Association of allergen-specific regulatory T cells with the onset of clinical tolerance to milk protein

Wayne G. Shreffler, MD, PhD,[a,b] Niya Wanich, MD,[a] Marla Moloney, MD,[a] Anna Nowak-Wegrzyn, MD,[a] and Hugh A. Sampson, MD[a,b] *New York, NY*

Background: About 70% of children with milk allergy tolerate extensively heated milk (HM) products and outgrow their allergy earlier than those who react to HM.
Objective: To test the hypothesis that HM-tolerant children have a higher precursor frequency of adaptive allergen-specific regulatory T (Treg) cells.
Methods: Allergic, HM-tolerant, outgrown, or control subjects were defined by oral food challenge. PBMCs were cultured with purified caseins and controls for 7 days, and proliferating $CD25^+CD27^+$ Treg cells were identified by flow cytometry. Proliferating cells were also characterized for their expression of FoxP3, CTLA 4, CD45RO, and CD127. Allergen-specific Treg cell origin and function were assessed by depletion of $CD25^{hi}$ cells before culture.
Results: There was a higher percentage (median [25th% to 75th%], 16.85% [7.1-31.7]) of proliferating allergen-specific $CD25^+CD27^+$ T cells from cultures of HM-tolerant subjects (n = 18) than subjects with allergy (n = 8; 4.91% [2.6-7.5]; $P <$ .01). Control subjects with no history of milk allergy (n = 7) also had low percentages of these cells (2.9% [2.4-6.0]), whereas outgrown subjects (n = 7) had intermediate percentages (9.0% [2.7-16.4]). There were no significant differences between the patient groups in the frequency of polyclonal Treg cells or allergen-specific effector T cells. Allergen-specific Treg cells were found to be $FoxP3^+CD25^{hi}CD27^+$, cytotoxic T lymphocyte–associated antigen 4^+, $CD45RO^+CD127^-$ and were derived from circulating $CD25^{hi}$ T cells. Depletion of the $CD25^{hi}$ cells before *in vitro* culture significantly enhanced allergen-specific effector T-cell expansion.
Conclusion: A higher frequency of milk allergen-specific Treg cells correlates with a phenotype of mild clinical disease and favorable prognosis. (J Allergy Clin Immunol 2009;123:43-52.)

Key words: *Treg, FoxP3, CTLA-4, immune tolerance, oral tolerance, food allergy, milk allergy*

Cow's milk allergy affects approximately 2.5% of young children. Most of these immune-mediated reactions are IgE-dependent, although predominantly T_H2-mediated cellular inflammatory conditions (eg, atopic dermatitis, allergic eosinophilic gastrointestinal disorders) are also common, and some patients have manifestations of both IgE-mediated and cell-mediated reactivity to milk antigens.[1] Despite this high prevalence, the majority of affected children achieve clinical tolerance spontaneously.[2-4]

Oral tolerance, whereby the immune system is rendered hyporesponsive to antigen after oral feeding, has been extensively characterized in animal models. Several distinct mechanisms have been proposed, including the induction of 1 or more populations of regulatory T (Treg) cells, generally associated with low antigen doses,[5] and either clonal anergy or deletion, associated with high dose tolerance.[6,7]

The relationship between oral immune tolerance and tolerance to food antigens in human beings is not well established. Several recent reports have supported the hypothesized role for Treg cells, including the $CD4^+CD25^+$ subset, in the development of normal tolerance or the spontaneous resolution of milk allergy.[8-10]

Thymus-derived $CD4^+CD25^+FoxP3^+$ Treg cells are well known to maintain self-tolerance. However, there is growing evidence that peripherally-induced, adaptive Treg cells regulate both T_H1 and T_H2 immune responses to exogenous antigens.[11,12] There has been an intensive effort to define this regulatory population better by flow cytometry. Reports, first of the expression of CD27, and subsequently of the lack of CD127 (IL-7Rα) expression, have demonstrated a correlation with FoxP3 expression and functional suppression.[13-17]

We investigated the relationship between the frequency of milk allergen–specific Treg cells and the phenotype of milk allergy in a group of 40 subjects characterized with respect to their clinical tolerance of extensively heated (HM)—that is, baked—or unheated (NHM) cow's milk. Patients were defined by strict clinical criteria as outgrown (previously reactive and now clinically nonreactive) , HM-tolerant (unreactive to baked milk products, eg, muffins, cakes, and so forth), allergic (reactive to all forms of milk products), or nonallergic control subjects.[18] HM-tolerant children have milder disease with a better prognosis than children

From [a]the Jaffe Food Allergy Institute, Division of Allergy and Immunology, Department of Pediatrics, and [b]the Immunology Institute, Mount Sinai School of Medicine.

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Reprint requests: Wayne G. Shreffler, MD, PhD, Division of Pediatric Allegry and Immunology, Department of Pediatrics, Box 1198, One Gustave L. Levy Place, New York, NY 10029. E-mail: wayne.shreffler@mssm.edu.

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Abbreviations used
CTLA: Cytotoxic T lymphocyte–associated antigen
HM: Heated milk
NHM: Nonheated milk
Treg: Regulatory T

with allergy, and we hypothesized that they represent a tolerizing phenotype.

We used *ex vivo* carboxyfluorescein succinimidyl ester (CFSE) dilution of allergen-stimulated or control-stimulated cells, which allowed us to identify milk allergen–specific T cells and compare the frequency of those cells that have a Treg phenotype between these pediatric patient groups. We extensively characterized the phenotype, parent population, and function of these expanded cells to show that they are derived from circulating allergen-specific adaptive Treg cells. We believe that these results offer strong evidence that allergen-specific Treg cells play a role in the development of clinical tolerance to a group of major food allergens in human beings, and we demonstrate the feasability of characterizing these allergen-specific cells in a pediatric study.

METHODS

Subjects

Subjects were recruited cross-sectionally from a larger clinical study on the natural history of milk allergy, in which they were characterized by placebo-controlled food challenges as outgrown (n = 7), having a positive history of milk allergy and milk-specific IgE but currently tolerant to both HM and NHM; HM-tolerant (n = 18), able to tolerate HM but not NHM; and allergic (n = 8), reactive to HM.[18] Control subjects (n = 7) with no history of milk allergy were also recruited from a separate study of egg allergy. HM-tolerant subjects were obtained both from initial baseline challenge (n = 7/18) and after introduction of a HM-containing diet (n = 11/18). Further subject characterization is provided in Table I. All research protocols were approved by the Mount Sinai Institutional Review Board, and informed consent was obtained for all subjects.

TABLE I. Study subject characteristics

Group	Diet	Age (y)	PST mm wheal	sIgE (kU/L)
Allergic	Avoiding	14.3	7.5	0.3
Allergic	Avoiding	13.0	NA	23.5
Allergic	Avoiding	8.9	8.0	2.0
Allergic	Avoiding	6.2	NA	13.0
Allergic	Avoiding	5.7	NA	101.0
Allergic	Avoiding	4.6	NA	54.6
Allergic	Avoiding	4.3	12.5	0.6
Allergic	Avoiding	3.3	0.0	0.3
Median		**5.7**	**7.8**	**2.0**
Control	Ingesting	8.2	NA	NA
Control	Ingesting	7.8	NA	NA
Control	Ingesting	7.4	0.0	0.3
Control	Ingesting	7.0	NA	NA
Control	Ingesting	7.0	3.0	NA
Control	Ingesting	6.8	NA	NA
Control	Avoiding	2.8	0.0	1.0
Median		**7.0**	**NA**	**NA**
HM-tolerant	Ingesting	12.8	10.0	2.7
HM-tolerant	Ingesting	10.3	6.0	1.4
HM-tolerant	Ingesting	9.1	4.0	1.3
HM-tolerant	Ingesting	8.8	5.0	7.2
HM-tolerant	Ingesting	8.0	11.0	4.6
HM-tolerant	Ingesting	6.0	8.0	0.4
HM-tolerant	Ingesting	6.0	8.0	3.7
HM-tolerant	Ingesting	5.9	6.0	2.1
HM-tolerant	Ingesting	5.5	7.0	3.4
HM-tolerant	Ingesting	5.5	4.0	1.2
HM-tolerant	Ingesting	5.1	7.0	12.4
HM-tolerant	Avoiding	10.9	11.0	28.5
HM-tolerant	Avoiding	8.6	7.0	0.7
HM-tolerant	Avoiding	7.2	6.0	13.9
HM-tolerant	Avoiding	5.8	11.0	NA
HM-tolerant	Avoiding	5.5	8.0	0.7
HM-tolerant	Avoiding	5.5	9.0	2.8
HM-tolerant	Avoiding	5.3	13.0	1.3
Median		**6.0**	**7.5**	**2.7**
Outgrown	Ingesting	18.3	10.0	0.8
Outgrown	Ingesting	15.2	NA	NA
Outgrown	Ingesting	8.7	6.0	1.0
Outgrown	Ingesting	7.6	NA	NA
Outgrown	Ingesting	6.3	4.0	2.5
Outgrown	Ingesting	3.0	NA	0.6
Outgrown	Avoiding	2.4	0.0	NA
Median		**7.6**	**5.0**	**0.9**

PST, Prick skin test (mm wheal); *sIgE,* milk-specific IgE; *NA,* not available.

CFSE-based characterization of antigen specific phenotype

PBMC samples were labeled with 5 μmol/L CFSE[19] and cultured for 7 days with media alone (AIM-V, Invitrogen, Carlsbad, Calif), 50 μg/mL purified α-caseins, β-caseins, and κ-caseins (Sigma, St Louis, Mo), or anti-CD3/CD28 beads (Dynal, Carlsbad, Calif). Cultures were supplemented from day 0 with recombinant human IL-2 (20 U/mL; R&D Systems, Minneapolis, Minn) and expanded as necessary. The phenotype and proliferation were characterized by flow cytometry by using mAbs for CD3 (Sk7), CD4 (Sk3), CD25 (M-A251), CD27 (M-T271), CD127 (hIL-7R-M21), and CD45RO (UCHL1; all BD Biosciences, San Jose, Calif), and ethidium monoazide bromide was used as a live/dead discriminator (Molecular Probes, Carlsbad, Calif). For FoxP3 staining, surface staining was performed first, followed by fixation/permeabilization and staining (PCH101) as per the manufacturer's protocol (eBioscience, San Diego, Calif). Fluorescence data were acquired on an LSR II running Diva 3.0 software (BD Biosciences). Raw data were analyzed using FlowJo 8.5.3 (TreeStar, Ashland, Ore).

CD25 depletion

Where indicated in the Results, PBMCs were CFSE-labeled and then partially depleted of $CD25^+$ cells by incubation with anti-CD25 paramagnetic beads as per the manufacturer's protocol (StemCell Technologies, Vancouver, British Columbia, Canada) modified to use half of the anti-CD25 reagent to deplete the highest CD25-expressing cells semiselectively. By using this protocol, we typically depleted approximately 50% of the $CD25^{hi}$ CD4 T cells (see Fig E1 in this article's Online Repository at www.jacionline.org). Control PBMCs were mock-treated by carrying through the same protocol without the addition of anti-CD25 reagent. Proliferation and phenotype were assessed by flow cytometry as discussed. Cells were then cultured as discussed with the addition of a tetanus toxoid control (5 μg/mL).

Proliferation assays

PBMCs from serial HM-tolerant subjects were cultured as discussed in AIM-V media with 50 μg/mL purified α-caseins, β-caseins, and κ-caseins (Sigma) and 20 U/mL recombinant human IL-2 (R&D Biosystems). After peak proliferation (day 10-11), $CD25^+$ cells were selected by using anti-CD25 paramagnetic beads (StemCell Technologies) as described. These cells were cultured in triplicate (5000, 10,000 or 20,000 per well in 96-well *U*-bottom plates; Falcon, San Jose, Calif) with autologous PBMCs that had been pulsed

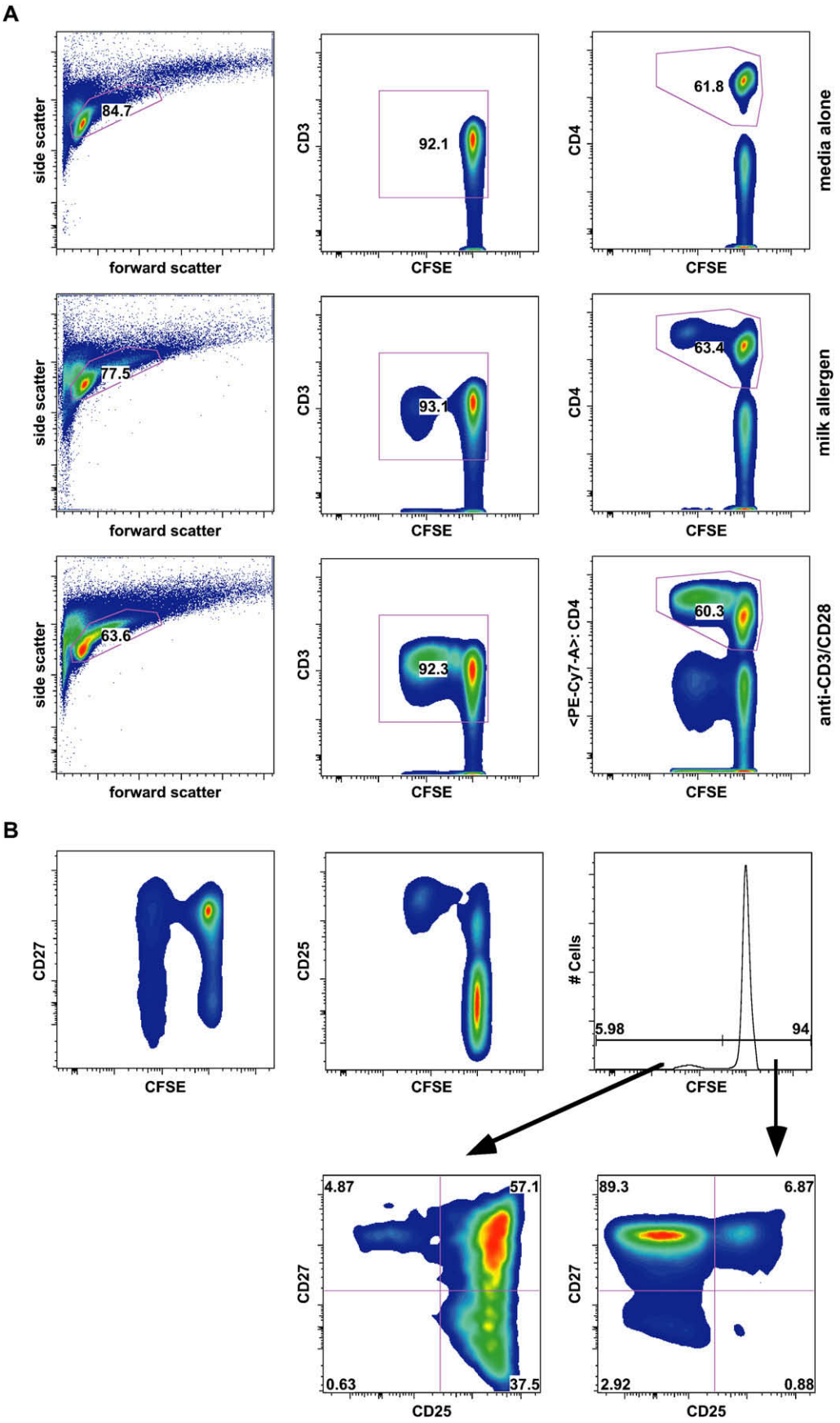

FIG 1. Gating strategy for the identification of $CD25^{+}CD27^{+}$ putative Treg cells. **A**, Each column shows successively gated populations (ethidium monoazide bromide negative gate for exclusion of dead cells not shown). Stimulation conditions: IL-2 alone *(top row)*, IL-2 plus caseins *(middle)*, or anti-CD3, anti-CD28 *(bottom)*. **B**, CD25 and CD27 expression of live $CD3^{+}CD4^{+}$ events. Data shown are from a HM-tolerant subject.

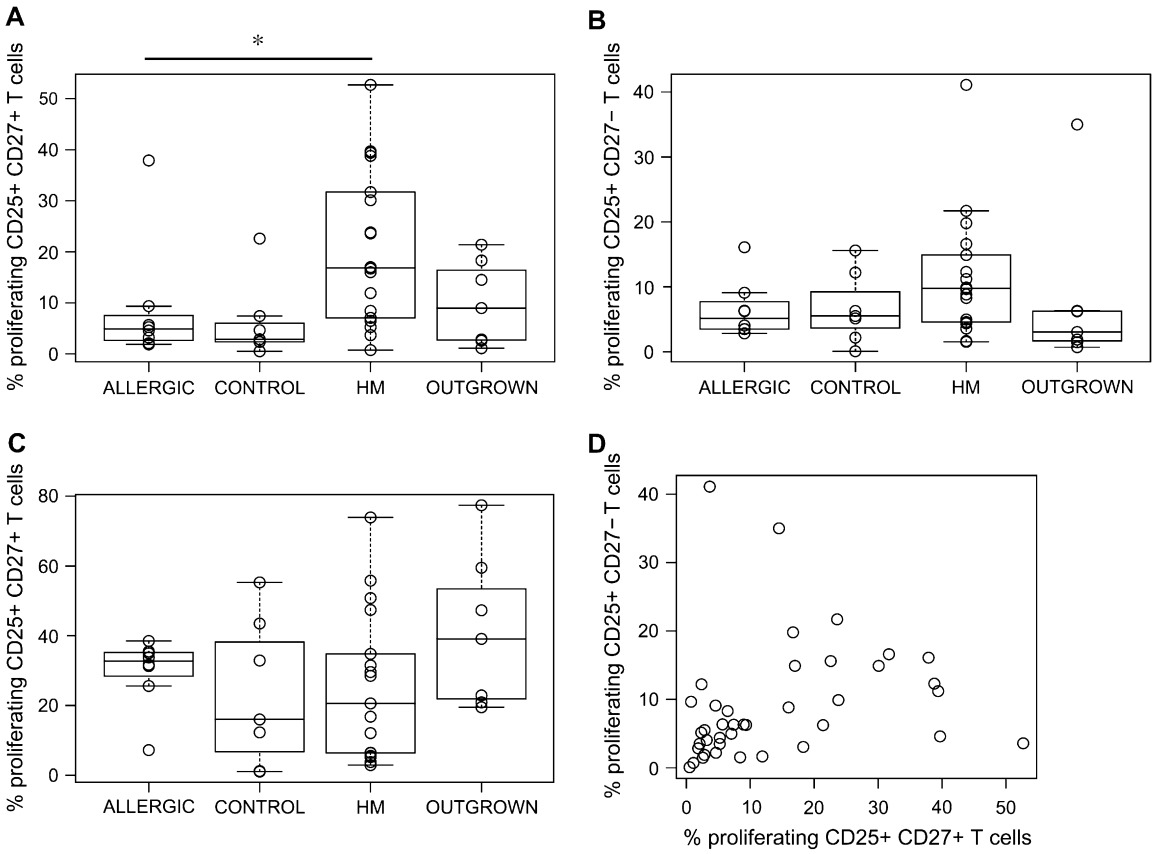

FIG 2. HM-tolerant patients have higher frequencies of $CD25^+CD27^+$ allergen-specific T cells. **A**, Percentage of live $CD3^+CD4^+$ events by clinical phenotype that are $CFSE^{low}CD25^+CD27^+$ (Treg) after 7-day culture with milk allergens. $*P = .0094$. Percentage of live $CD3^+CD4^+$ events by clinical phenotype that are $CFSE^{low}CD25^+CD27^-$ (T effector) after 7-day culture with (**B**) milk allergens or (**C**) anti-CD3/-CD28 mAbs. D, Percentage of live $CD3^+CD4^+$ Treg vs T-effector cells.

overnight with 50 μg/mL purified α-caseins, β-caseins, and κ-caseins (Sigma) irradiated and cryopreserved at day 0 (5000/well) and allogeneic $CD4^+$ T cells (10^5/well; >95% purity from magnetic bead selection; StemCell Technologies). Control cultures lacked either Treg cells or allogeneic $CD4^+$ responder cells. On day 5, ^{3}H-thymidine was added for 16 hours. Results were analyzed from experiments with an adequate control allogeneic response (cpm > 20,000; n = 4).

Statistical analyses

Proliferation index, defined as the product of the percentage of proliferating cells and the average number of divisions, was calculated by using the proliferation analysis platform of FlowJo 8.5.3 (TreeStar). Graphical display and statistical analyses were performed by using R analysis 2.5.0 (www.R-project.org). For comparisons of phenotype percentages and proliferation index, the unpaired or paired Wilcoxon rank-sum test was used for between-group comparisons or pre/post–CD25 depletion analysis, respectively. R analysis script and raw data are available in this article's supplementary text in the Online Repository at www.jacionline.org.

RESULTS

Heated milk–tolerant subjects have a higher frequency of casein-specific $CD25^+CD27^+CD4^+$ T cells in circulation. Casein-induced proliferation measured at day 7 was almost entirely restricted to $CD4^+$ T cells (see Fig 1 for example). The majority of these cells were $CD25^+$, whereas coexpression of CD27 varied greatly between patients (see this article's Table E1 in the Online Repository at www.jacionline.org). We observed that the frequency of proliferating CD25, CD27 double positive ($CFSE^{low}CD3^+CD4^+CD25^+CD27^+$) T cells from HM-tolerant subjects' PBMCs, while variable, was significantly greater than that of other patient groups (Fig 2, *A*). This difference was greatest for the HM-tolerant group compared with either subjects with allergy or control subjects ($P < .01$ for both), whereas in comparison with the outgrown group, the difference was smaller but still significant ($P < .05$). There was no significant difference among the other groups. This was not the case for $CFSE^{low}CD25^+CD27^-$ (Fig 2, *B*) or other non-Treg populations ($CFSE^{low}CD25^-CD27^+$ or $CFSE^{low}CD25^-CD27^-$; Table E1). There was also no difference in the frequency of $CD25^+CD27^+$ T cells proliferating to polyclonal control stimulation (anti-CD3 and anti-CD28) among any of the groups (Fig 2, *C*). Proliferation from IL-2 media–alone control conditions was negligible (less than 1% $CFSE^{low}CD3^+CD4^+CD25^+CD27^+$ of total $CD3^+CD4^+$ for all subjects), and there were no differences between groups (data not shown). There was no correlation observed between the proliferation of $CD25^+CD27^-$ and $CD25^+CD27^+$ T cells (Fig 2, *D*).

Unexpectedly, there was no significant difference in the frequency of $CD25^+CD27^+$ T cells between the allergic and control groups. In addition, the outgrown subjects were found to have

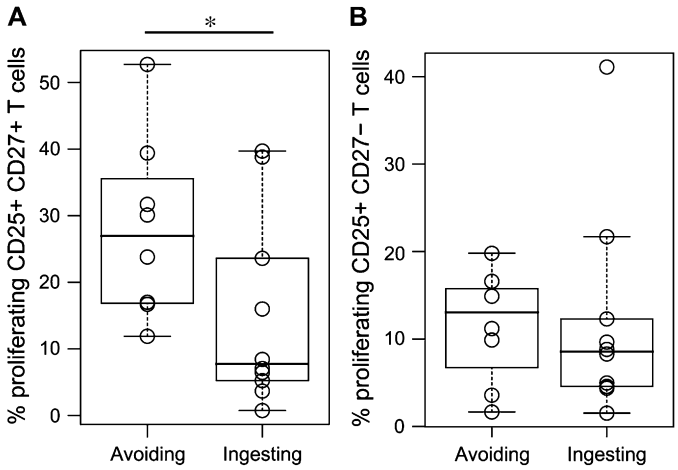

FIG 3. HM-tolerant patients without dietary milk exposure have the highest Treg cell frequency. Percentage of live $CD3^+CD4^+$ events by milk diet status within the HM-tolerant group that are Treg cells ($CD25^+CD27^+$) (**A**), or T effector cells ($CD25^+CD27^-$) (**B**) after 7-day culture with milk allergens. $*P = .022$.

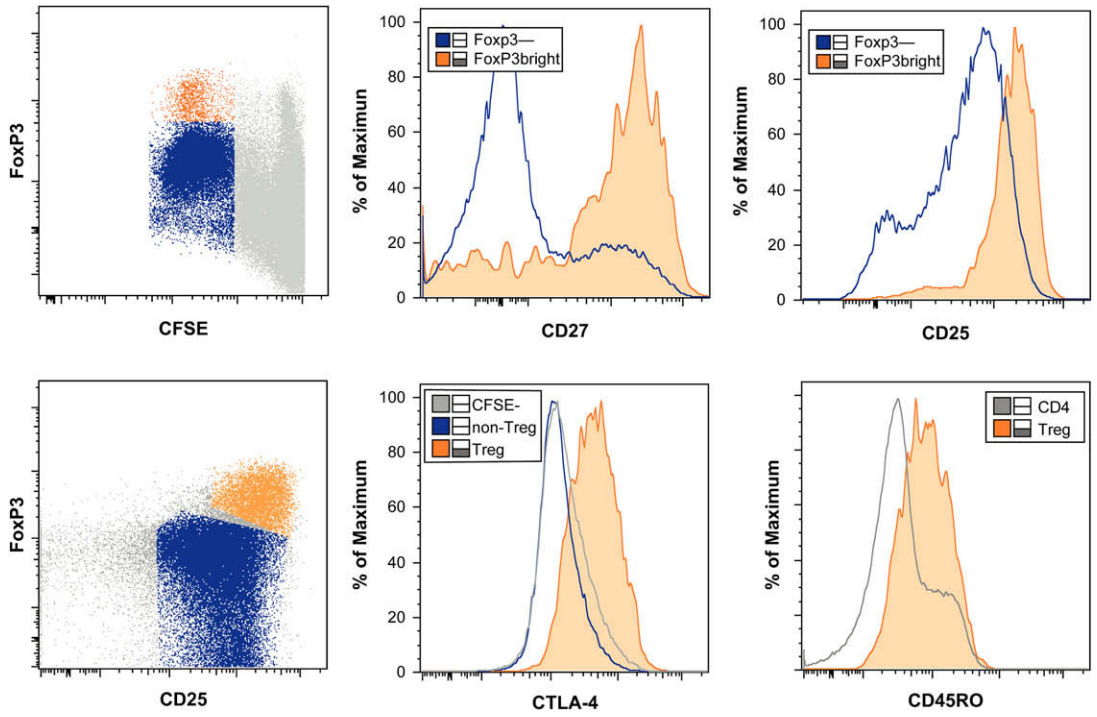

FIG 4. $FoxP3^{hi}$ Treg cells are memory $CD25^{hi}$ $CD27^+CTLA\text{-}4^+$ Overlay contour graphs showing $CFSE^{low}$ T-effector cells in *blue* and $FoxP3^{hi}$ cells in *orange*.

a frequency of $CD25^+CD27^+$ proliferation that was intermediate to the HM-tolerant subjects and subjects with allergy (Fig 2, *A*), suggesting that the high frequency of Treg cells may be transient. Interestingly, 1 of the 7 nonallergic control subjects who was an outlier (>3-fold higher frequency of $CD25^+CD27^+$ than any other individual in that group) was a child who did not ingest milk protein by parental preference, suggesting that the presence of dietary milk protein may be an important variable affecting the frequency of Treg cells in the peripheral blood.

Therefore, we tested the hypothesis that inclusion of dietary milk protein is associated with a lower frequency of casein-specific $CD25^+CD27^+$ T cells present in peripheral blood. Subjects from the HM-tolerant group who were either avoiding or ingesting milk in their diet were compared (Fig 3). We found that expansion of $CD25^+CD27^+$ T cells was significantly greater in the subjects who had just passed challenge, before inclusion of milk into their diet, than from those who had been ingesting HM products for 3 months or more ($P < .05$). Consistent with this observation, the difference between the HM-tolerant and allergic groups was more significant ($P < .001$) when only subjects excluding milk were compared (7 of 15 HM-tolerant and 7 of 7 with milk allergy).

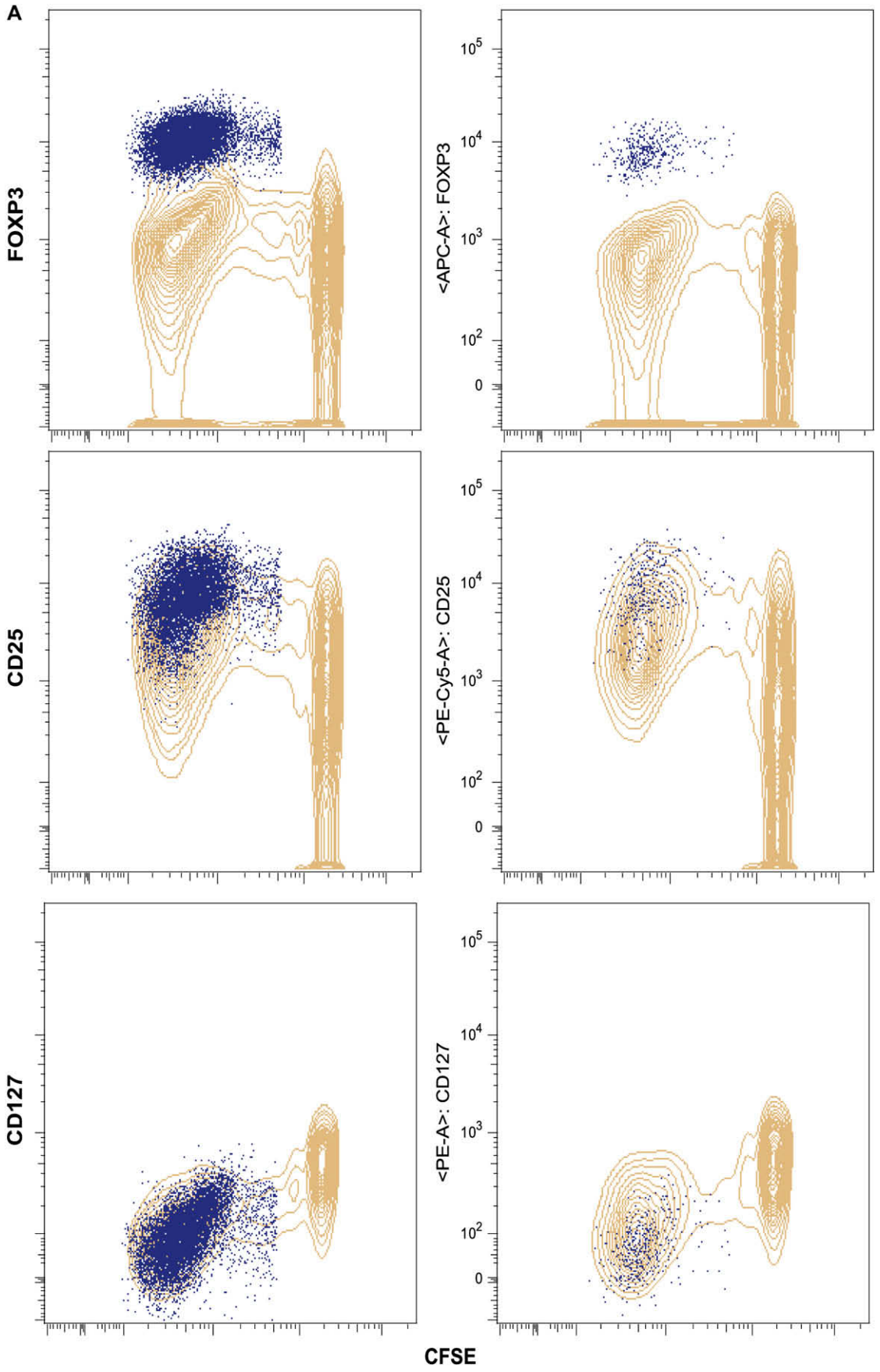

FIG 5. *(Continued)*

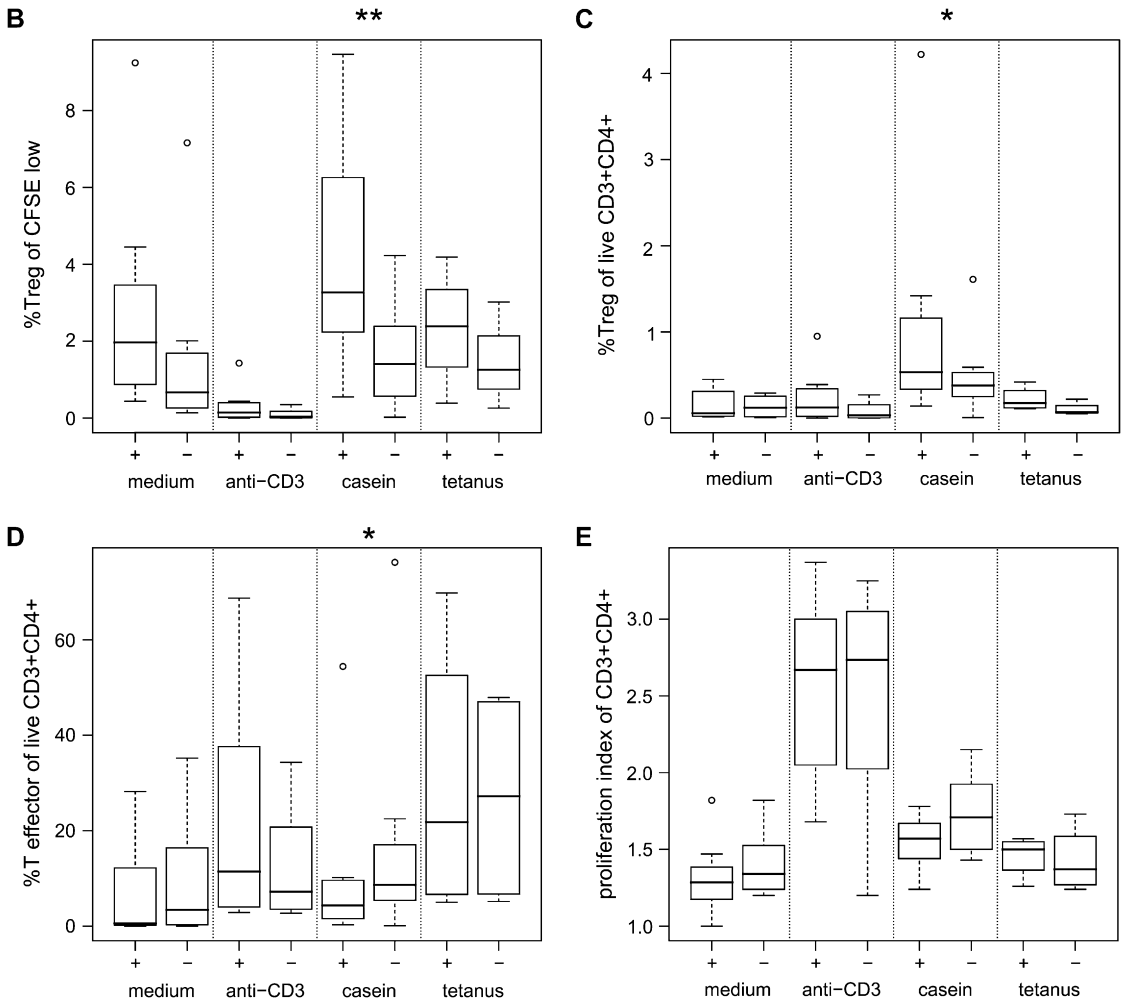

FIG 5. *In vitro* expansion of FoxP3^{hi} is from the circulating CD25^{hi} cell population, and their depletion enhances proliferation of effector T cells to casein. **A**, Overlay of CFSE^{low}FoxP3^{hi}CD25^{hi}CD127^{low} cells *(blue)* on total live CD4 T cells *(gray)* after 7-day culture of either mock-treated *(left column)* or CD25-depleted *(right column)* PBMC with milk antigens. **B-E**, Summary comparison of 7-day HM-tolerant PBMC culture mock treated (+) or CD25-depleted *(−)* and cultured with medium, anti-CD3/CD28, caseins, and tetanus toxoid as indicated (n = 8). **B**, Percent Treg cells of CFSE^{low} live CD4 T cells. *P = .015. **C**, Percent Treg cells of total live CD4 T cells. **D**, Perccent T-effector cells of total live CD4 T cells. **E**, Proliferation index (the average number of cell divisions that a cell in the original population has undergone, including the undivided cells).

Allergen-specific Treg cells are CD25$^+$CD27$^+$ cytotoxic T lymphocyte–associated antigen 4$^+$ CD45RO$^+$

We next characterized the phenotype of FoxP3-expressing cells from a subset of subjects for expression of other reported markers of the CD4$^+$CD25$^+$ Treg cells, including those known to be directly regulated by FoxP3, such as cytotoxic T lymphocyte–associated antigen (CTLA)–4, glucocorticoid-induced TNF-related protein (GITR), and CD127.[16,17,20] Consistent with recent reports of FoxP3 expression in activated T cells,[21] we observed that the expression of FoxP3 in casein-specific, proliferating cells was bimodal with dim expression in many cells and bright expression in a smaller distinct population. Compared to the total allergen-specific CD4 population, FoxP3^{hi} cells were unique for the highest levels of CD25 and CTLA-4 and coexpression of CD27$^+$ (Fig 4). In contrast, all casein-specific cells (CFSE^{low}) were predominantly CD45RO$^+$CD127$^-$, regardless of FoxP3 expression (Figs 4 and 5, *A*). We did not detect increased GITR expression on FoxP3$^+$ cells (data not shown).

Ex vivo expanded Treg cells are derived from circulating CD25$^+$ cells

We hypothesized that CFSE^{low} cells with a regulatory phenotype from cultured PBMCs are derived from committed Treg cells—that is, present in the circulating CD25$^+$ population. Alternatively, expression of Treg markers could have been induced *in vitro* with potentially no relationship to the precursor frequency of committed antigen-specific Treg cells.

To test this, HM-tolerant patient PBMCs (n = 8) were cultured with or without previous partial depletion of CD25$^+$ cells. CD25^{hi} cells were reduced by 40% to 60% as assessed by flow cytometry (see this article's Fig E1 in the Online Repository at www.jacionline.org). PBMCs were CFSE-labeled as discussed, split for CD25 depletion or mock treatment, and then cultured under identical conditions. Depletion of CD25$^+$ cells significantly reduced the percentage of CFSE^{low} putative Treg cells present after culture with milk antigens (Fig 5). As a percentage of dividing CD3$^+$CD4$^+$ cells in response to casein, FoxP3^{hi} CD25^{hi} CD127$^-$ cells were reduced from a median of 3.3% (range,

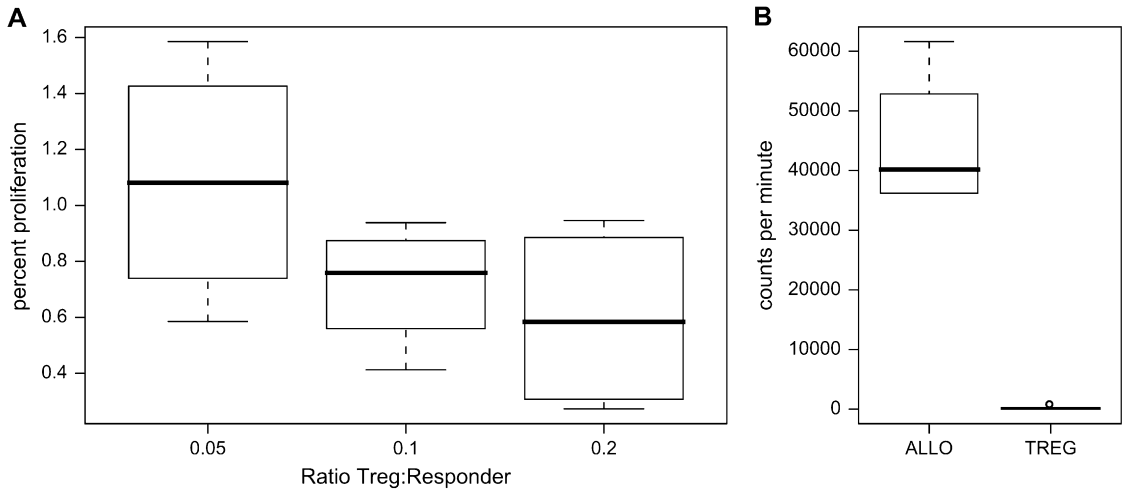

FIG 6. Casein-expanded $CD25^{hi}$ cells suppress CD4 T-cell proliferation to alloantigen. **A**, $CD25^{hi}$ cells, after expansion to caseins from HM-tolerant subject PBMCs, were cultured with autologous APCs and allogeneic $CD4^+$ T cells at ratios of 1:20, 1:10, and 1:5. **B**, Control cultures with APCs and allogeneic $CD4^+$ responder cells alone *(ALLO)* or APCs and Treg cells alone *(TREG)*; n = 4, $P < .1$ for difference in cpm of proliferation of $CD4^+$ responder cells alone versus with 1:5 Treg cells (paired Wilcoxon rank-sum test, $P = .0625$).

0.55-9.5) to 1.4% (0.02-4.2) as a result of predepletion of $CD25^{hi}$ (Fig 5, *B*; $P < .01$). This decrease was also significant as a percentage of all $CD3^+CD4^+$ cells (Fig 5, *C*; median, 0.54 versus -0.38; $P < .05$).

$CD25^+CD27^+$ T cells are functionally suppressive. Although the depletion of $CD25^+$ cells before *in vitro* culture markedly decreased the expansion of putative Treg cells, milk allergen–specific proliferation within the T-effector populations was enhanced (Fig 5, *D*). The frequency of casein-specific effector T cells ($FoxP3^-CD25^+$) increased from a median of 4.3 to 8.6% of total $CD3^+CD4^+$ ($P < .05$). There was also a trend toward an increased casein-specific CD4 T-cell proliferation index as a result of CD25 depletion ($P = .1$). There was no correlation between Treg cell and T-effector proliferation in undepleted cultures (see this article's Fig E2 in the Online Repository at www.jacionline.org).

CD25 depletion appeared to reduce the frequency of expanded Treg cells for all stimulation conditions (Fig 5, *B and C*); however, the effect was greatest for the casein-stimulated cultures, and there was not a consistent enhancement of T-effector proliferation to other stimulants after $CD25^{hi}$ depletion (Fig 5, *D and E*).

Casein-expanded $CD25^{hi}$ T cells are anergic and suppressive

To evaluate further whether the casein-expanded $CD25^{hi}$ T cells are Treg cells, increasing numbers of $CD25^{hi}$ T cells (5×10^3, 1×10^4, or 2×10^4) from consecutive HM-tolerant subjects were selected after expansion and cocultured with irradiated autologous antigen-presenting cells and 10^5 allogeneic responder CD4 T cells, resulting in ratios from 1:20 to 1:5 $CD25^{hi}$ to responder CD4 allogeneic T cells. Casein-expanded $CD25^{hi}$ cells suppressed the mixed lymphocyte response of responder CD4 T cells in a dose-dependent manner (median proliferation 60% at 1:5 Treg cell to responder ratio; Fig 6, *A*; n = 4). Casein-expanded $CD25^{hi}$ Treg cells were anergic when stimulated with casein-pulsed autologous APCs, whereas allogeneic CD4 responder cells proliferated strongly (Fig 6, *B*).

DISCUSSION

We have shown in this study that before introduction of baked milk products, HM-tolerant subjects have a higher frequency of milk-specific $CD25^+CD27^+$ cells in their peripheral blood than those children who experience allergic symptoms to all forms of milk protein. These markers define a population that includes $FoxP3^{hi}CTLA\text{-}4^+CD127^-$ cells that are present in the resting, constitutively $CD25^+$ cells in circulation. Their depletion causes the enhanced proliferation of allergen-specific, effector T-cell proliferation, demonstrating that they are functionally suppressive. Finally, they are anergic and capable of suppressing the proliferation of $CD4^+$ T cells in a mixed lymphocyte response.

Several reports have addressed the potential role of regulatory T cells, including $CD25^+$ Treg cells, in the regulation of allergic disease generally,[11,22] and milk allergy specifically.[8-10,23] Most findings of regulatory T-cell activity in allergic disease come from studies of immunotherapy rather than spontaneous tolerance, and the most convincing data suggest a role for the IL-10–secreting, so-called T regulatory type 1(Tr1), subset of regulatory T cells.[22,24]

Consistent with the importance of this subset for milk tolerance as well, Tiemessen et al[10] have reported increased IL-10 secretion from milk-specific T-cell clones from atopic children with tolerance of milk. The potential role of IL-10 in the resolution of milk (and other) allergy is also indirectly supported by the observation made by many investigators that an elevation of IgG_4 (and ratio of IgG_4/IgE) is associated with tolerance and that *in vitro* IL-10 can modulate T_H2 cytokine-induced B-cell class switching in favor of IgG_4 over IgE.[25-27] The relationship, if any, between the $CD25^+$ Treg cell subset and Tr1 cells in these patients is unknown. We were unable to detect a clear IL-10–positive T-cell population by intracellular cytokine staining in pilot experiments with milk-tolerant subjects (data not shown).

Karlsson et al[8] have implicated the $CD25^+$ Treg cell in the resolution of milk allergy. They found that PBMCs from children with allergy who tolerated reintroduction of milk after an avoidance period of 2 or more months had higher percentages of total

$CD4^+CD25^+$ directly *ex vivo* and were less proliferative to milk allergen than their counterparts who still had allergy. They also found that depletion of $CD25^+$ cells before culture enhanced proliferation, although this was seen only after reintroduction of milk into the diet. Comparison of that study to our own is complicated by the fact that patients with IgE-mediated milk allergy were specifically excluded in the former study. Furthermore, those authors measured responses only to β-lactoglobulin, an allergen we specifically avoided because of its well known heavy contamination with endotoxin.[9,28] Taken together with a more recent study specifically examining children with IgE-mediated milk allergy, which showed that CD25-depletion enhanced milk allergen–specific proliferation from tolerant children but not children with allergy,[9] the data do support a role for this subset in the milk allergen–specific T-cell response and its regulation.

In contrast, Tiemessen et al[23] did not find a functional defect of $CD25^+$ cells in individuals with milk allergy. They compared the proliferation to both milk allergen and anti-CD3 in the $CD25^+$ and $CD25^-$ subsets between controls with and without milk allergy and found that proliferation to both milk allergen and anti-CD3 was equally enhanced regardless of milk-allergic status. We also found that CD25 depletion enhanced milk allergen–induced proliferation of effector T cells of patients with allergy (see this article's Fig E3 in the Online Repository at www.jacionline.org), although we do not know whether this enhancement is less pronounced in patients with allergy compared with HM-tolerant individuals. Our data suggest that there may be no functional defect of the $CD25^+$ Treg-cell subset in individuals with allergy, but that a higher frequency of these cells is associated with tolerance. Our findings, that HM-tolerant individuals actively ingesting milk allergen have peripheral Treg-cell frequencies that are similar to subjects with allergy, are potentially consistent with Tiemessen's data.[23] However, as we show here, the interpretation of bulk assays (eg, proliferation by thymidine incorporation) is complicated by the differential effects on distinct cell populations. Enhancement of T-effector cell proliferation was much more significant than the overall change in CD4 proliferation (Fig 5). Furthermore, the interpretation of functional differences in any PBMC assay is complicated by the unknown relationship between changes in circulating populations versus other potentially more relevant anatomical compartments.

Interestingly, associated with the introduction of milk into the diet, we found a decrease in the frequency of Treg cells present in the peripheral blood that was concomitant with an increasing IgG_4/IgE ratio[18] and declining basophil response (N. Wanich, A. Nowak-Wegrzyn, H. A. Sampson, W. G. Shreffler, unpublished data, October 2008). Although it must be emphasized that this is a cross-sectional study, the interpretation that this change is driven by the introduction of dietary antigen is also consistent with the intermediate frequency of Treg cells seen in children who recently outgrew milk allergy and with the observation of a high Treg-cell frequency seen in the 1 of 7 controls without milk allergy who weas avoiding milk. However, the avoidance of dietary milk alone is not the sole cause of higher circulating Treg-cell frequency, because children with milk allergy who are also strictly avoiding the antigen have a low frequency of Treg cells as a group. We hypothesize that after the ingestion of milk antigen, Treg cells are recruited into the gastrointestinal tract by the presence of antigen. One paradoxical question raised by these findings is how such a milk-specific population might be induced or expanded in children who are actively avoiding the antigen. However, intermittent low-dose exposure of such a ubiquitous antigen is highly likely, and the subsequent anatomical compartmentalization of any induced adaptive memory Treg cells would be expected to include the peripheral blood from which these cells could be recruited to tissue after antigen exposure.

The major advantage of the CFSE-based methodology used here is in the identification of antigen-specific cells. Multiple studies have now demonstrated that Treg cells may be expanded *in vitro* in an IL-2–dependent manner with stable expression of the Treg cell phenotype.[14,29] However, it is also known that the specificity of many markers associated with Treg cells is poor because they may be upregulated by activation—a feature that is now known to extend to FoxP3 expression in human cells.[21] By depleting the $CD25^{hi}$ population before culture, we have shown that the $CFSE^{low}$ Treg cell population characterized in this study is derived from circulating committed Treg cells and not induced *in vitro* from T effectors. Our results demonstrating overlap of $CD25^+CD27^+$ expression with other markers of Treg cells also confirm other findings. Ruprecht et al[15] found that ~40% of $CD25^+CD27^+$ expressed high levels of FoxP3 and were suppressive, whereas $CD25^+CD27^-$ cells were not. They also demonstrated that cultured $CD25^+CD27^+$ maintained a regulatory phenotype.

We believe our observation of bimodal FoxP3 expression in the proliferating population is consistent with the literature that activation may induce low-level FoxP3 expression[21] and hypothesize that the $FoxP3^{hi}$ subset, which also has the highest CD25 expression, has the highest CTLA-4 expression, and is preferentially affected by our CD25 depletion experiments, represents the true regulatory population that is expanding from a circulating $CD25^+$ Treg-cell population. Based on oral tolerance models in mice, this population is most likely a TGF-β–dependent, antigen-induced population, distinct from the thymus-derived natural Treg cells.[30]

We hypothesize that individuals who are tolerant to HM products represent a milder milk allergy phenotype with evidence of greater immune regulation, such as elevated specific IgG_4, lower specific IgE, and skin tests and basophil reactivity. Anecdotal observations and early longitudinal data[18] suggest that HM-tolerant children outgrow their milk allergy earlier than children who react to HM. The present observation that HM-tolerant subjects have a higher frequency of allergen-specific T cells fits this pattern.

Clinical implications: Milk allergen–specific regulatory T cells may play a role in the resolution of milk allergy and therefore may be important targets for therapy and immune monitoring.

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APPENDIX.

Analysis script

```
# Copyright 2008, Wayne G. Shreffler. Free non-comercial use is welcome.
# This is a R analysis script for analysis of data set produced in collaboration with Anna Nowak-Wegrzyn, Hugh A. Sampson and others.
#R is by the R Foundation for Statistical Computing and is freely available at http://www.R-project.org. R Development Core Team (2006). R: A language and
  environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL.
# THIS WILL RUN WITHOUT MODIFICATION ON OS X but will require editing of pipe functions and substitution of quartz functions (e.g. with 'pdf') for
  use on Windows OS.
# Direct questions or suggestions about this script to the author at wayne.shreffler@mssm.edu.
#functions
BATCH <-function(directory, exportname="", export=TRUE) {
dir <- readLines(pipe(paste("ls", directory)))
pathname <- paste(directory, "/", sep="")
FILENUM <- 0
non_data <- 0
cv <- function(x) 100*((sqrt(var(x)/length(x)))/mean(x)) #define CV
non_Ag <- 0
cat("processing . . .\n")
for (step in 1:length(dir)) {
  filename <- paste(pathname, dir[step], sep="")
  shortname <- dir[step]; corename <- substring(shortname, 1, nchar(shortname)-3)
  csv_test <- substring(filename, nchar(filename)-2, nchar(filename))
  boo <- any(as.logical(grep(csv_test, "csv")))
  if (boo) {
    if (FILENUM == 0) FILENUM<-1 else FILENUM <- 2 # used below for define of data_array
    TABLE <- read.table(filename, sep=",",check.names=FALSE, header=TRUE, fill=TRUE, strip.white=TRUE)
    cat(paste(filename, "\n"))
    data_sum <- data.frame(subject=corename, sample=TABLE[,1], CFSE_TREG_FREQ=TABLE[,2], TREG_FREQ=TABLE[,3],
  CFSE_TREG_FREQ_PARENT=TABLE[,4], PROLIF_INDEX=TABLE[,5], PERCENT_DIVIDED=TABLE[,6], CLINICAL=TABLE[,7],
  INGESTING=TABLE[,8], CFSE_nTreg_FREQ=TABLE[,9])
    if (FILENUM==1) {data_array <<- data_sum}
    else {data_array <<- rbind(data_array, data_sum)} }
  else non_Ag <- non_Ag + 1
  }
cat(paste("total number of files:", paste(step, "\n")))
cat(paste("total number of data files:", paste(step - non_Ag, "\n")))
if (export) {
  exportpath <- paste(readLines(pipe("pwd")),"/",sep="")
  write.csv(data_array, paste(exportpath, exportname, sep = ""))
  cat(paste("writing file", paste(exportpath, paste(exportname, "\n"), sep="")))
  }
}
current_dir <- readLines(pipe("pwd"))
download.file("http://www.iisinai.org/shreffler/Treg_JACI_data.zip", "milk_data.zip")
pipe("unzip 'milk_data.zip'", "w")
#Figures2-3
milk <- read.table("Treg_JACI_data/R_DATA_frequency/CD25CD27_frequency.csv", header=TRUE, sep=",")
x <- subset(milk, stim == "casein" & IL.2 == "TRUE")
y <- subset(milk, stim == "CD3" & IL.2 == "TRUE")
z <- subset(milk, stim == "casein" & IL.2 == "FALSE")
#figure 2A - casein Treg
quartz(3,7); plot(x$CD25posCD27pos ~ x$group, ylab="% proliferating CD25+ CD27+ T cells", xlab="", cex.lab=1.4,cex.axis=1.1, cex=1.5,
  outline=FALSE, par(mar=c(5,5,5,5)))
points(x$CD25posCD27pos ~ x$group, pch=21, col=1, cex=1.5)
#Wilcoxon analysis for HM tolerant vs. Allergic
HM <- subset(milk, group == "HM" & IL.2 == "TRUE" & stim == "casein")
Allergic <- subset(milk, group == "ALLERGIC" & IL.2 == "TRUE" & stim == "casein")
Control <- subset(milk, group == "CONTROL" & IL.2 == "TRUE" & stim == "casein")
Outgrown <- subset(milk, group == "OUTGROWN" & IL.2 == "TRUE" & stim == "casein")
#Wilcoxon tests for group differences – – one tailed
wilcox.test(Allergic$CD25posCD27pos, HM$CD25posCD27pos, "less")
wilcox.test(Control$CD25posCD27pos, HM$CD25posCD27pos, "less")
wilcox.test(Outgrown$CD25posCD27pos, HM$CD25posCD27pos, "less")
#figure 2B - casein Teffector
quartz(3,7); plot(x$CD25posCD27neg ~ x$group, ylab="% proliferating CD25+ CD27- T cells", xlab="", cex.lab=1.4,cex.axis=1.1, cex=1.5,
  outline=FALSE, par(mar=c(5,5,5,5)))
points(x$CD25posCD27neg ~ x$group, pch=21, col=1, cex=1.5)
```

```
#figure 2C - anti-CD3/CD28 Treg
quartz(3,7); plot(y$CD25posCD27pos ~ y$group, ylab="% proliferating CD25+ CD27+ T cells", xlab="", cex.lab=1.4,cex.axis=1.1, cex=1.5, outline=FALSE, par(mar=c(5,5,5,5)))
points(y$CD25posCD27pos ~ y$group, pch=21, col=1, cex=1.5)
#figure 2D - relationship between casein Treg and Teffector
quartz(5,5);plot(x$CD25posCD27pos, x$CD25posCD27neg, xlab="% proliferating CD25+ CD27+ T cells", ylab="% proliferating CD25+ CD27- T cells", cex.lab=1.4, cex.axis=1.1, cex=1.5)
#figure 3A - influence of diet on casein Treg frequency in HM tolerant group
quartz(3,4); plot(HM$CD25posCD27pos ~ as.factor(HM$diet), ylab="% proliferating CD25+ CD27+ T cells", xlab="", cex.lab=1.4,cex.axis=1.1, cex=1.5, outline=FALSE, par(mar=c(5,5,5,5)))
points(HM$CD25posCD27pos ~ as.factor(HM$diet), pch=21, col=1, cex=1.5)
wilcox.test(subset(HM, diet == "Avoiding")$CD25posCD27pos, subset(HM, diet == "Ingesting")$CD25posCD27pos, "greater")
#figure 3B - influence of diet on casein Teffector frequency in HM tolerant group
quartz(3,4); plot(HM$CD25posCD27neg ~ as.factor(HM$diet), ylab="% proliferating CD25+ CD27- T cells", xlab="", cex.lab=1.4,cex.axis=1.1, cex=1.5, outline=FALSE, par(mar=c(5,5,5,5)))
points(HM$CD25posCD27neg ~ as.factor(HM$diet), pch=21, col=1, cex=1.5)
# group differences with casein stim by ratio of Treg/ Teffector
quartz(3,7); plot(x$CD25posCD27pos/x$CD25posCD27neg ~ x$group, ylab="ratio Treg/Teffector", xlab="", cex.lab=1.4,cex.axis=1.1, cex=1.5, outline=FALSE, par(mar=c(5,5,5,5)))
points(x$CD25posCD27pos/x$CD25posCD27neg ~ x$group, pch=21, col=1, cex=1.5)
# group differences with anti-CD3/CD28 stim by ratio of Treg/ Teffector
quartz(3,7); plot(y$CD25posCD27pos/y$CD25posCD27neg ~ y$group, ylab="ratio Treg/Teffector", xlab="", cex.lab=1.4,cex.axis=1.1, cex=1.5, outline=FALSE, par(mar=c(5,5,5,5)))
points(y$CD25posCD27pos/y$CD25posCD27neg ~ y$group, pch=21, col=1, cex=1.5)
# plot of casein Treg and poly Treg
quartz(); plot(subset(x, Sample != "PP470")$CD25posCD27pos, y$CD25posCD27pos, xlab="Casein Treg", ylab="Polyclonal Treg")
# plot of casein Treg and milk-specific IgE
quartz(); plot(x$CD25posCD27pos, x$IgE, xlab="Casein Treg", ylab="sIgE")
# plot of casein Treg and milk PST
quartz(); plot(x$CD25posCD27pos, x$wheal, xlab="Casein Treg", ylab="sIgE")
# plot of casein Teffector and milk PST
quartz(); plot(x$CD25posCD27neg, x$wheal, xlab="Casein T effector", ylab="sIgE")
#Figure 5B-E
BATCH(paste(current_dir, "/Treg_JACI_data/R_DATA_depletion", sep=""), exportname="Treg_JACI_data/milk.csv")
milk <- read.csv("Treg_JACI_data/milk.csv")
#remove egg white as n is small
milk <- subset(milk, sample != "E+"); milk <- subset(milk, sample != "E-")
samplef <- factor(milk$sample)
quartz(,10,10); layout(matrix(c(1,2,3,4),2,2,byrow=TRUE))
labels<-c("+","-","+","-","+","-","+","-")
#5B
plot(milk$CFSE_TREG_FREQ_PARENT ~ samplef, ylab="%Treg of CFSE low", xlab="", names=labels, cex.lab=1.2, cex.axis=1.2); abline(v=c(2.5, 4.5, 6.5), lty=3); mtext("medium", 1, 3, at=1.5); mtext("anti-CD3", 1, 3, at=3.5); mtext("casein", 1, 3, at=5.5); mtext("tetanus", 1, 3, at=7.5); mtext("**", 3, at=5.5, cex=2); mtext("B", 3, at=-0.5, cex=4.5)
#5C
plot(milk$CFSE_TREG_FREQ ~ samplef, ylab="%Treg of live CD3+CD4+", xlab="", names=labels, cex.lab=1.2, cex.axis=1.2); abline(v=c(2.5, 4.5, 6.5), lty=3); mtext("medium", 1, 3, at=1.5); mtext("anti-CD3", 1, 3, at=3.5); mtext("casein", 1, 3, at=5.5); mtext("tetanus", 1, 3, at=7.5); mtext("*", 3, at=5.5, cex=2); mtext("C", 3, at=-0.5, cex=4.5)
#5D
plot(milk$CFSE_nTreg_FREQ ~ samplef, ylab="%T effector of live CD3+CD4+", xlab="", names=labels, xlab="", cex.lab=1.2, cex.axis=1.2); abline(v=c(2.5, 4.5, 6.5), lty=3); mtext("medium", 1, 3, at=1.5); mtext("anti-CD3", 1, 3, at=3.5); mtext("casein", 1, 3, at=5.5); mtext("tetanus", 1, 3, at=7.5); mtext("*", 3, at=5.5, cex=2); mtext("D", 3, at=-0.5, cex=4.5)
#5E
plot(milk$PROLIF_INDEX ~ samplef, ylab="proliferation index of CD3+CD4+", xlab="", names=labels, xlab="", cex.lab=1.2, cex.axis=1.2); abline(v=c(2.5, 4.5, 6.5), lty=3); mtext("medium", 1, 3, at=1.5); mtext("anti-CD3", 1, 3, at=3.5); mtext("casein", 1, 3, at=5.5); mtext("tetanus", 1, 3, at=7.5); mtext("E", 3, at=-0.5, cex=4.5)
quartz(); plot(milk$CFSE_TREG_FREQ, milk$CFSE_nTreg_FREQ, main="frequency of Treg vs. non-Treg", ylab="% T effector of total live CD3+CD4+", xlab="% Treg of total live CD3+CD4+")
#test for decrease in Treg within the prolfierating population (casein)
wilcox.test(subset(milk, sample == "C+")$CFSE_TREG_FREQ_PARENT, subset(milk, sample == "C-")$CFSE_TREG_FREQ_PARENT, "greater", paired=TRUE)
#test for increase in T effector as percent of CD3CD4 (casein)
wilcox.test(subset(milk, sample == "C+")$CFSE_nTreg_FREQ, subset(milk, sample == "C-")$CFSE_nTreg_FREQ, "less", paired=TRUE)
#test for increase in proliferation index (casein)
wilcox.test(subset(milk, sample == "C+")$PROLIF_INDEX, subset(milk, sample == "C-")$PROLIF_INDEX, "less", paired=TRUE)
#Figure 6 and analysis
```

```
pro <- read.table("Treg_JACI_data/R_DATA_proliferation/PROLIFERATION.csv", header=TRUE, sep=",")
allo <- subset(pro, TEFF == "ALLO")
quartz(,8,4); lo <- layout(matrix(c(1,1,2),1,3))
allo <- subset(allo, RATIO != 0)
Ratiof <- factor(allo$RATIO)
agg <- tapply(allo$PERCENT, Ratiof, fivenum)
boxplot(agg, names=rownames(agg), cex.lab = 1.5, ylab="percent proliferation", xlab="Ratio Treg:Responder"); mtext("A", at=0.2, cex=2.5, line=0.1)
control <- subset(pro, RATIO == 0)
Tf <- factor(control$TEFF)
agg <- tapply(control$CPM, Tf, fivenum)
boxplot(agg, cex.lab=1.5, ylab="counts per minute"); mtext("B", at=0.1, cex=2.5, line=0.5)
t.test(subset(allo, RATIO == 0.2)$PERCENT, subset(allo,RATIO ==0.05)$PERCENT)
allo <- subset(pro, TEFF == "ALLO")
wilcox.test(subset(allo, RATIO == 0)$CPM, subset(allo, RATIO ==0.2)$CPM, paired=TRUE)
```

TABLE E1. Detailed T-cell phenotype data

Sample	% $CFSE^{low}$	$CD25^+CD27^+$	$CD25^+CD27^-$	$CD25^-CD27^+$	$CD25^-CD27^-$	$CD25^+CD27^+$ of $CFSE^{low}$	$CD25^+$	non-Treg	Group	Diet
PP454	46.5	23.6	21.7	0.95	0.17	50.9	45.3	22.8	HM	Ingesting
PP455	23.1	18.3	3.05	1.6	0.19	79	21.3	4.85	Outgrown	Ingesting
PP456	49.1	31.7	16.6	0.64	0.18	64.5	48.3	17.4	HM	Avoiding
PP457	47.1	30.1	14.9	1.71	0.48	63.7	45	17.1	HM	Avoiding
PP458	58.2	14.5	35	6.57	2.09	24.9	49.5	43.7	Outgrown	Ingesting
PP462	15	5.67	6.34	2.21	0.75	37.9	12	9.31	Allergic	Avoiding
PP464	70.9	3.67	41.1	3.47	22.6	5.18	44.8	67.3	HM	Ingesting
PP465	23.7	0.75	9.65	1.01	12.3	3.16	10.4	23	HM	Ingesting
PP466	52.9	38.8	12.3	1.22	0.58	73.3	51.1	14.1	HM	Ingesting
PP467	31.1	21.4	6.23	2.42	1.12	68.6	27.6	9.77	Outgrown	Ingesting
PP468	6.23	2.6	1.47	0.8	1.36	41.7	4.07	3.63	Outgrown	Avoiding
PP469	64.4	39.4	11.2	12.9	0.97	61.1	50.5	25	HM	Avoiding
PP470	34.6	23.8	9.89	0.79	0.1	68.8	33.7	10.8	HM	Avoiding
PP471	60.1	39.7	4.59	14.1	1.77	66	44.2	20.4	HM	Ingesting
PP472	41.4	16.7	19.8	1.48	3.37	40.4	36.5	24.6	HM	Avoiding
PP473	1.46	0.52	0.086	0.26	0.6	35.3	0.6	0.95	Control	Ingesting
PP474	8.35	2.09	3.51	1.29	1.47	25	5.6	6.27	Allergic	Avoiding
PP475	21.7	7.06	4.98	4.34	5.37	32.4	12	14.7	HM	Ingesting
PP476	79.5	52.7	3.58	19.6	3.65	66.3	56.3	26.8	HM	Avoiding
PP477	14.6	11.9	1.66	0.89	0.093	81.8	13.6	2.64	HM	Avoiding
PP478	9.87	5.25	4.38	0.22	0.018	53.2	9.63	4.62	HM	Ingesting
PP479	16.7	9.36	6.26	0.56	0.51	56.1	15.6	7.34	Allergic	Avoiding
PP485	38.8	8.98	6.31	16.7	6.79	23.1	15.3	29.8	Outgrown	Ingesting
PP486	63.8	37.9	16.1	7.49	2.22	59.5	54	25.8	Allergic	Avoiding
PP487	3.98	1.12	0.71	1.24	0.91	28	1.83	2.86	Outgrown	Ingesting
PP488	4.71	1.87	2.84	0	0	39.7	4.71	2.84	Allergic	Avoiding
PP489	5.97	2.83	1.89	0.83	0.43	47.3	4.71	3.14	Outgrown	Ingesting
PP490	14.3	4.58	9.09	0.53	0.11	32	13.7	9.73	Allergic	Avoiding
PP491	9.04	5.24	3.48	0.27	0.047	58	8.72	3.8	Allergic	Avoiding
PP495	32.9	17	14.9	0.62	0.3	51.8	31.9	15.8	HM	Avoiding
PP496	14.9	2.39	12.2	0.23	0.14	16	14.5	12.5	Control	Ingesting
PP499	36	4.64	2.19	29	0.19	12.9	6.83	31.4	Control	Ingesting
PP508	15.6	7.42	6.29	0.6	1.33	47.5	13.7	8.21	Control	Ingesting
PP514	14.6	2.86	5.51	0.65	5.53	19.7	8.37	11.7	Control	Ingesting
PP523	56.8	22.6	15.6	8.58	9.99	39.9	38.2	34.1	Control	Avoiding
PP526	8.63	2.34	5.14	0.61	0.55	27.1	7.47	6.29	Control	Ingesting
PP541	13	8.43	1.53	2.07	0.93	65	9.96	4.53	HM	Ingesting
PP542	30.7	16	8.82	3.22	2.69	52	24.8	14.7	HM	Ingesting
PP580	15.8	6.47	8.29	0.61	0.47	40.9	14.8	9.37	HM	Ingesting
PP582	7.79	3.2	4.05	0.3	0.24	41.1	7.25	4.59	Allergic	Avoiding

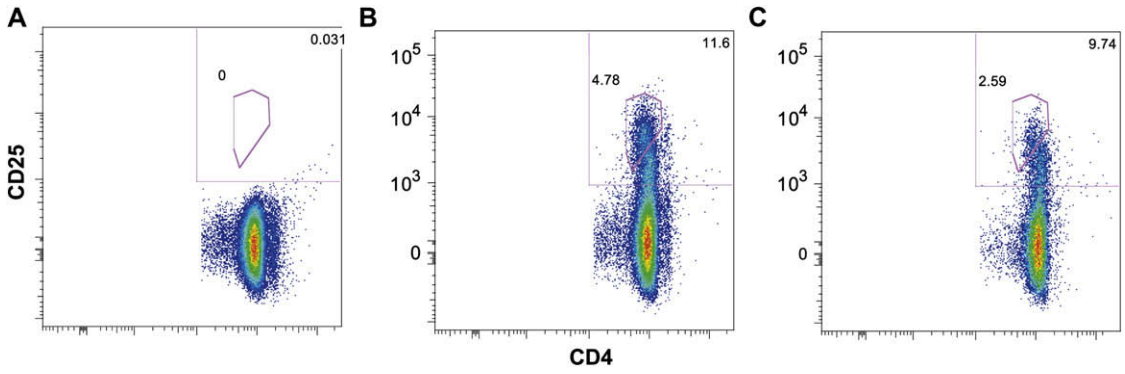

FIG E1. Partial depletionof $CD25^{hi}$ T cells. All plots show live $CD3^+$ events from the same patient PBMC plotted as CD4 vs CD25. **A,** Gating control with no anti-CD25 staining. **B,** Mock-treated PBMCs showing 11.6% $CD25^+$ and 4.8% $CD25^{hi}$, with the characteristic slightly lower CD4 expression previously described. **C,** Anti-CD25–treated PBMCs from the same donor showing >45% fewer $CD25^{hi}$ T cells after depletion with magnetic beads.

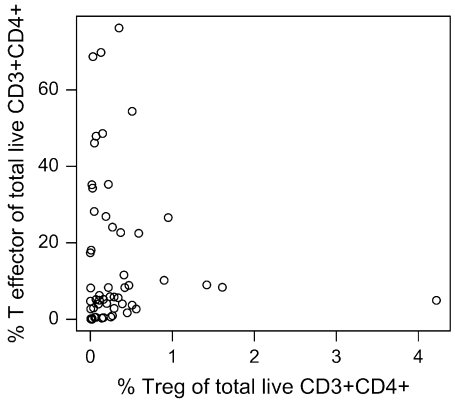

FIG E2. Relationship of casein-induced proliferation between regulatory and effector populations. PBMCs from HM-tolerant subjects (n = 8) were expanded for 7 days, and the percentage of total $CD3^{+}CD4^{+}$ of proliferating Treg cells ($CFSE^{low}$ $CD25^{hi}$ $FoxP3^{hi}$) was plotted against the percentage of proliferating T-effector cells ($CFSE^{low}$ $CD25^{+}$ $FoxP3^{-}$).

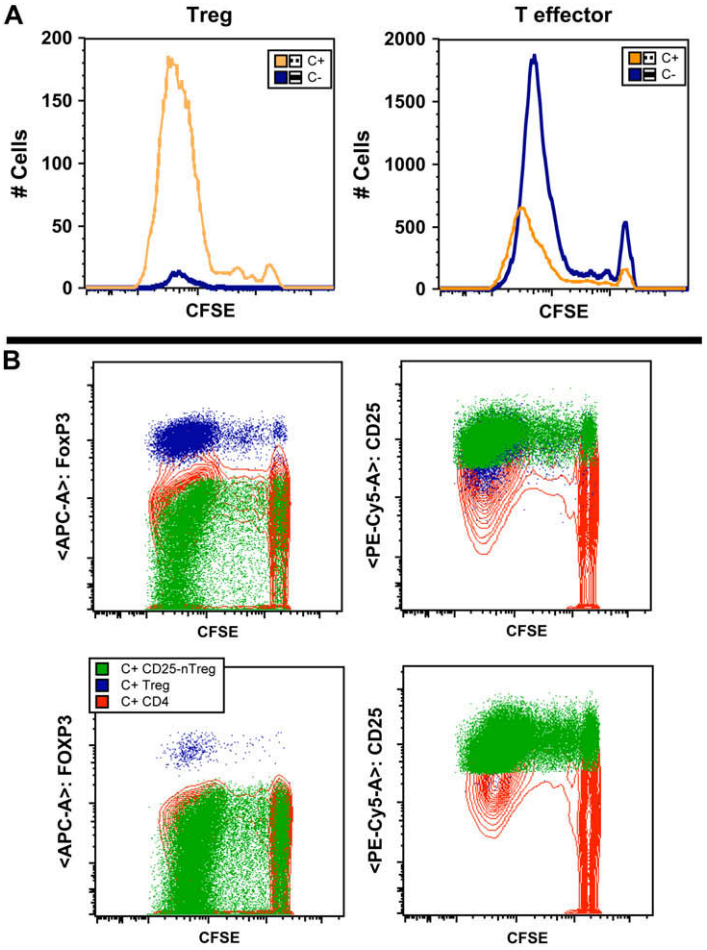

FIG E3. Functional competence of allergen-specific Treg cells from a donor with allergy. **A,** Histograms showing absolute number by CFSE of Treg cells *(left)* or T-effector cells *(right)* after expansion without *(yellow)* or with *(blue)* baseline depletion of $CD25^{hi}$ cells. **B,** FoxP3 *(left)* and CD25 *(right)* expression of Treg cells *(blue)* or T-effector cells *(green)* superimposed on total live $CD3^{+}CD4^{+}$ cells *(red)*. Without *(top row)* or with *(bottom row)* baseline $CD25^{hi}$ depletion.