

Lymphoproliferative responses to dendritic cell presentation of sensitizing allergens in atopic children with multiple allergies

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ABSTRACT

Background: Peripheral blood mononuclear cells (PBMCs) proliferate inconsistently, rendering current lymphoproliferation assays unreliable in diagnosis.

Objective: To investigate the utility and nature of proliferation responses in allergy by comparison of the standard lymphoproliferation with a new dendritic cell (DC) stimulated assay.

Methods: Monocyte-derived DCs were pulsed with allergens and incubated with autologous T cells for 7 days. DC-stimulated and standard PBMC proliferation responses to 3 common dietary allergens in children with allergy and without atopy were measured by incorporation of tritiated thymidine and reduction of carboxyl fluorescein succinimidyl ester staining.

Results: The DC presentation of sensitizing allergens induced significantly higher proliferative responses than PBMC stimulation ($P = .04$) and greater distinction between normal and allergic responses. DC-induced stimulation indices of children without sensitivity and those with allergy were significantly different with all 3 foods ($P < .001$). All children with allergy presented with peanut allergy and 12 of 14 (86%) β -lactoglobulin-pulsed DC preparations proliferated more than 3.3-fold above un-pulsed cells, but 8 of 18 children (44%) with ovalbumin egg allergy showed proliferation below this level. The stimulation index of DC tritiated thymidine incorporation correlated closely with carboxyl fluorescein succinimidyl ester reduction ($P < .001$). Sensitivity of detection of peanut, milk, or egg allergy was 100%, 85.7%, or 55.6% and specificity was 60%, 88.9%, or 86.7%, respectively. DC-stimulated T cells expressed increased levels of CD45 RO and CD25 and most produced interferon- γ . DC-stimulated proliferation correlated with total immunoglobulin E and peanut antigen-stimulated proliferation correlated with peanut specific immunoglobulin E ($P = .03$).

Conclusion: The DC-induced lymphoproliferation had higher sensitivity, specificity, and reproducibility than the standard assay and caused increased memory and activated T-cell proliferation in children with food allergy.

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Introduction

Limited and contradictory information is available on the usefulness of lymphoproliferation as an indicator of allergic sensitization. Lymphocytes from children with immunoglobulin E (IgE)-mediated cow's milk allergy have been found to proliferate significantly more in the presence of milk allergens compared with those from control subjects.^{1,2} Strong proliferative responses found in children with asthma compared with the absence of lymphoproliferation to house dust mite in healthy children, correlating

with serum allergen-specific IgE levels and eosinophil counts,³ does support the concept that activation of allergen-specific T lymphocytes in blood might reflect the intensity of allergic sensitization in childhood atopy.

However, the degree and frequency of lymphoproliferative responses rarely correspond with the clinical severity of allergy. A large proportion of healthy children had lymphoproliferative responses to common allergens within the ranges of children with allergy,^{4–6} whereas no difference between proliferative values of individuals with and without sensitization to common foods has been found.^{7–9} These conflicting results have resulted in confusion as to the molecular mechanism and the diagnostic significance of the proliferative response in individuals with allergy.

Skewed T-cell response to specific allergens are the foundation of allergic sensitization.¹⁰ We previously documented the association of T-helper cell type 2 (T_H2) cytokine-skewed CD4⁺ memory

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T cells in the allergen-specific responses of a cohort of children with food allergy.¹¹ By eliminating extraneous factors present in assorted peripheral blood mononuclear cells (PBMCs) in the conventional lymphoproliferation assay, purified T-cell interaction with allergen-pulsed antigen-presenting cells could be more directly indicative of the level of sensitization. To determine whether defined cell populations could better denote the association of lymphoproliferation with allergic sensitization, we used allergen-loaded dendritic cells (DCs) to stimulate purified T cells in this group of children and compared results with the use of carboxyl fluorescein succinimidyl ester (CFSE) staining¹² and the standard proliferative assay based on incorporation of tritiated thymidine into PBMCs.

Methods

Blood was obtained from 23 children attending the allergy outpatient clinics of the Great Ormond Street Hospital NHS Trust (London, United Kingdom). Clinical symptoms of children with allergy included asthma, eczema, gastrointestinal symptoms, and anaphylaxis (Table 1). Age ranged from 3 years 4 months to 13 years 11 months (mean 7 years 6 months); 11 were girls and 12 were boys. Diagnosis was based on recent blinded food challenge test results or persistent clinical history of reactivity to foods, IgE measurements (Unicap; Phadia AB, Uppsala, Sweden), and skin prick test (SPT) results (Alk Abello, Hungerford, United Kingdom) performed to the manufacturers' recommendations. Five of the 23 children were allergic to 1 of the 3 foods, 5 were allergic to 2 foods, and 13 were allergic to egg, milk, and peanut. Total serum IgE varied from 11.4 to 14,600 kU/L (average 2,052 kU/L). Prior local medical ethics committee approval and informed parental consent were obtained for the study subjects and for samples taken from 20 healthy age-matched control children in whose sera no IgE specific to food allergens was detected.

Hybridoma OKT3 to human CD3 (CRL-8001; American Type Culture Collection [ATCC], Manassas, Virginia), 60bca (anti-human CD14, HB-247; ATCC), L243 and L203 (anti-HLA DR, HB-55, and HB-171; ATCC), IF3 (anti-human CD20, HB-9645; ATCC), and BU12 (anti-human CD19; from Dr Debbie Hardie, University of

Birmingham, Birmingham, United Kingdom) were cultured to high density in 20% fetal calf serum and HybriCare medium (46-X; ATCC), and the supernatant was collected, clarified, filtered (0.4 μ m), and frozen. The amount of hybridoma supernatants required to remove more than 95% of positive cells with greater than 95% specificity with magnetic beads (M101 Dynal; Invitrogen Ltd, Paisley, United Kingdom) was evaluated by flow cytometry. Blocking antibodies were concentrated from culture supernatant by affinity chromatography (protein G sepharose, 10-1242; Invitrogen, La Jolla, California) and reconstituted at 2 mg/mL in RPMI-1640.

Peanut flour (SPF Inc, Austin, Texas) was defatted repeatedly with hexane and extracted with 0.1 mol/L of NH_4HCO_3 and protein was precipitated with 60% $(\text{NH}_4)_2\text{SO}_4$ at 4°C for 2 hours.¹³ Protein precipitate was dissolved in phosphate buffered saline (PBS), dialyzed extensively, filtered (0.2 μ m), and lyophilized. Bovine serum albumin, β -lactoglobulin (BLG), ovalbumin (OVA), and ovomucoid (B6917, A5503, L3908 and T2011, respectively; Sigma-Aldrich, Poole, Dorset, United Kingdom) were received as premium quality lyophilized powders and reconstituted in PBS. Food antigens were tested by the Limulus Amoeba Lysate kit (Quadrant Ltd, Epsom, Surrey, United Kingdom) and contained lipopolysaccharides below reliably detectable levels.

Cell Purification and Culture

Blood was extracted with 5 U/mL of heparin and mixed with an equal volume of RPMI-1640 medium (without glutamine + 25 mmol/L of HEPES; catalog number 42401-42, Gibco, Grand Island, New York) and monocyctic cells (PBMCs) collected over a cushion of a half volume of Lymphoprep (Alere Ltd, Stockport, United Kingdom) after 30 minutes' centrifugation at 280g. Upper plasma supernatant was collected, clarified twice at 320 and 700g for 10 minutes, and used as a growth medium supplement. PBMCs were used directly or were washed twice at 220g to reduce platelets and were frozen in cold 10% dimethyl sulfoxide and 50% autologous plasma at a density of 5×10^6 cells/mL. T cells were prepared from PBMCs, ensuring greater than 95% viability of thawed cells, by negative selection; extraction twice with 250 μ L of HB-247, 400 μ L

Table 1
Patient IgE and Clinical Symptoms

Patient	Sex	Age	Peanut IgE	Milk IgE	Egg IgE	Total IgE	Other sensitizations or allergies	Clinical presentation
1	F	3 y 4 mo	14.6	>100	6.92	3,246	3	asthma rhinitis
2	F	3 y 6 mo	—	—	23.6	868	1	eczema
3	M	3 y 11 mo	13.5	6.3	10.2	1,099	1	
4	M	4 y 4 mo	>100	>100	>100	12,768	2	asthma, eczema
5	M	4 y 9 mo	>100	49.7	12.4	4,748	1	eczema
6	F	5 y 3 mo	9.4	26.4	10.7	2,319	2	rhinitis
7	F	5 y 7 mo	46.5	42	8.1	1,824	—	gastritis
8	M	5 y 10 mo	28.6	24.9	11.5	3,602	2	eczema
9	M	6 y 6 mo	—	1.4 ^a	0.7	11.4	—	
10	F	6 y 9 mo	1.09	—	11.6	900	—	eczema
11	M	6 y 10 mo	5.06	10.8	18.2	712	1	
12	F	6 y 1 mo	54	>100	>100	11,647	2	eczema, rhinitis, gastritis
13	M	7 y 1 mo	>100	—	—	478	1	
14	F	7 y 4 mo	>100	>100	>100	14,600	2	asthma, rhinitis
15	M	8 y 10 mo	7.3	—	10.7	1,287	1	eczema
16	M	8 y 11 mo	29.9	—	87.9	953	—	
17	M	9 y 11 mo	62.7	30.6	16.6	1,648	2	eczema
18	M	9 y 8 mo	20.9	11.8	9.1	1,681	5	eczema, rhinitis
19	F	10 y 6 mo	13.2	—	—	1,949	—	eczema
20	F	10 y 7 mo	—	—	15.7	825	1	
21	F	11 y 7 mo	—	8.9	18.7	692	—	asthma
22	M	12 y 4 mo	11.7	9.9	5.6	2,132	2	eczema
23	F	13 y 11 mo	17.1	9.1	—	217	1	

Abbreviations: F, female; IgE, immunoglobulin E; M, male.

^aDecreasing titer resolved clinical symptoms.

of L242, and 350 μ L of BU12 hybridoma supernatants in 2 mL of RPMI medium and 10 μ L of magnetic beads generated cells in which CD3⁺ positivity exceeded 96% (mean 96.6%, range 92.1–99.8%, $n = 34$).

Functional DCs were generated from adherent PBMCs cultured for 7 to 12 days in 20% autologous plasma and RPMI supplemented with 100 ng/mL of human granulocyte-macrophage colony-stimulating factor and 50 ng/mL of human interleukin (IL)-4 (JR-1900 and IB-1016; Insight Biotechnology, Wembley, United Kingdom). The DCs were depleted of contaminating cells by magnetic anti-IgG-coated beads after incubation with 350 μ L of BU12, 200 μ L of OKT3, and 450 μ L of HB-247 hybridoma supernatants in 2 mL of RPMI. DCs were irradiated at 35 Gy in a gamma irradiator, pulsed with food allergens (100, 10, and 1 μ g/mL) for 24 hours, washed, and used in proliferation assays.

Lymphoproliferation Assays

Proliferative activity was assessed by quantification of [³H]-thymidine uptake and by flow cytometry of carboxy fluorescein diacetate succinimidyl ester (CFSE; 21888, Sigma-Aldrich) reduction in replicating cells. Autologous PBMCs were incubated in triplicate at 1×10^5 per well in autologous plasma with a range of food allergens directly and compared with purified autologous T cells mixed in a 10:1 ratio with allergen-pulsed DCs. The same concentrations of bovine serum albumin, BLG, OVA, peanut protein, and ovomucoid were added to PBMC cultures as used to pulse DCs before addition to purified T cells. PBMCs and DCs without allergen exposure served as negative controls, and PBMCs with IL-2 (5 U/mL; BI-1014, 10^7 U/mg; Insight Biotechnology) and phytohemagglutinin (10 μ g/mL; L9132; Sigma-Aldrich) were used as positive controls. Culture was continued for 7 days in 20% autologous plasma and RPMI-1640 in round-bottom 96-well plates (Costar 3596; Corning International, Corning, New York) and proliferation was measured by tritiated thymidine (TRK637; Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom) uptake measured 18 hours after addition of 37 kBq/well and by analysis of CFSE reduction in replicating cells. Tritiated cellular DNA was captured onto glass-fiber filters (Wallac 1450-421; Perkin Elmer, Waltham, Massachusetts) with a TomTec Cell Harvester 96, overlaid with wax scintillant (Meltex A, Wallac 1450-441) and incorporated thymidine was measured by scintillation counter (Wallac 145, MicroBeta Trilux). Proliferation in tritiated cells was expressed in counts per minute (cpm) or as the stimulation index (SI), the ratio between the mean cpm values of unstimulated and allergen-stimulated PBMCs, or the ratio of T cells incubated with un-pulsed or allergen-pulsed DCs, measured in triplicate. CFSE reduction assay¹² was performed with T cells treated with 5 μ mol/L of filtered CFSE in RPMI-1640 for 20 minutes before culture. Cultured cells were washed in cold PBS and 0.01% sodium azide and cells gated on size and FL1 (CFSE) intensity were acquired on a FACSCalibur flow cytometer (Becton Dickinson, Oxford, United Kingdom). The SI of CFSE-stained cells was calculated as the mean percentage of replicating cells multiplied by the mean number of cycles per replicating cell of allergen-stimulated cultures divided by unstimulated cultures. In accordance with proposed limits for defining positivity,¹⁴ the proliferative response with an SI higher than 3.3 above background, the level of un-pulsed DC stimulated proliferation, was regarded as positive. Cell surface markers were assessed in CFSE-stained cells incubated in CD3 phycoerythrin (340662; PharMingen, San Diego, California), CD45 RO Cy-Chrome (31308X; PharMingen), and CD25 Cy-Chrome (30798X; PharMingen) in PBS and 0.01% sodium azide for 20 minutes or incubated for 2 hours with brefeldin A (10 μ g/mL), ionomycin (1 μ g/mL), and phorbol 12-myristate 13-acetate (10 ng/mL), washed, fixed, made permeable, and stained with mouse

anti-human CD3 Cy-Chrome (30118X; PharMingen) and anti-cytokine phycoerythrin conjugated antibodies against IL-2, IL-4, interferon- γ (340450, 340451, 340452, respectively; PharMingen), or IL-10 (MCA1531PE; Serotec, Raleigh, North Carolina). Blocking antibodies were added to PBMC and mixed T-cell and DC cultures at initiation at a concentration of 2 μ g/mL unless otherwise stated. Sensitivity and specificity of the DC proliferation assay as a predictor of allergic sensitization were calculated as true positives/(true positives + false negatives) and true negatives/(false positives + true negatives), respectively, using combined clinical tests, specific IgE, and SPTs as indicators of allergy.

Statistical Analyses

Statistical analyses were performed using SPSS 8.0 for Windows (SPSS, Inc, Chicago, Illinois) using an independent-sample 2-tailed *t* test. Mean and SD of group SIs were compared by *F* test. Coefficient of variation was calculated as SD/mean $\times$ 100. Correlation was assessed using the Spearman rank correlation coefficient. *P* values less than .05 were considered significant.

Results

Comparison of Conventional PBMC Assay With DC-Stimulated T-Cell Proliferation

Factors that influence proliferation were investigated with monoclonal antibodies. PBMCs with or without 100 μ g/mL of peanut protein (Fig 1A, upper panel) or isolated T cells mixed in a 10:1 ratio with autologous monocyte-derived DCs un-pulsed or pulsed with 100 μ g/mL of peanut protein (Fig 1A, lower panel) were incubated for 1 week without antibody or anti-HLA-DR antibody, anti-CD20 antibody, anti-CD14 antibody, or nonspecific IgG. Peanut-pulsed DCs stimulated over 9-fold greater proliferation (mean 14,907 cpm of 4 experiments) than occurred in PBMCs (mean 1,512 cpm, $n = 4$). Antibody to CD20 reduced proliferation by 15% to 35% in PBMCs of different children (mean 21.7% reduction, $P = .036$) but had no effect on DC-stimulated proliferation. Antibody 60bca to CD14 affected the 2 assays (PBMC mean decrease in proliferation $30.4 \pm 4.7\%$, $P = .016$), with less effect on DC:T-cell proliferation (decrease $18.9\% \pm 9.1\%$, $P = .04$). PBMC- and DC-stimulated proliferation responses were profoundly blocked by MHC class II DR antibody (L203, $50.8 \pm 9.9\%$, $P = .001$; $53.9 \pm 7.5\%$, $P = .002$, respectively), whereas IgG antibody promoted proliferation ($3.9 \pm 8.3\%$ increase in PBMC and $5.9 \pm 3.6\%$ increase in DC:T-cell proliferation). Proliferation in PBMC culture was affected by different factors, whereas DC-stimulated proliferation was largely restricted by MHC.

The effect of MHC class II DR antibody was examined more closely (Fig 1B). PBMCs incubated in 100 μ g/mL of peanut protein or T cells cultured with DCs pulsed with peanut protein were cultured without antibody or with increasing concentrations of anti-HLA-DR antibody for 1 week. The reduction of DC-stimulated proliferation of proliferation was linearly related to the concentration of DR antibody, whereas PBMC proliferation was inhibited irregularly by higher concentrations of antibody.

The PBMC- and DC-stimulated proliferation responses of 5 children with food allergy assayed with sensitizing and non-sensitizing antigens demonstrated similar positive and negative responses (Table 2), but DCs pulsed with higher allergen concentrations stimulated over 5-fold higher tritium incorporation than allergen incubation of PBMCs (mean 11,484 vs 2,137 cpm, respectively). Baseline proliferation with un-pulsed DCs (mean of 4 replicates from 5 children without sensitization and those with allergy; 497 and 522 cpm, respectively) also was higher than PBMCs cultured without allergens (mean 304 and 282 cpm in children without sensitization and those with allergy, respectively)

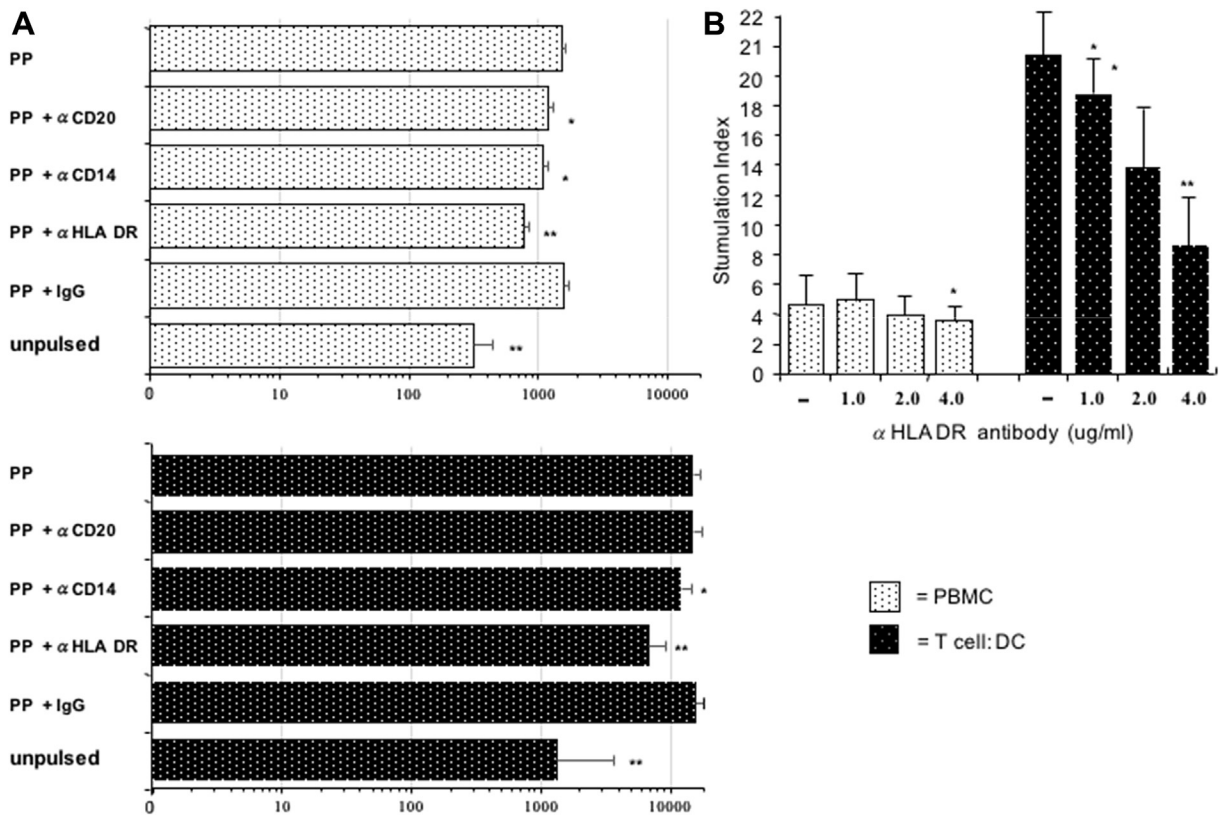


Figure 1. Proliferation of peripheral blood mononuclear cells (PBMCs) and allergen-presented T cells in the presence of blocking antibodies. (A) PBMCs with or without 100 μ g/mL of peanut protein (PP; upper panel) or T cells mixed in a 10:1 ratio with un-pulsed autologous monocyte-derived dendritic cells (DCs) or DCs pulsed with 100 μ g/mL of PP (lower panel). Anti-HLA-DR antibody (hybridoma L203), anti-CD20 antibody (hybridoma 1F3), anti-CD14 antibody (hybridoma 60bca), or nonspecific mouse immunoglobulin G (IgG) was added to equivalent cultures. Proliferation results (mean count per minute of quadruplicate wells) are representative of 4 experiments of children with peanut sensitization. (B) PBMCs exposed to 100 μ g/mL of PP (gray bars) or T cells mixed with PP-pulsed autologous monocyte-derived DCs (black bars) were cultured without antibody or with increasing concentrations of anti-HLA-DR antibody. * $P < .05$, ** $P < .01$ by unpaired Student t test.

but baseline variation was much less to incubation with un-pulsed DCs, with 3.2-fold variation in responses of 5 individuals ($235\text{--}739 \pm 206$ cpm [42%] and $354\text{--}853 \pm 206$ cpm [38%] for children without sensitization and those with allergy) vs 8.4-fold variation in range of PBMC responses without antigens ($140\text{--}552 \pm 172$ cpm [56%] and $80\text{--}672 \pm 228$ cpm [81%] for non-sensitized and allergic PBMCs, respectively). The DC-stimulated and PBMC lymphoproliferation of sensitizing and non-sensitizing allergens in the 5 children with food allergy were significantly different (DC mean $11,484 \pm 3,817$ and 996 ± 477 cpm, $P < .01$ vs PBMC mean $2,137 \pm 809$ and 908 ± 762 cpm, $P = .04$, for sensitizing and non-sensitizing allergens, respectively) but the difference with DC stimulation (11-fold) was greater than with PBMC (2.4-fold difference). The coefficients of variation for proliferation (Table 2) measured in the conventional PBMC assay (range 37.9–95.7) were much higher than those induced by DC allergen presentation (range 15.1–47.9), indicating that the assay using DC allergen presentation is more reproducible.

Proliferative Response to Sensitizing Foods and Relation to IgE

All children with peanut allergy ($n = 18$; Fig 2, top panel) and most children with milk allergy (12 of 14, 86%; Fig 2, middle panel) had proliferative responses above 3.3 SI when stimulated with DCs pulsed with the relevant food allergen, whereas children without food allergy, including 9 of 15 (60%) without peanut allergy and 16 of 18 (88.9%) without milk allergy, had proliferation reactions below 3.3 SI. One of the 2 children non-sensitized to milk (Fig 2, middle panel, right) who did not show significant proliferation to BLG had recently outgrown a clinical response to milk ingestion but

retained specific IgE at a decreasing level. Five of the 6 children not allergic to peanut showed a reaction to peanut protein and the single child without milk allergy showed a reaction to BLG; these children had atopy and allergy to other foods.

The lymphoproliferative responses of children with egg allergy were lower than those of children with peanut or milk allergy and 8 of 18 children (44%) with egg allergy showed proliferation below a level of 3.3 SI when exposed to DCs pulsed with OVA (Fig 2, lower panels). The proliferative responses of children non-sensitized and allergic to peanut exposed to DCs pulsed with 100 μ g/mL of peanut protein (SI mean $\pm$ SD, 6.7 ± 11.4 and 13.0 ± 8.8 , respectively, $P = .04$) and with 10 μ g/mL of allergen (4.1 ± 4.4 and 7.4 ± 5.5 , respectively, $P = .03$) were significantly different. The SIs of children without sensitization and those with allergy also were significantly different when presented with DCs pulsed with 100 μ g/mL of OVA (2.2 ± 2.4 and 5.8 ± 6.4 , respectively, $P = .02$) but not with 10 μ g/mL of OVA-pulsed cells (2.2 ± 2.8 and 3.8 ± 3.8 , respectively, $P = .08$). The proliferative values for children non-sensitized and allergic to milk to 100 μ g/mL of BLG-pulsed DCs, with a single exceptionally high non-sensitized response, were not significantly different (3.6 ± 6.2 and 5.8 ± 3.7 , respectively, $P = .11$), but SIs of milk-sensitized T cells were significantly different when exposed to DCs pulsed with 10 μ g/mL of BLG (2.4 ± 2.0 and 5.2 ± 3.9 , respectively, $P = .01$).

When T cells were stained with CFSE and incubated with and without food allergen-pulsed DCs (Fig 3), more than 50% of cells of most patients proliferated with mitogenic stimulation with more than 4 cell divisions on average. Survival of stained cells was low and variable (mean survival $21.9 \pm 18.8\%$, range 12.1–72.3%) but despite this the CFSE reduction SI values were in close agreement

Table 2

Tritium Incorporation and SI of PBMCs and Purified T Cells Presented With Sensitizing and Non-sensitizing Antigens

Patient	Antigen	PBMC assay					DC assay				
		No antigen	100 ng	1 μ g	10 μ g	100 μ g	No antigen	100 ng	1 μ g	10 μ g	100 μ g
1 ^a	sensitizing (cpm)	176	176	341	1,794	3,174	576	649	857	3,443	18,147
	SI	—	1.6	1.9	10.2	18	—	1.1	1.5	6.1	32
	non-sensitizing (cpm)	297	140	276	376	441	471	397	346	508	475
2 ^a	SI	—	0.5	0.9	1.3	1.5	—	0.8	0.7	1.1	1
	sensitizing (cpm)	231	166	236	492	1,284	354	897	946	3,149	9,475
	SI	—	0.7	1	2.1	5.6	—	2.5	2.7	8.9	26.8
3 ^a	non-sensitizing (cpm)	140	172	212	328	525	379	440	407	513	738
	SI	—	1.2	1.5	2.3	3.8	—	1.2	1.1	1.4	1.9
	sensitizing (cpm)	672	519	1,327	1,591	2,672	489	972	1,482	5,404	11,153
4 ^b	SI	—	0.8	2	2.4	4	—	2	3	11.1	22.8
	non-sensitizing (cpm)	552	554	645	1,867	2,256	662	552	561	514	1,158
	SI	—	1	1.2	3.4	4.1	—	0.8	0.8	0.8	1.7
5 ^b	sensitizing (cpm)	80	443	441	783	2,145	349	827	1,786	1,909	9,761
	SI	—	5.5	5.5	9.8	26.8	—	2.4	5.1	5.5	28
	non-sensitizing (cpm)	151	113	315	145	744	235	451	522	415	885
5 ^b	SI	—	0.7	2.1	1	4.9	—	1.9	2.2	1.8	3.8
	sensitizing (cpm)	251	392	586	1,875	1,408	853	751	997	2,415	8,885
	SI	—	1.6	2.3	7.5	5.6	—	0.9	1.2	2.8	10.4
Mean $\pm$ SD	non-sensitizing (cpm)	381	241	253	951	573	739	381	416	871	1,726
	SI	—	0.6	0.7	2.5	1.5	—	0.5	0.6	1.2	2.3
	sensitizing (cpm)	282 $\pm$ 228	359 $\pm$ 140	586 $\pm$ 434	1,307 $\pm$ 628	2,137 $\pm$ 809	552 $\pm$ 207	819 $\pm$ 126	1,213 $\pm$ 402	3,264 $\pm$ 1,340	11,484 $\pm$ 3,817
Mean $\pm$ SD	SI	—	2.0 $\pm$ 2.0	2.6 $\pm$ 1.7	6.4 $\pm$ 3.9	12.0 $\pm$ 10.0	—	1.8 $\pm$ 0.7	2.7 $\pm$ 1.8	6.9 $\pm$ 3.2	24.0 $\pm$ 8.3
	non-sensitizing (cpm)	304 $\pm$ 172	244 $\pm$ 180	340 $\pm$ 174	734 $\pm$ 702	908 $\pm$ 762	497 $\pm$ 210	444 $\pm$ 67	450 $\pm$ 88	564 $\pm$ 177	996 $\pm$ 477
	SI	—	0.8 $\pm$ 0.3	1.3 $\pm$ 0.6	2.1 $\pm$ 1.0	3.2 $\pm$ 1.6	—	0.8 $\pm$ 0.2	0.8 $\pm$ 0.2	1.0 $\pm$ 0.3	1.7 $\pm$ 0.5
CV (%)	sensitizing	80.8	47.2	74.0	48.1	37.9	39.5	15.3	33.1	41.1	33.2
	non-sensitizing	56.4	73.7	51.3	95.7	83.9	41.3	15.1	19.6	31.3	47.9

Abbreviations: CV, coefficient of variation; DC, dendritic cell; OVA, ovalbumin; PBMC, peripheral blood mononuclear cell; SI, stimulation index.

^aPeanut sensitizing antigen.^bOVA sensitizing antigen.

with lymphoproliferation to DCs pulsed with sensitizing allergens evaluated by tritium incorporation (correlation R^2 values for CFSE reduction with DC-stimulated tritium incorporation for BLG, OVA, and peanut were 0.626, 0.727, and 0.595, respectively, $P < .001$). Proliferation of CFSE-stained T cells from children without sensitization and those with allergy incubated with DCs pulsed with peanut protein, BLG, and OVA ($3.85 \pm 1.8\%$ and $14.6 \pm 3.1\%$; $4.19 \pm 2.7\%$ and $11.22 \pm 4.1\%$; $5.38 \pm 2.9\%$ and $12.51 \pm 5.2\%$, respectively) were significantly different ($P = .02$, $P = .05$, $P = .06$, respectively), whereas non-sensitized and allergic CFSE-stained PMBCs were not significantly different for any food. The magnitude of DC-stimulated proliferation was related to IgE, with correlation between the SI and total IgE ($r = 0.601$, $P = .04$), in children with allergy. Peanut antigen-stimulated proliferation and peanut-specific IgE were associated ($P = .03$) but specific IgE values of milk or egg in children with allergy were not ($P = .07$, $P = .11$, respectively). Anaphylactic reactivity to food antigens, or any clinical symptom of atopy, was not consistently associated with higher proliferation responses.

The sensitivity of a positive lymphoproliferative response of DC presentation of allergen as a predictive test in food allergy, calculated as true positives as a proportion of true positives and false negatives, was 82.3% overall for the 3 food allergies. Specificity of DC presentation of allergen, calculated as true negatives as a proportion of true negatives and false positives, was 78.5% to indicate the correct food allergy. Table 3 presents the predictive values of positive proliferative responses to peanut, BLG, and OVA allergens independently, which correctly indicated individual sensitizations of 75.0%, 85.7%, and 83.3%, respectively, of the time in children with multiple allergies. A lack of proliferative response correctly indicated non-sensitization to peanut, milk, or egg at 100%, 88.9% and 61.9% of occasions, respectively.

To examine whether children with egg allergy who showed no proliferation response to OVA showed reactivity to other egg antigens, 4 of 6 children with egg allergy who showed no reaction to

OVA, 4 children with egg allergy who reacted to OVA, 4 children with atopy and allergy to other foods, and 4 adults without atopy were tested with albumin and ovomucoid. There was little response to albumin (data not shown) but 2 of 4 children with egg allergy without response to OVA, 3 children with egg allergy with response to OVA, and 1 of the 4 children without sensitization responded to ovomucoid above a level of 3.3 SI (Fig 4). However, responses of children with egg allergy to ovomucoid (mean SI $\pm$ SD, 5.0 ± 2.7 , range 2.2–10.4, $n = 8$) were lower than those to OVA (5.8 ± 6.4 , range 1.3–27.0, $n = 19$). Inclusion of ovomucoid responses in the predictive evaluation of DC-directed lymphoproliferation (Table 2) improved the sensitivity of detection of egg allergy with either antigen from 55.6% to 77.2% and the negative predictive values of the DC-stimulated proliferation test increased from 61.9% to 77.2% of allergic samples. Confidence interval estimates (Table 2) indicate that using DC-stimulated proliferation to diagnose food allergy generates population parameters of specificity, sensitivity, positive and negative predictive values that fall within the intervals of 67.4% or 68.6% to 91% 95% of the time.

Characterization of Proliferating Cells

The proportion of T_H2 , memory, and activated T cells that proliferated in response to food antigens was explored by labeling cultured CFSE-stained cells with cytokine, CD45 RO, and CD25 antibodies. Interferon- γ expression was evident in most proliferating cells (Fig 5A), although the intensity of expression and the proportion of interferon- γ positivity decreased compared with non-CFSE-stained cells. Substantial IL-4 expression ($>10\%$) was detected in some samples of sensitized cells (Fig 5B); however, variability was high and the difference between children without sensitization and those with allergy was not consistent ($P = .08$). CD45 RO expression was marginally higher on sensitized T cells ($P = .063$), initially low in nonproliferating cells (mean 12%, range 3 to 35%), and increased in proportion and intensity of expression

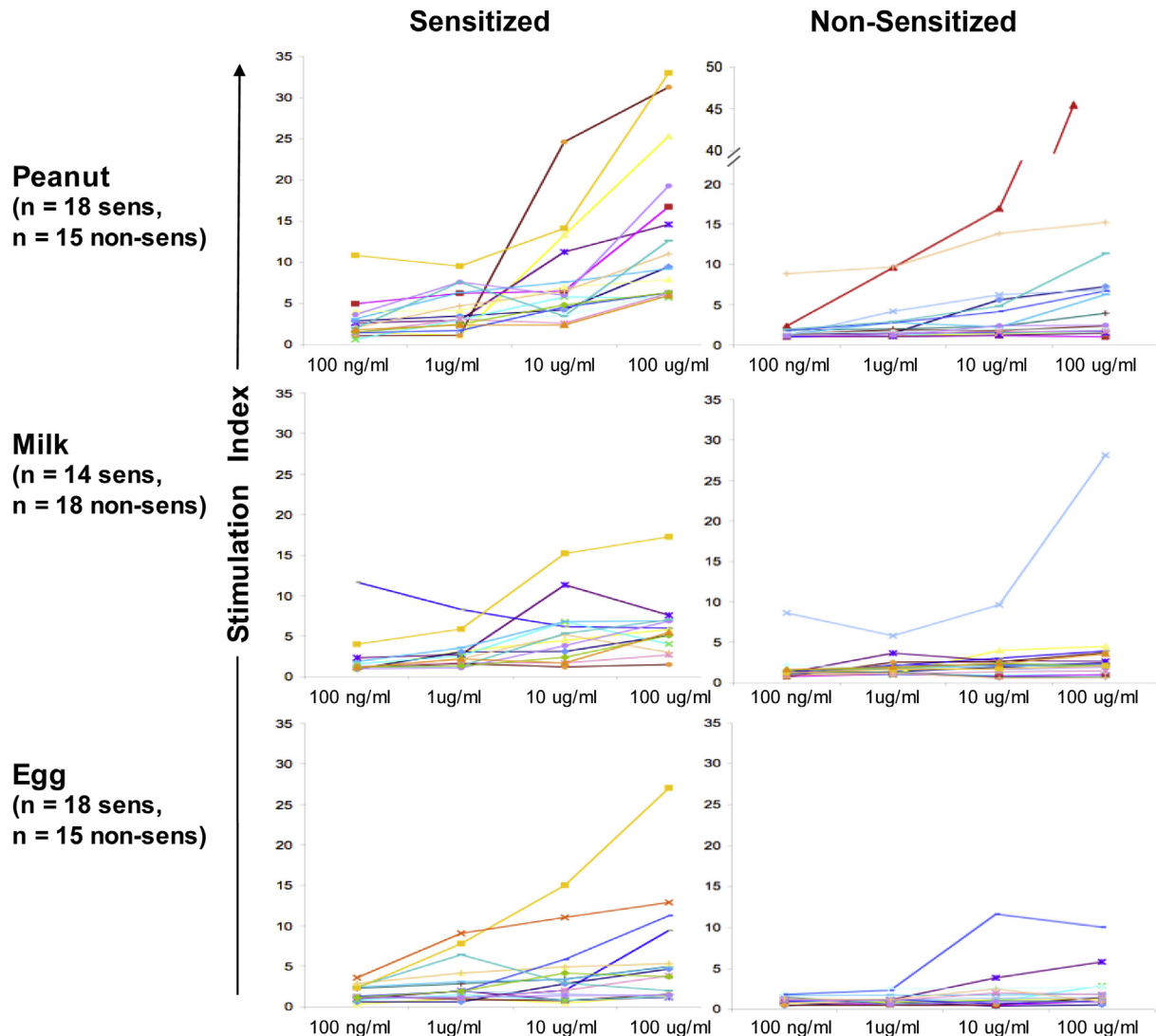


Figure 2. Proliferation of dendritic cell presentation of food antigens in children with allergy and those without sensitization. Purified T cells were mixed in a 10:1 ratio with cytokine-matured autologous monocytes pulsed with defatted peanut protein (upper panels), β -lactoglobulin (middle panels), or ovalbumin (lower panels) in 4 log dilutions from 100 ng/mL to 100 μ g/mL. Stimulation indices = tritiated thymidine incorporation in T cells exposed to by allergen pulsed dendritic cells/T cells mixed with un-pulsed dendritic cells.

(Fig 5C) with replication (cycle 3 mean 98%, range 88–100%). The presence of CD25 was slightly higher on unstimulated cells (mean $12.5 \pm 6.8\%$ vs $9.4 \pm 3.6\%$, $P = .07$, $n = 15$) and on non-sensitized cells compared with those from children with allergy (mean $10.1 \pm 3.9\%$ vs $11.4 \pm 7.9\%$, $P = .11$) but expression decreased with proliferation (Fig 5D) and the difference between sensitized and non-sensitized cells was not significant (Figs 4 and 5).

Discussion

T-cell activation to sensitizing allergens is the determining factor in the establishment of allergy and in maintaining IgE-mediated reactions through cytokine polarization.¹⁵ We examined the response to antigen presentation by autologous DCs in proliferation of purified T cells to sensitizing and non-sensitizing food allergens in children with multiple allergies compared with a conventional mixed PMBC proliferation assay. The isolation of T cells and the use of autologous plasma and a single aliquot of allergen-pulsed DCs cells enhanced individually pertinent stimulation. Responses of children without sensitization and those with allergy to DC presentation of allergen were phenotypically similar to the

conventional PBMC and correlated with proliferation measured by CFSE but generated over 5-fold higher proliferative responses than PBMCs and greater distinction between sensitizing and non-sensitizing allergens.

The difference between proliferation responses of children without sensitization and those with allergy to allergen presentation by monocyte-derived DCs was consistently significant with all 3 food allergens, whereas PBMC lymphoproliferation was not. Allergen-pulsed monocyte-derived DCs used with autologous T cells allergic to inhalant allergens and dust mite proteins generated T_H2 cytokines and greater proliferation in sensitized cells.^{16,17} DCs generated from monocytes of individuals allergic to birch and grass pollen produced increased levels of IL-5 and IL-13 and $CD4^+$ T-cell proliferation when stimulated with Bet v 1 and Phl p 5.¹⁶ Der p 2-pulsed DCs used to stimulate autologous $CD14^+$ cells induced allergen-specific T-cell proliferation, marker expression, and T_H2 cytokine production in patients allergic to dust mite.¹⁷ Culture conditions affect DC phenotype and function¹⁸ with pro-inflammatory T-cell responses promoted by monocyte differentiation in granulocyte-macrophage colony-stimulating factor¹⁹ that could negatively affect other investigations. However, monocyte-derived

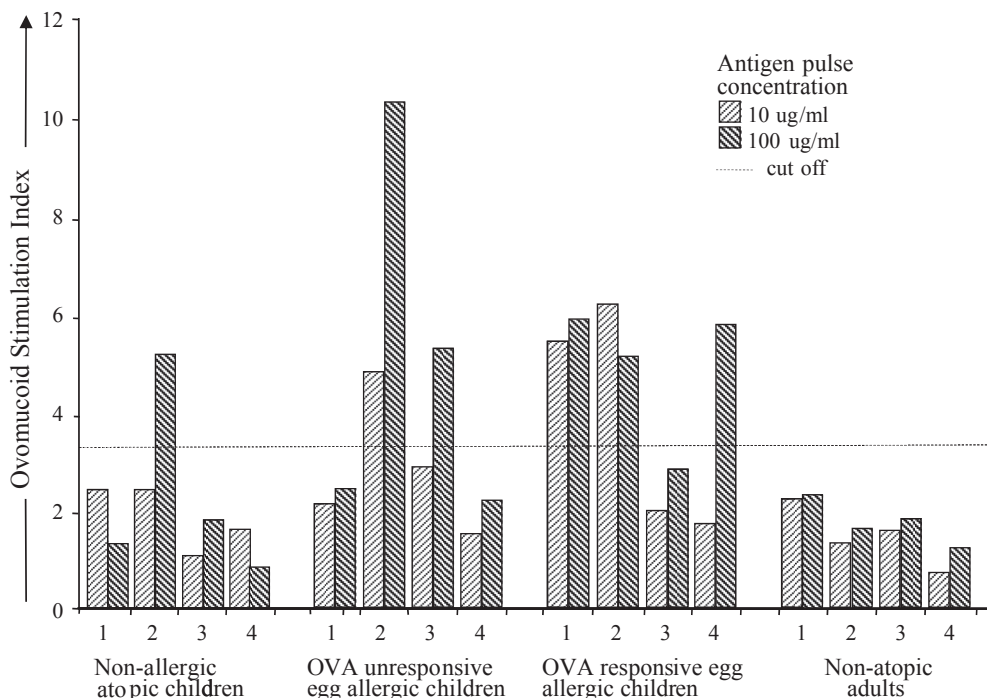


Figure 3. Comparison of proliferation of dendritic cell presentation of ovomucoid in children and adults with egg allergy and those without sensitization. Purified T cells of 4 individuals of 4 groups (children sensitized to egg who responded to ovalbumin [OVA] exposure, children with egg allergy who were unresponsive to OVA, children without sensitization with atopy, and adults without atopy) were mixed in a 10:1 ratio with cytokine-matured autologous monocytes pulsed with 2 concentrations (10 and 100 $\mu\text{g/mL}$) of ovomucoid. Stimulation indices = tritiated thymidine incorporation by T cells exposed to allergen pulsed dendritic cells/tritiated thymidine in T cells exposed to un-pulsed dendritic cells.

DCs express fewer receptors for stimulating T-regulatory cells,²⁰ inducing more memory cells, which could explain the lower background and relatively consistent induction of lymphocytic proliferation. Variation in cell populations could underlie inconsistent PBMC lymphoproliferation responses because PBMC proliferation was influenced by a number of cellular markers, whereas DC proliferation was linearly related to an MHC class II antibody, which interferes with antigen presentation.²¹

The sensitivity of DC-stimulated proliferation to correctly indicate allergy varied with different foods. Peanut and milk allergy was correctly determined more than 90% of the time but egg allergy was relatively poorly designated in approximately two thirds of children with allergy by OVA. Results with outgrown milk allergy could indicate that proliferation to DC-presented allergens could distinguish between clinical allergy and residual sensitization but further investigation is required. However, the level of

accuracy in diagnosing allergy compares well with previous evaluations of sensitivity by lymphocyte proliferative responses and the sensitivity of other tests, with basophil histamine release, for example, having a general positive predictive value of approximately 29% and a negative predictive value of less than 80%.²² In combination with different allergy tests, 60% to 88% of children with milk allergy were correctly identified by convention proliferation testing.^{23,24} PBMC proliferation sensitivity for definitive allergens in contact dermatitis²⁵ and drug hypersensitivity²⁶ have been reported at approximately 60%. The relatively low specificity found in the present study with DC presentation of BLG (88.9%), OVA (86.7%), and peanut protein (60%) reflects the in vitro reaction of children with atopy to foods not detected by IgE or SPT. Proliferative responses to allergens defined as non-sensitizing have been reported in individuals with atopy⁶ and could indicate that proliferation is a more sensitive indicator of sensitization

Table 3
Sensitivity, Specificity, and Predictive Values of Dendritic Cell Allergen Presentation Proliferation Test for Indicating Allergy

	Sensitivity, %	Specificity, %	Positive predictive value, %	Negative predictive value, %
Peanut	100	60.0	75.0	100
Milk	85.7	88.9	85.7	88.9
Egg				
OVA ^a	55.6	86.7	83.3	61.9
OVA + ovomucoid ^b	72.2	80.0	80.0	72.2
Mean ^c	82.3	78.5	81.3	83.6
Mean ^d	86.0	76.3	80.2	87.0
CI				
Upper limit	91.4	91.1	91.4	91.1
Lower limit	68.6	67.4	68.6	67.4

Abbreviations: CI, confidence interval OVA, ovalbumin.

^aPredictive values for egg allergy assessed with OVA.

^bPredictive value for egg allergy with assessed OVA and ovomucoid.

^cMean predictive value for peanut, milk, and egg allergy assessed with OVA.

^dMean predictive value for peanut, milk, and allergy assessed with OVA and ovomucoid.

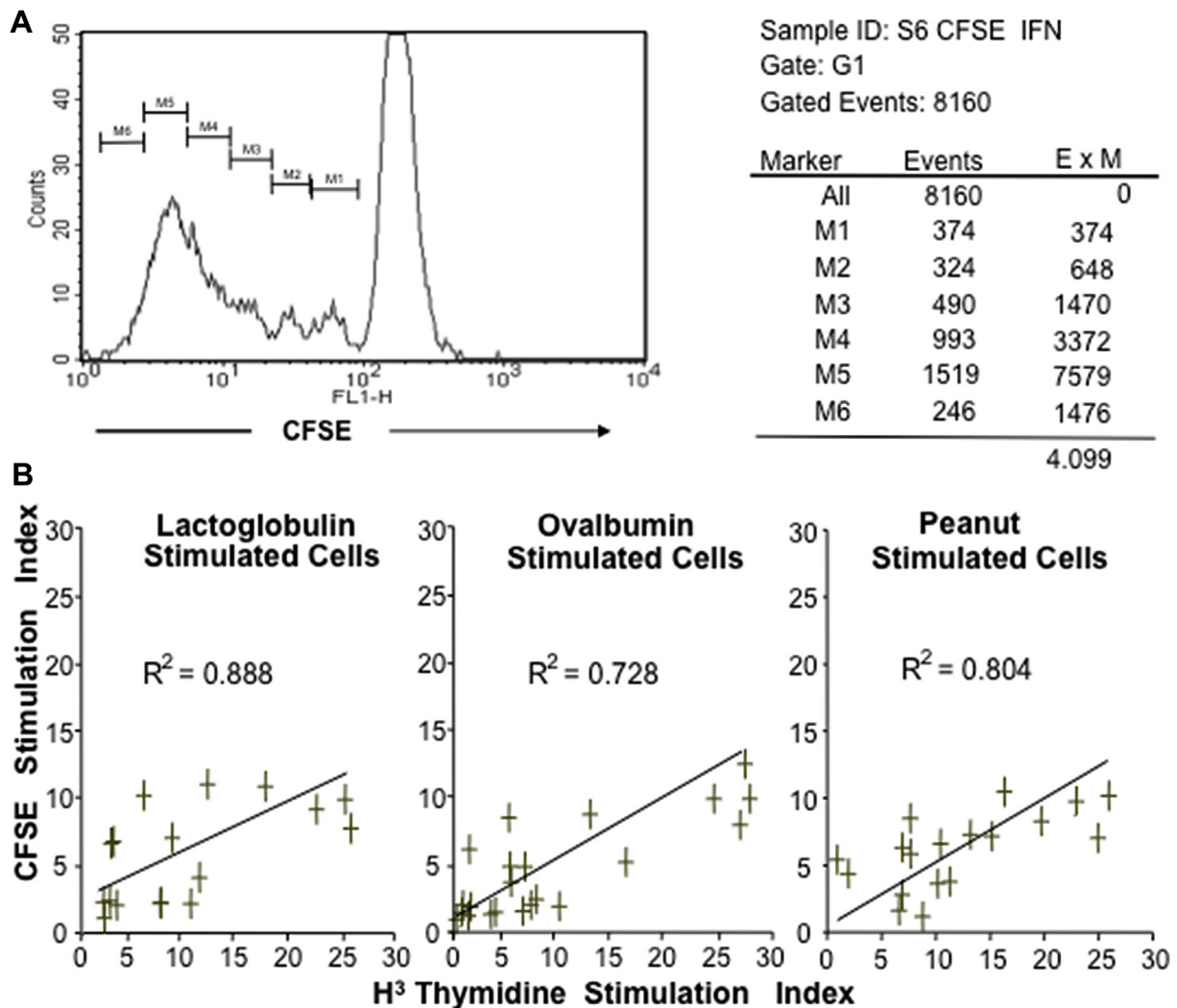


Figure 4. Proliferation of T cells measured by carboxyl fluorescein succinimidyl ester (CFSE) reduction. (A) Flow cytometric analysis of purified CFSE-treated T cells. Cell division is indicated by 50% reduction in fluorescent membrane dye. Proliferative cells and the number of cumulative cell divisions are calculated for each gate. The stimulation index = mean number of cell divisions of stimulated cells/mean cell division of unstimulated cells. (B) Correlation of proliferation responses of children with allergy and those without sensitization stimulated with β -lactoglobulin ($n = 19$), ovalbumin ($n = 20$), and peanut protein ($n = 18$) of tritium incorporation and CFSE reduction. Correlation values (R^2) were generated using Spearman rank coefficient.

than available specific IgE detection methods. If undiagnosed sensitization to food antigens presented by DCs is the explanation for proliferation in children with atopy in this study, then this would indicate that the DC antigen presentation test is more sensitive than IgE detection and that the predictive values generated for the DC test are underestimated.

The higher sensitivity of DC-stimulated proliferation for peanut allergy could be due to the complex allergens present in the peanut protein preparation. Previously, reactions with more peanut peptides recognized by sera of children with allergy correlated with clinical severity of peanut allergy, without direct association of any epitope with allergy.²⁷ Similarly, whole peanut extract generated significantly higher proliferation in children with allergy,^{9,28} whereas a recombinant preparation of the dominant peanut protein Ara h 2 did not.²⁹ Likewise, a combination of casein proteins improved the discrimination between patients with milk allergy and atopic dermatitis and controls without sensitization.² Although proliferative responses were greater to OVA, which better represents clinical egg allergy,^{9,30} higher sensitivity for egg allergy was achieved in this study with additional ovomucoid tests, which

elucidated responses from half the children with egg allergy not responsive to OVA. A combination of component allergens augments the proportion of lymphoproliferative responses and could enhance predictive values of DC-stimulated lymphoproliferative tests in children with sensitization.

There was no association of lymphoproliferation with age or sex. Lymphoproliferation SIs did correlate with total IgE and peanut allergic responses with specific IgE. This finding supports the previous correlation of proliferation with peanut-specific IgE in children with peanut allergy.¹² However, no association occurred with milk or egg specific IgE, cytokine production, or SPT wheal size. T_H1 or T_H2 cytokines in proliferating cells in children without sensitization and those with allergy were affected by CFSE staining and not statistically different, although we previously documented higher $CD3^+ T_H2$ responses to DC stimulation in a similar cohort of children with food allergy.¹¹ Correlation among proliferation, allergen-specific serum IgE levels, and eosinophil counts was found in young children with asthma,³ but specific IgE concentrations in children with milk and egg allergy³¹ or peanut allergy³² have not consistently correlated with clinical severity or

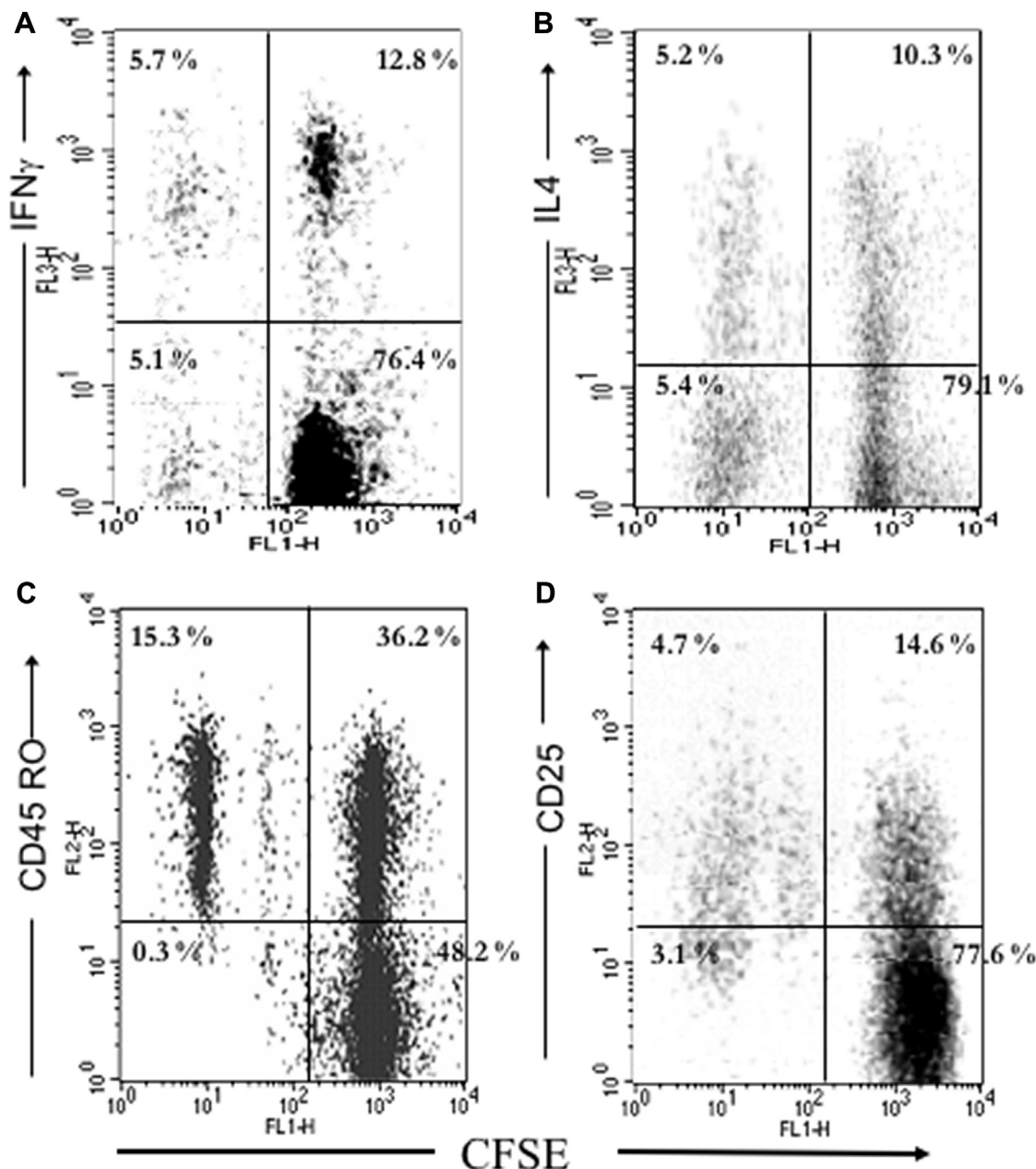


Figure 5. Surface markers and cytokine production in carboxyl fluorescein succinimidyl ester (CFSE)-stained cells. Isolated T cells stained with CFSE were cultured with food antigen-pulsed autologous dendritic cells for 1 week before assessment of expression of (A) CD45 RO, (B) CD25, (C) interferon- γ (IFN- γ), and (D) interleukin-4 (IL-4). Intracellular cytokines were measured using intracellular staining with phycoerythrin-labeled antibodies on cells re-stimulated on day 7 of culture with phorbol myristate acetate and ionomycin in the presence of brefeldin A. Percentages apply to marker or cytokine expression in proliferative cells.

lymphoproliferation in the PBMC assay. Our data suggest that selection of cell populations, for example, extraction of regulatory cells, could overcome some of the individual variability in proliferation assays providing information on important immunologic parameters in allergy, as in other clinical situations.^{33,34} Monocyte-derived DC allergen presentation to purified T cells provides a higher and more consistent evaluation of lymphoproliferation than the conventional mixed PBMC assay and offers a more effective means of discriminating between children without sensitization and those with allergy. It is expected that further selection of antigen-presenting and cell populations sensitized to component

allergens, by reducing non-MHC restricted parameters and indicating immunodominant epitopes, will improve the detection of sensitization by lymphoproliferation and define the signal components of individual allergens.

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