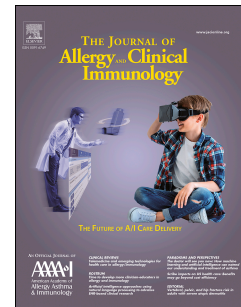


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Regulation of peanut-specific CD8⁺ T cells from nonallergic individuals.

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1 Title: Regulation of peanut-specific CD8⁺ T cells from nonallergic individuals.

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Short summary: Peanut-specific CD8⁺ T cells in nonallergic individuals are not deleted, but have an expansion block that can be released by impairing regulatory T cell associated signaling pathways.

Key words: human CD8⁺ T cells, food allergy, food immune tolerance.

To the Editor:

It is postulated that loss of immune tolerance to food can lead to food allergies. CD8⁺ T cells are known to participate in pathologic immune responses to food antigen, such as seen in eosinophilic esophagitis and celiac disease.¹⁻⁴ In light of this, we recently reported that peanut specific CD8⁺ T cells are increased in the blood of peanut-allergic individuals and express the T_H2 associated chemokine receptor CCR4.¹ We also identified an Ara h 1 derived peptide that triggered the activation of nearly one percent of CD8⁺ T cells from one HLA-A*02:01⁺ peanut-allergic individual, and derived Ara h 1 specific CD8⁺ T cell clones from the same person by *in vitro* expansion.¹

In this follow-up study, we first wanted to better characterize the phenotype of the peanut-specific CD8⁺ T cell clones described above in the setting of food allergy. Five Ara h 1 specific CD8⁺ T cell clones were incubated for 2 days with or without phytohemagglutinin (PHA), after which their cytokine expression profile was measured by BD cytokine bead array assay. Supernatants from all five clones contained increased levels of IL-5, IL-13, and GM-CSF after stimulation (Fig E1, A). The same cytokines were increased after a 5 day incubation with the Ara h 1 peanut peptide, but not a control peptide (Fig E1, B). Thus, peanut-specific CD8⁺ T cells may contribute to food allergy by becoming activated and expressing T_H2 cytokines after exposure to allergen. GM-CSF enables antigen cross presentation by dendritic cells, which would promote further allergen recognition by CD8⁺ T cells.⁵

The identification of a peanut epitope recognized in food allergy opened the opportunity to study the behavior of food-specific CD8⁺ T cells in *nonallergic* individuals. Doing so is challenging not only because one must first identify a food epitope that is recognized in disease, but the frequency of cells specific for a given food epitope is also low, making detection difficult. We addressed this issue by employing tetramer enrichment with HLA-A*02:01 tetramers loaded with the Ara h 1 peptide, and conjugated to phycoerythrin (PE).⁶ In this technique, tetramer⁺ cells are incubated with paramagnetic beads coated with anti-PE antibody, then enriched using a magnetized column before detection by flow cytometry. Peptide-specific cells at frequencies less than one cell per a million total CD8⁺ T cells can be detected by flow cytometry in this way.⁶

To determine if peanut-specific CD8⁺ T cells are present in nonallergic individuals, we performed tetramer enrichment on PBMCs from HLA-A*02:01⁺ blood donors from the Stanford Blood Center. The prevalence of peanut allergy is approximately 2.3% among adults, so it is unlikely that multiple donors are peanut allergic.⁷ We found that the frequency of Ara h 1 peptide-specific CD8⁺ T cells in eight different blood donors is approximately one cell in 10⁵ to 10⁶ total CD8⁺ T cells (Fig 1, A). We conclude that peanut-specific CD8⁺ T cells are not deleted (as a mechanism of immune tolerance) in peanut tolerant individuals and are found in peripheral blood at frequencies consistent with CD8⁺ T cells specific for other antigens, both foreign and self.⁶

To measure the activation of Ara h 1 specific CD8⁺ T cells in *nonallergic* individuals, we incubated tetramer enriched cells with autologous feeder cells, anti-CD28 antibody, and peanut peptide – the same conditions which previously activated nearly one percent of

CD8⁺ T cells from a HLA-A*02:01⁺ peanut allergic individual.¹ After one week, we measured the number of activated CD8⁺ T cells by the expression of CD25 and CD38. In five blood bank donor samples, the number of activated CD8⁺ T cells after stimulation was less than the original input of Ara h 1 specific CD8⁺ T cells (Fig 1, B). This poor activation is similar to what we have previously observed in CD8⁺ T cells specific for self antigen,⁶ and is consistent with the need for immune tolerance to both food antigens and endogenous antigens. We conclude that Ara h 1 specific CD8⁺ T cells are poorly activated by stimulation through the TCR and CD28 costimulatory receptor in nonallergic individuals, consistent with a mechanism of tolerance to food.

We hypothesized that CD4⁺ regulatory T cells (Tregs) might contribute to the suppression of Ara h 1 specific CD8⁺ T cells in nonallergic individuals. To test this, we mixed Ara h 1 specific CD8⁺ T cells with either whole autologous PBMCs (as before) or CD25-depleted, autologous PBMCs before stimulating with peanut peptide and anti-CD28 antibody (Fig 2, A). Because FOXP3⁺ Tregs are CD25^{hi}CD127^{lo}, removing CD25⁺ cells by bead depletion should lift suppression due to this cell type. An analysis of 8 blood donor samples showed two outcomes: in the case of 4 samples there was a 3.4 to 10.5-fold increase in the number of Ara h 1 specific CD8⁺ T cells when CD25⁺ cells were depleted; in the case of the other samples there was little change (Fig 2, A, middle and right). We conclude that in a proportion of individuals, CD25⁺ cells suppress the activation of Ara h 1 specific CD8⁺ T cells.

We sought to determine which intercellular signals could relieve the activation block in Ara h 1 specific CD8⁺ T cells. Tregs use multiple mechanisms to suppress other cell

types including IL-2 sequestration, the inhibitory coreceptor CTLA-4, and IL-10 secretion.⁸ To test these mechanisms in food specific CD8⁺ T cells, we added IL-2 (50 to 100U/ml) as well as blocking antibodies against CTLA-4 (20ug/ml) and IL10 (40ug/ml) to the standard *in vitro* assay of Ara h 1 CD8⁺ T cells, peanut peptide, anti-CD28 antibody, and autologous PBMCs (including CD25⁺ Tregs). Blocking IL-10 and CTLA-4 signaling while adding IL-2 resulted in robust proliferation of Ara h 1 specific CD8⁺ T cells from 8 blood bank donor samples, again consistent with a model where Tregs contribute to the suppression of peanut specific CD8⁺ T cells (Fig 2, B, left and middle, $p = 0.0078$).

Because previously we had shown that self-specific CD8⁺ T cells fail to expand after stimulation with peptide and anti-CD28 antibody,⁶ we tested whether self-specific CD8⁺ T cells would proliferate (as peanut specific CD8⁺ T cells do) after the addition of IL-2 combined with blocking antibodies to CTLA-4 and IL-10. We used tetramer enrichment to isolate CD8⁺ T cells specific for endogenous peptides derived from human preproinsulin (PPI). An analysis of 5 blood bank samples showed minimal expansion of self-specific CD8⁺ T cells even with the addition of IL-2 combined with blocking antibodies to CTLA-4 and IL-10 (Fig 2, B, left and right). We conclude that despite the need to develop CD8⁺ T cell tolerance to two parallel classes of antigens - food or endogenous – different mechanisms are used to achieve this goal.

In summary, these initial results show that Ara h 1 specific CD8⁺ T cells from nonallergic individuals are not deleted, but expand poorly after stimulation through the TCR and CD28 coreceptor. Their expansion is augmented in some individuals by the deletion of

CD25⁺ cells, and more generally by the addition of IL-2 combined with a block on CTLA-4 and IL-10. These interventions are consistent with a role for Treg cells. Interestingly, in one mouse food allergy model, blocking CTLA-4 during sensitization with peanut protein and cholera toxin enhanced the IgE response against Ara h 1, while CTLA-4 blockade combined with peanut exposure resulted in increased T_H2 cytokines.⁹ We propose a model where food antigen specific CD8⁺ T cells are susceptible to suppression by Tregs in an antigen independent manner via a combination of IL-10, CTLA-4 and IL-2 signaling. A breakdown of this tolerance mechanism would enable CD8⁺ T cells to augment an allergic response to food via the secretion of T_H2 cytokines and the cross presentation of food allergen. The expansion of this study to a larger population will provide additional confirmation of these mechanisms.

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Figure Legends:**FIG 1. A, Ara h 1 specific CD8⁺ T cells are not clonally deleted in *nonallergic* individuals.**

Tetramer enrichment using HLA-A*02:01 tetramers loaded with Ara h 1 peptide was performed on PBMCs from HLA-A*02:01⁺ blood bank donors, after which peanut (Ara h 1) tetramer⁺ CD8⁺ T cells were enumerated by flow cytometry. Each point represents one blood bank sample.

FIG 1. B, Poor activation of Ara h 1 specific CD8⁺ T cells in *nonallergic* individuals.

Left: Ara h 1 specific CD8⁺ T cells were isolated from a HLA-A*02:01⁺ blood bank donor by tetramer enrichment followed by FACS. Ara h 1 specific CD8⁺ T cells were incubated one week with Ara h 1 peptide (1.5ug/ml), anti-CD28 antibody (5ug/ml), and autologous PBMCs as feeder cells before analysis. Flow cytometry panels are gated on CD8⁺ T cells.

Right: Cumulative results from 5 blood bank samples. Each pair of points connected by a line represents one sample. In some cases, FACS was not performed and instead 1/11th of the tetramer enriched cells was analyzed by flow cytometry to calculate the number of Ara h 1 specific CD8⁺ T cells, and the remainder used for tissue culture. The p value for a 2 tailed Wilcoxon signed rank test was 0.0625, which is the minimum value possible for n=5.

FIG 2. A, Enhanced expansion of CD8⁺ T cells specific for Ara h 1 after the depletion of CD25⁺ cells from PBMC feeders in the case of some *nonallergic* individuals, but not others.

Left: Example depletion of CD25^{hi}CD127^{lo} CD4⁺ regulatory T cells from autologous PBMC feeder cells. Flow cytometry plots gated on CD4⁺ T cells.

Middle: CD8⁺ T cells specific for Ara h 1 were isolated by tetramer enrichment from a HLA-A*02:01⁺ blood bank donor as in Figure 1B. An equal number of Ara h 1 specific CD8⁺ T cells was then incubated for one week with cognate peptide, anti-CD28 antibody, and either whole autologous PBMCs or autologous PBMCs that had been depleted of CD25⁺ cells. Flow cytometry plots gated on CD8⁺ T cells.

Right: Cumulative results from 8 blood bank donors. Each pair of points connected by a line represents one sample. Blue points represent samples for which donors were confirmed peanut IgE negative. P value calculated by Wilcoxon signed rank test, 2 tailed.

FIG 2. B, CTLA-4 and IL-10 blockade combined with the addition of IL-2 promote the expansion of Ara h 1 (i.e., food specific) CD8⁺ T cells, but not self antigen specific CD8⁺ T cells.

Left: CD8⁺ T cells specific for Ara h 1 or human preproinsulin peptide(s) (PPI 2:10 and 15:24) were isolated by tetramer enrichment from a HLA-A*02:01⁺ blood bank donor. An equal number of enriched cells was then incubated with cognate peptides, anti-CD28 antibody, and autologous PBMCs (*without* CD25 depletion) +/- IL-2 with blocking antibodies to both CTLA-4 and IL-10. Flow cytometric plots gated on CD8⁺ T cells show analysis after one week.

246 **Middle and Right:** Cumulative results of CD8⁺ T cells specific for either Ara h 1
247 (middle) or PPI (right). Each pair of points connected by a line represents one blood
248 bank sample. Blue points represent samples for which donors were confirmed peanut
249 IgE negative. P value calculated by Wilcoxon signed rank test, 2 tailed.

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Figure E1 (peanut allergic).

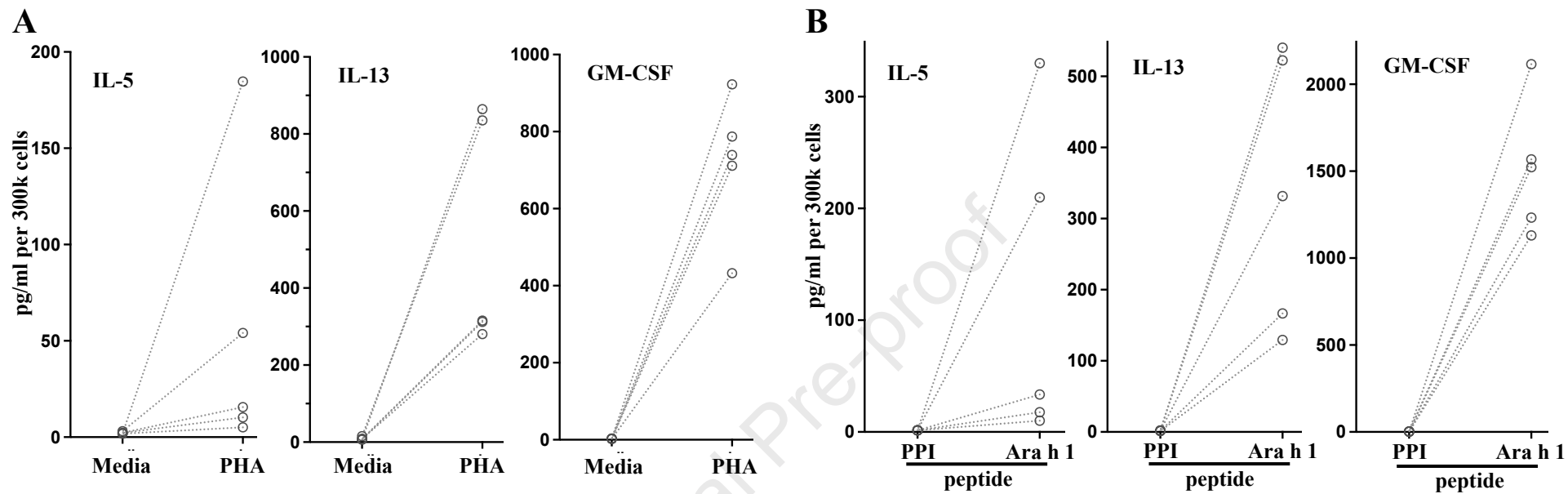


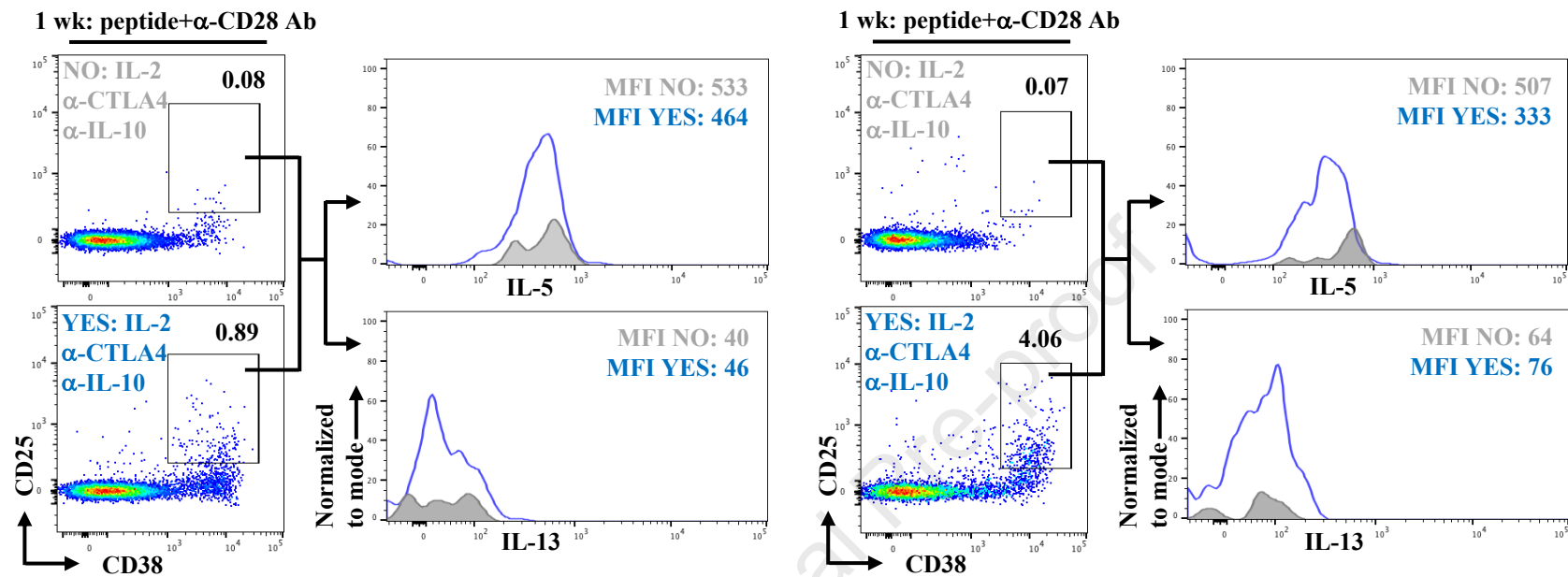
Figure E2 (*non*allergic).

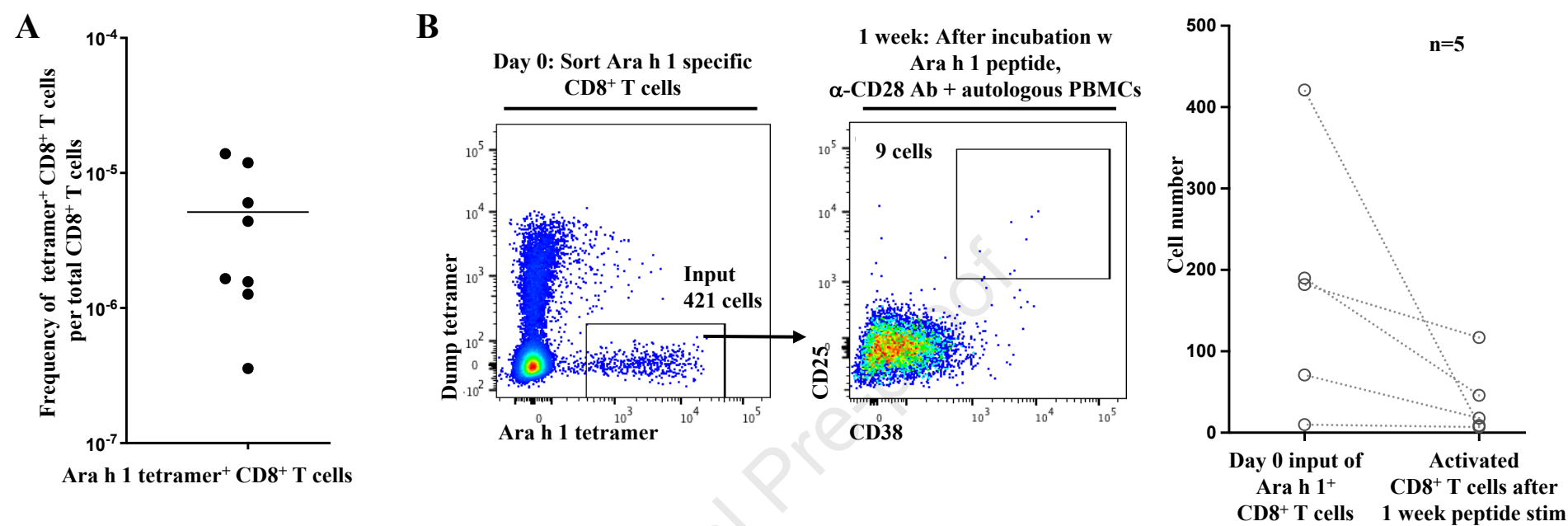
Figure 1 (*non*allergic)

Figure 2 (*nonallergic*)1 week: After incubation w peptide and α -CD28 Ab