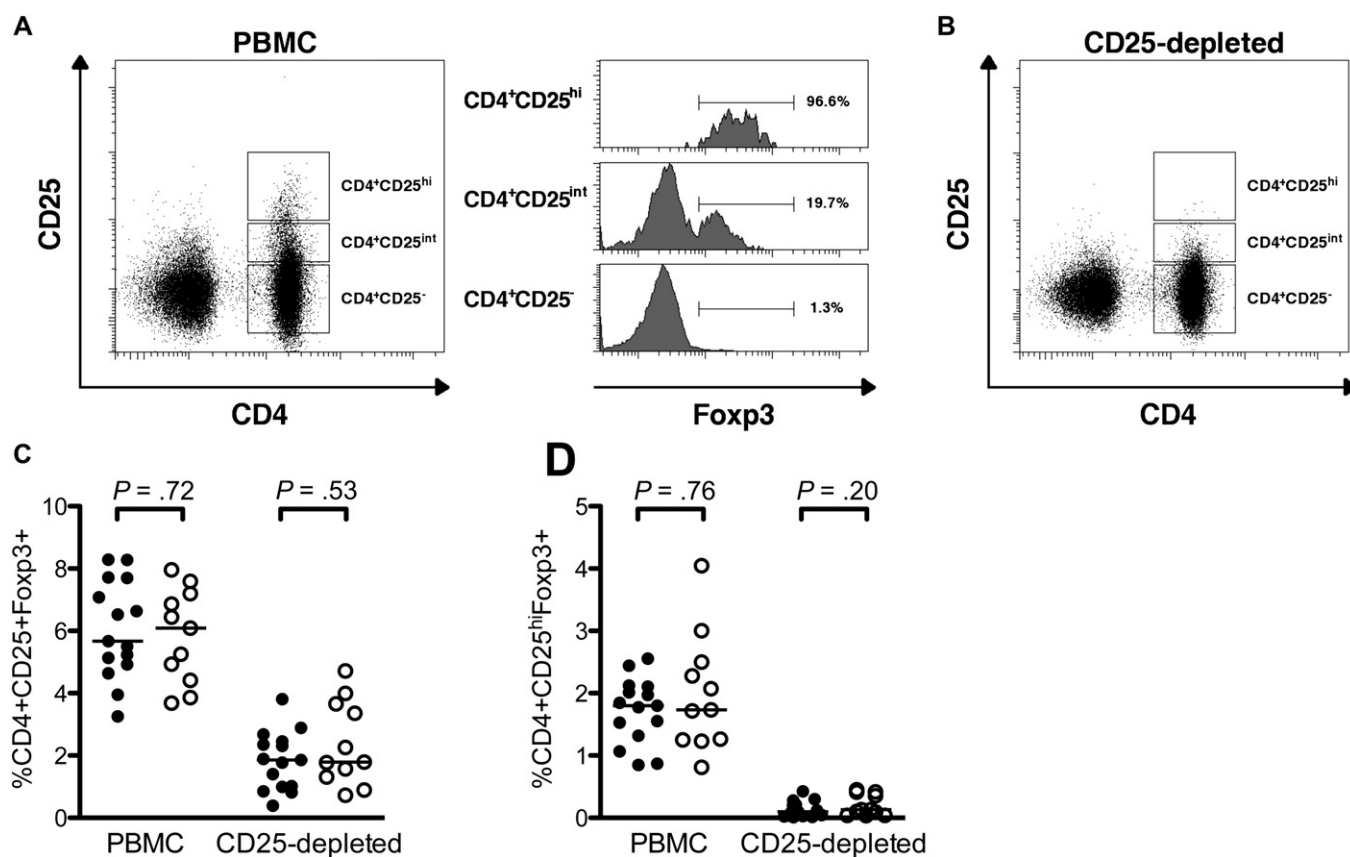


FIG E2. Identification of CD4⁺CD25⁺Foxp3⁺ Treg cells in PBMCs and their depletion. CD4⁺CD25⁻, CD4⁺CD25^{int}, and CD4⁺CD25^{hi} cells were identified as shown (**A**). CD4⁺CD25^{hi} cells were defined as those cells with a higher CD25 expression than that on the CD4⁻ population. Foxp3 expression within each population was assessed by intracellular staining. Depletion of CD25^{hi} by anti-CD25-coated magnetic beads (**B**). The percentages of CD4⁺CD25⁺Foxp3⁺ (**C**) and CD4⁺CD25^{hi}Foxp3⁺ (**D**) cells in the PBMCs of subjects with (●) and without allergy (○) before and after CD25 depletion.

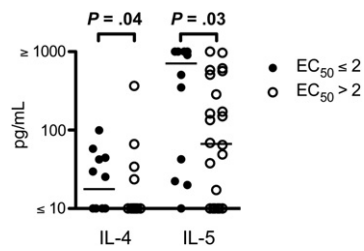


FIG E3. Production of IL-4 and IL-5 by higher-avidity ($EC_{50} \leq 2 \mu\text{g/mL}$, ●) and low-avidity ($EC_{50} > 2 \mu\text{g/mL}$, ○) T cells from subjects with allergy. Cytokine production was measured from the supernatants of cultures stimulated with $100 \mu\text{g/mL}$ Can f 1.