

Association between CD62 ligand on naïve and memory T cells and history of cow's milk hypersensitivity in atopic patients

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Background: Both IgE and non-IgE cell-mediated reactions can contribute to the immunopathogenesis of food hypersensitivity.

Objective: To study L-selectin expression in naïve (CD45RA⁺) and memory (CD45RO⁺) T cells in atopic patients with a history of cow's milk allergy and CD69 expression.

Methods: We evaluated 12 children with a history of cow's milk allergy. All 12 children underwent clinical history, examination, in vivo skin prick testing to inhalants and milk allergen, and in vitro radioallergen sorbent testing for milk and total serum IgE levels. L-selectin levels on naïve and memory T cells from patients and controls were analyzed by flow cytometry with fluorochrome-conjugated monoclonal antibodies after 4 hours or 7 days of incubation with medium alone, 1 μ g/mL of pokeweed mitogen, or 50 μ g/mL of casein.

Results: The percentage of CD45RA⁺ 62L⁺ cells with casein was 56% $\pm$ 11% vs 38% $\pm$ 7.7% in healthy controls ($P < .009$). The percentage of CD45RO⁺ 62L⁺ cells was 22% $\pm$ 7.1% vs 39% $\pm$ 7.6% in healthy controls ($P < .001$), whereas the expression of CD62L⁺ in CD45RA⁺ and CD45RO⁺ cells decreased 149% $\pm$ 33% vs 540% $\pm$ 36% for healthy controls ($P < .001$) and 89% $\pm$ 16% vs 402% $\pm$ 103% for healthy controls ($P < .001$), respectively.

Conclusions: The results of the present study demonstrate the presence of CD62 ligand on naïve and memory T cells, which might serve as a predictive marker for atopic dermatitis with other overlapping atopic disorders, such as asthma and allergic rhinitis.

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INTRODUCTION

Pathophysiologic mechanisms and immune responses related to food hypersensitivity reactions and clinical evaluation have been previously reviewed.¹ Food allergy or food hypersensitivity is the result of abnormal immune reaction to certain food allergens mediated by IgE or non-IgE mechanisms.² The humoral immune system plays an important role in mucosal immunity through IgE- and IgA-bearing cells in lymphoid tissue.^{3,4} The basic regulatory mechanisms of oral tolerance have been shown to involve a suppressive cytokine, such as transforming growth factor β (TGF- β) secreting CD8⁺ cells, which can then act on other immune cells that are naïve to the antigen and suppress their reactivity.¹

Cellular immunity and cytokines have also been suggested to play some roles in the pathophysiology of food hypersen-

sitivity.⁴ In a previous study from our division, we evaluated 15 atopic children and adult patients with atopic dermatitis (AD) and other allergic conditions. A history of food hypersensitivity and milk or egg-protein positivity through skin test or specific IgE test was examined. Milk or egg specific interleukin 2 (IL-2)-triggered T-cell proliferation was demonstrated to decline in several patients after elimination diets for 3 weeks in children and up to 2 months in adults. Diet modification improved cutaneous and gastrointestinal symptoms, and all patients had decreased serum IL-4 levels.⁵

Further studies from our division investigated the role of cellular and serum interferon- γ (IFN- γ), serum IL-4, and IgE levels in several patients with AD.⁶ In mitogen or antigen-stimulated cell cultures, IL-4 levels were detected in a few patients, but decreased levels of IFN- γ were noted compared with healthy controls. In contrast, a study by Schade et al⁷ noted high levels of IFN- γ in AD patients without cow's milk allergy. Thus, cellular and humoral IL-4 and IFN- γ production can be dysregulated in certain patients with AD.⁶

Intact rather than intestinally processed cow's milk proteins have been shown to stimulate peripheral blood mononuclear cells (PBMCs) to release proinflammatory cytokines in patients with cow's milk allergy.⁸ Majamaa and Isolauri⁹ hypothesized that oral introduction of probiotics, defined as a live microbial feed supplement that beneficially affects the host by improving the intestinal microbial balance, may

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prove to be a useful tool for the treatment of food allergy by alleviating intestinal inflammation. Systemic immune response was evaluated by determining the concentration of eosinophilic cationic protein in sera and by determining the cytokine production [IL-4, IFN- γ , tumor necrosis factor α (TNF- α)] of PBMCs.⁹

In a preliminary study in 6 children with AD, we noted dust mite sensitivity from 4+ or class V in 4 children. Three AD children with either cow's milk allergy or egg allergy had increased antigen-stimulated IL-4 levels mostly to cow's milk antigen, which decreased with *Lactobacillus casei* GG, vs 3 AD controls,¹⁰ and IFN- γ levels were negative with food antigens.¹⁰

An important homing receptor on naive T cells is L-selectin (also called CD62L), which has a molecular structure of 74 to 95 kDa. CD62L is expressed on the surface of most circulating leukocytes, including lymphocytes, neutrophils, monocytes, eosinophils, basophils, hematopoietic progenitor cells, and immature thymocytes. L-selectin also mediates the binding of lymphocytes to nonlymphoid vascular endothelium, which is high-endothelial venules at sites of inflammation and can play a significant role in cutaneous inflammation such as AD.

Beyer et al¹¹ noted that severe allergic reactions to foods are predicted by increases of CD4⁺ CD45RO⁺ T cells and loss of L-selectin expression. PBMCs were prepared from 19 children with AD and stimulated in vitro with the suspected allergen (cow's milk, hen's egg), tetanus toxoid, and pokeweed mitogen (PWM). Surface marker expression was evaluated by flow cytometry, and results were compared with the clinical outcome of a subsequent oral food challenge.¹¹ After stimulation with the allergen, a significant increase of CD4⁺ CD45RO⁺ T cells ($P < .05$) was detected selectively for patients who showed severe clinical reactions. The combined evaluation of CD4⁺ CD45RO⁺ and CD4⁺ L-selectin expression on T cells was more sensitive for the prediction of the clinical outcome of the food challenge ($P < .01$) than was measurement of cytokines.¹¹ The purpose of this study was to study L-selectin expression in naive (CD45RA⁺) and mem-

ory (CD45RO⁺) T cells in atopic patients with a history of cow's milk allergy and CD69 expression.

MATERIALS AND METHODS

Patient Demographics

The demographics of the patient population are given in Table 1. Twelve patients evaluated for cow's milk allergy (age range, 6 months to 18 years; 5 males and 7 females) were evaluated by history, physical examination, and skin prick or specific IgE test for cow's milk allergen and total IgE levels. A double-blind food challenge was not performed, since patients were obtained from an ambulatory clinic. Eight patients had either asthma or allergic rhinitis. Six had AD and 3 had urticaria. Positive radioallergosorbent test (RAST) results for cow's milk allergen were noted in 8 patients. Total IgE levels ranged from 25 to 6,252 IU/mL.

Specific and Total IgE

Specific IgE test or RAST for milk protein was performed by a standardized automated enzyme-linked immunosorbent assay (ELISA; Diagnostic Products Corporation, Los Angeles, CA). Total IgE was measured by a standardized ELISA (Bio Merieux, Hazelwood, MO). Samples were added into wells with mouse anti-IgE conjugated with alkaline phosphatase. After washing, the fluorescent substrate, 4-methylumbelliferyl phosphate, was added. The bound enzyme catalyzed the conversion of the substrate and intensity of fluorescence measured by an optical scanner.

Culture and Immunophenotyping of Whole Blood

Heparinized blood was divided into 3 tubes. Cells at 1×10^6 cells/mL were incubated with RPMI medium alone, 50 μ g/mL of casein, or 1 μ g/mL of PWM. Cultures were incubated with 5% carbon dioxide at 37°C for 4 to 6 hours followed by the addition of monoclonal antibodies conjugated to allophycocyanin (APC), fluorescein isothiocyanate conjugated (FITC), or phycoerythrin (PE) as follows: CD4, APC; CD45RA, FITC; CD45RO, FITC; CD62L, PE; and CD69, PE or APC. Cultures were incubated for 30 minutes in the dark at room temperature,

Table 1. Patient Demographics

Age, y	Asthma	AD	Rhinitis	Urticaria	RAST class	Total IgE level, IU/mL
3	Y	N	Y	N	0	65
8	Y	N	Y	N	IV	2,589
5	Y	Y	Y	N	III	711
2	Y	Y	Y	N	III	1,520
2	N	Y	N	N	IV	110
3	Y	N	N	Y	0	28
4	Y	Y	Y	N	IV	1,056
3	N	N	N	Y	I	102
18	Y	Y	Y	N	III	1,956
0.5	N	Y	N	N	0	25
2	Y	N	Y	N	III	1,197
4	N	N	Y	Y	0	6,252

Abbreviations: AD, atopic dermatitis; N, no; RAST, radioallergosorbent test; Y, yes.

and FACS lysing solution was added followed by incubation for 15 minutes. After washing, cells were resuspended in 1% paraformaldehyde and analyzed on the FACS Vantage (Becton-Dickinson, San Jose, CA).

Culture and Immunophenotyping of PBMCs

PBMCs at 1×10^6 cells/mL were separated on Ficoll gradient and incubated with RPMI medium alone, 50 μ g/mL of casein, or 1 μ g/mL of PWM. Cells were washed after 7 days in culture with antigen (casein) or mitogen (PWM) re-added. The same monoclonal antibody panels for CD4, CD45RA/RO, CD62L, and CD69 were added to each tube. Cultures were incubated for 30 minutes in the dark at room temperature, washed, and resuspended in 1% paraformaldehyde. Cells were analyzed on the FACS Vantage (Becton-Dickinson).

Flow Cytometric Analysis

Cells were harvested and washed with phosphate-buffered saline (10 minutes, 1,500 rpm at room temperature) twice. Cells were resuspended in 1 mL of phosphate-buffered saline and incubated with monoclonal antibodies for 30 minutes at room temperature in the dark, washed, and resuspended in 1% paraformaldehyde. The same monoclonal antibody panels for CD4, CD45RA/RO, CD62L, and CD69 were added to each tube. Cells were analyzed on the FACS Vantage (Becton-Dickinson).

Statistical Analysis

The *t* test was applied to determine the differences between the CD45RA⁺ 62L⁺, CD45RA⁻ 62L⁺ expression on CD4⁺ T cells and CD69 expression on CD4⁺ T cells. Differences associated with $P < .05$ were considered statistically significant.

RESULTS

Flow cytometric analysis of whole blood for lymphocytes by forward scatter (FSC) and side scatter (SSC) for all cell

populations are illustrated in Figure 1A. The lymphocyte gate is illustrated in the left, lower, darkened quadrant. Mononuclear cells are illustrated above, reaching 800, and the neutrophil quadrant is illustrated by the larger concentric circular panel. CD4⁻ cells are noted in the lower left panel, and CD4⁺ cells are indicated by the triangular gate.

Flow cytometric analysis of whole blood for CD62L with either CD45RA (naïve cells) or CD4RO (memory cells) are illustrated in Figure 2A and B. The double-negative population is illustrated in the left, lower quadrants, CD62L cells alone in the left, upper quadrant, and the double-positive population for either CD62L/RA or RO in the right, upper quadrants. In Figure 2B, the right, lower quadrant indicates some CD45RO⁺ cells alone. Values are indicated in each quadrant. The percentage of CD45RA⁺ 62L⁺ cells with casein was $56\% \pm 11\%$ vs $38\% \pm 7.7\%$ in healthy controls ($P < .009$). The percentage of CD45RO⁺ 62L⁺ cells was $22\% \pm 7.1\%$ vs $39\% \pm 7.6\%$ in healthy controls ($P < .001$), whereas the expression of CD62L in CD45RA⁺ and CD45RO⁺ cells decreased $149\% \pm 33\%$ vs $540\% \pm 36\%$ for healthy controls ($P < .001$) and $89\% \pm 16\%$ vs $402\% \pm 103\%$ for healthy controls ($P < .001$), respectively.

Loss of L-selectin with either casein or PWM stimulation in whole blood is illustrated in Figure 3. A significant difference between unstimulated cells and PWM was noted ($P = .05$).

Flow cytometric analysis results of incubated cells for FSC and SSC for all lymphocytes are illustrated in Figure 4A. Nonviable cells are indicated in the left, lower quadrant, and activated lymphocytes are indicated in the drawn gate.

The CD4 lymphocyte gate for CD4⁻ cells is illustrated in the left, lower corner of Figure 4B. The left upper-gated population indicates activated CD4⁺ cells.

Flow cytometric analysis results of incubated cells for CD62L with either CD45RA or CD4RO are illustrated in Figure 5A and B. Similar to Figure 2A and B, Figure 5 shows

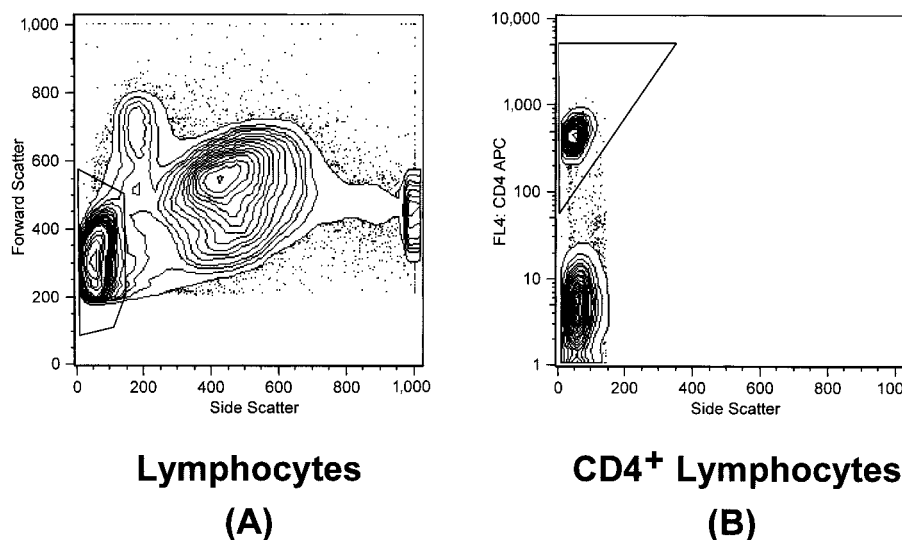


Figure 1. Flow cytometry gating in whole blood. FL4 indicates the intensity of emission of allophycocyanin (APC) used to label the anti-CD4 antibody.

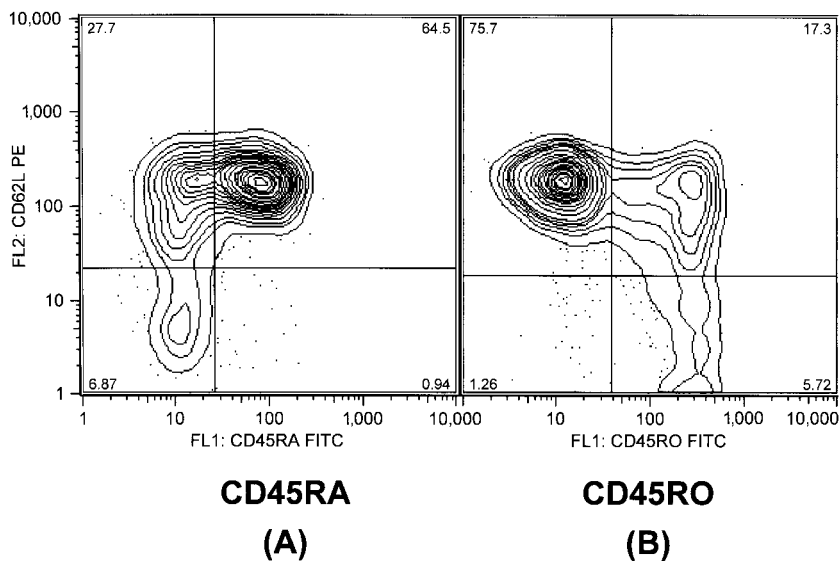


Figure 2. L-selectin expression in whole blood. FL1 and FL2 represent the intensity of emission of fluorescein isothiocyanate (FITC) and phycoerythrin (PE)-labeled anti-CD62L and anti-CD45RA/RO, respectively.

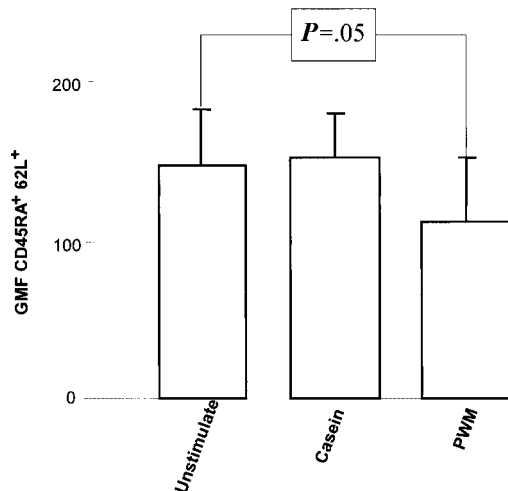


Figure 3. Loss of L-selectin with stimulation in whole blood. GMF indicates geometric mean fluorescence; PWM, pokeweed mitogen. Error bars indicate SEM.

double-negative populations, cells alone, or double-positive populations for either CD62L/RA or RO. Loss of L-selectin reflecting frequency of parent (FoP) with either casein or PWM stimulation in cultured lymphocytes is illustrated in Figure 6.

Similarly, loss of L-selectin reflecting geometric mean fluorescence with either casein or PWM in cultured lymphocytes is illustrated in Figure 7. A significant difference between unstimulated vs PWM stimulation was observed. L-selectin expression for FoP analysis for CD45RA⁺ 62L⁺ stratifying patients with low and high IgE levels is illustrated in Figure 8. A significant increase was noted for patients with high IgE levels ($P = .05$).

An increase in CD69 levels, reflecting FoP analysis for both patients and controls, is illustrated in Figure 9A and B. As indicated in Figure 9A, an increase in CD69 was noted with both casein and PWM; however, there was no change noted for casein and a much smaller response was noted for PWM (Fig 9B).

DISCUSSION

In this article, children with a history of cow's milk allergy had AD and coexisting asthma, rhinitis, and urticaria. Although a double-blind placebo food challenge would have been ideal, this method was not possible in an open ambulatory setting. The expression of L-selectin was lost with stimulation with casein and PWM. This loss of L-selectin was found in both fresh whole blood samples and in 7-day cultured cells in both populations of CD45RA⁺ and CD45RA⁻ cells (Figs 3–7).

Patients who had high total IgE had a higher expression of CD62L on their lymphocytes compared with patients with low total IgE level. CD69, which is a marker for activation, was higher in patients compared with controls and also the increase in the expression of CD69 was much higher with activation with casein and PWM in patients compared with controls (Fig 9A and B).

Children with cow's milk allergy and high total IgE levels had higher expression of CD62L than children with low total IgE levels. Similar to other allergic disorders in cow's milk allergy, lymphocytes became activated in vitro with stimulation with casein.

Sampson¹² reviewed immune intervention in food allergy. Several novel immunotherapeutic strategies are being examined, such as humanized anti-IgE monoclonal antibody and cytokine therapy, immunostimulatory sequence-modulated immunotherapy, plasmid DNA, "engineered" allergen protein, and peptide immunotherapy.¹³ Nucleotide sequences activate antigen-pre-

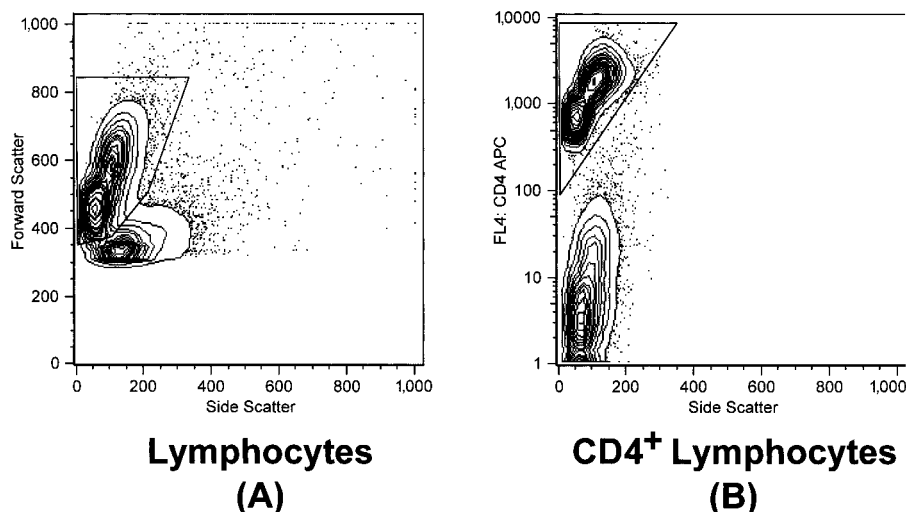


Figure 4. Flow cytometry gating in incubated cells. FL4 indicates the intensity of emission of allophycocyanin (APC) used to label the anti-CD4 antibody.

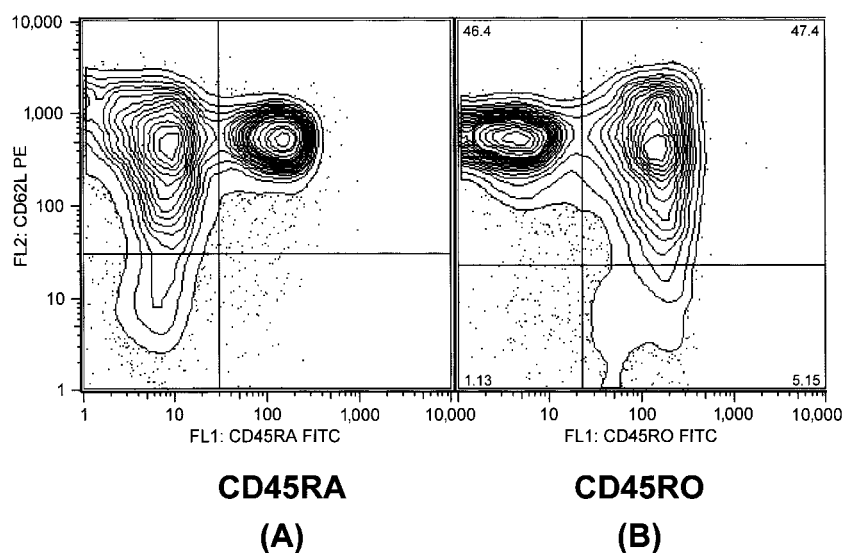


Figure 5. L-selectin expression in incubated cells. FL1 and FL2 represent the intensity of emission of fluorescein isothiocyanate (FITC) and phycoerythrin (PE)-labeled anti-CD62L and anti-CD45RA/RO, respectively.

senting cells (primary monocytes) to secrete IFN- α , IFN- β , IL-6, IL-12, IL-18, and natural killer cells to secrete IFN- γ , both of which promote T_H1 responses.¹³

Cell-mediated or delayed hypersensitivity reactions develop more than 24 to 48 hours when actively sensitized lymphocytes of the T_H1 subtype release various cytokines, such as IL-2, and IFN- γ interacts with specific antigen.⁴ In one study, the combination of cord blood IgE and the proliferative response of cord blood lymphocytes to food antigens appeared to be useful for the prediction of allergic disorders.¹⁴ IL-2 production of cord blood lymphocytes stimulated with food antigens in 24 newborn infants correlated well with proliferative response to ovalbumin or bovine serum albumin.¹⁴

In earlier studies, Kondo et al¹⁵ and Pene et al¹⁶ evaluated lymphocyte responses to food antigens in patients with AD sensitive to foods and IgE production by normal human

lymphocytes induced by IL-4 and suppressed by IFN and prostaglandin E₂.

In an open challenge study in food-hypersensitive adult patients, our division demonstrated that both serum and food antigen-stimulated IL-4 levels decline after dietary restriction.¹⁷ Open or single-blind challenges can be used to screen suspected food proteins, but these must be confirmed by a double-blind challenge.² Thus, an objective measure was identified as a possible biomarker for food allergy.

Cytokines and their role in the pathogenesis of severe food hypersensitivity reactions were evaluated by Jaffe and Metcalfe.¹⁸ Cytokine production from peripheral T cells was found to be abnormal after mitogenic stimulation, which may contribute to elevated IgE level and eosinophilia.

Benlounes et al¹⁹ demonstrated that intact rather than intestinally processed protein stimulated PBMCs to release

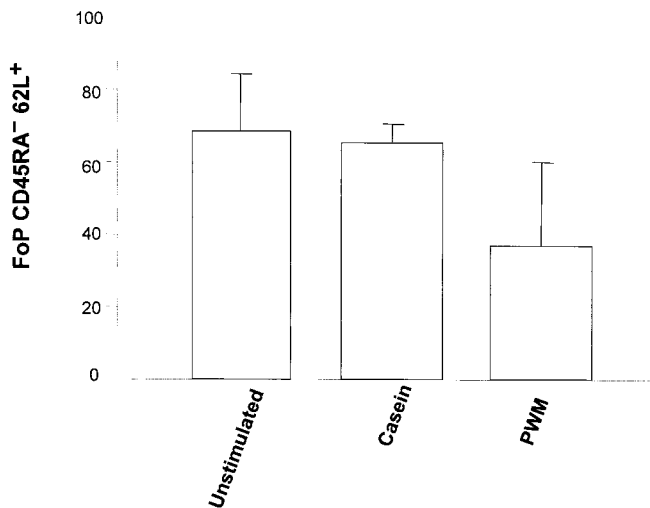


Figure 6. Loss of L-selectin with stimulation in cultured lymphocytes. FoP indicates frequency of parent; PWM, pokeweed mitogen. Error bars indicate SEM.

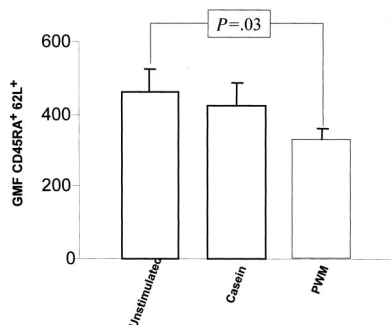


Figure 7. Loss of L-selectin with stimulation in cultured lymphocytes. GMF indicates geometric mean fluorescence; PWM, pokeweed mitogen. Error bars indicate SEM.

TNF- α and alter intestinal barrier capacity. The threshold for PBMC reactivity to milk antigens was also shown to decrease considerably during active cow's milk allergy with intestinal symptoms, suggesting TNF- α may be a good marker of immune reactivity to milk antigens.¹⁹

After passage through the mucosa and processing by antigen presenting cells, the antigen is presented to lymphocytes for subsequent reactions. Selected presentation in association with class I antigens may lead to specific CD8⁺ T suppressor cell activation, whereas class II antigens activate CD4⁺ T cells and lead to memory induction.¹ Thus, a regulatory interaction between CD4⁺ and CD8⁺ T cells is possible.²⁰ Activated CD8⁺ T cells can also provide a negative signal to autoreactive cells in affected target organs provided by TGF- β . In the absence of a costimulatory signal [eg, via CD28-CD8⁺ (B7 family)], T-cell anergy or tolerance could be induced.¹⁹

A specific T-cell-mediated immune response to casein was found in the blood of adolescent and adult patients with

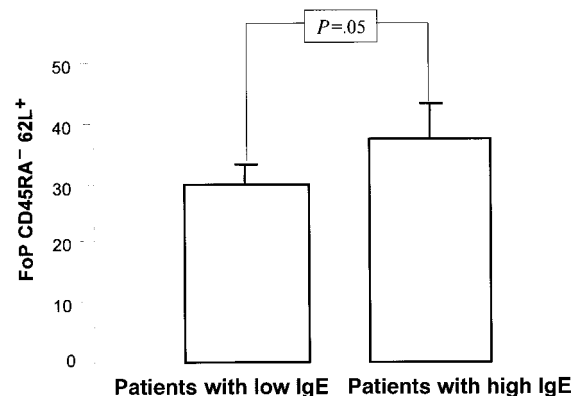


Figure 8. L-selectin expression in patients. FoP indicates frequency of parent. Error bars indicate SEM.

milk-related exacerbation of AD.²¹ In contrast to house dust mite-specific T cells, casein-specific T cells of adult patients who respond to cow's milk with worsening of AD produce little or no IL-4. Thus, in older patch test lesions and in chronic lesions of AD, type 2 cytokines, such as IL-4, are down-modulated and type I or IgE-mediated cytokines, such as IFN- γ , may be predominant.²²

Type IV, cell-mediated immune or non-IgE-mediated reactions have been reported in food-allergic patients.^{5,6,8,9,14,23,24} Although in vitro T-cell responses are commonly reported in healthy subjects,²⁵ studies suggest that activation of food antigen-specific lymphocytes in milk-induced eczema lead to a preferential homing of lymphocytes to specific target organs.²⁶ PBMCs from a group of children with eczema and type I milk hypersensitivity stimulated with the milk protein casein resulted in T-cell proliferation. The T cells in this study expressed the cutaneous lymphocyte antigen, but no expression was noted in milk-sensitive children with asthma or gastrointestinal allergy.²⁶

A study using lymphocyte transformation tests was performed in healthy adults to determine the mitogen-induced lymphocyte proliferative responses to bovine caseins hydrolyzed with pepsin and trypsin and bovine caseins hydrolyzed with enzymes derived from *L. casei*.²⁷ Lymphocyte proliferation was only stimulated with K-casein hydrolyzed with pepsin and trypsin. However, suppression of lymphocyte proliferation was noted with the bacterial hydrolyzed caseins.²⁷ This study established that degradation of food antigens with *L. casei*-derived enzymes modifies their immunomodulatory activity and leads to suppression of PBMCs in health. Such suppression of cell-mediated immunity, with simultaneous preservation of IgE production and IFN- γ producing T-cell reactivity, evolves after a phase of T-cell-mediated regulation as a result of continuous allergen exposure.²⁸

In conclusion, the results of the present study demonstrate that high total IgE levels correlate with increased levels of CD62L expression. Although the precise biologic significance of our findings is not entirely clear, they suggest an

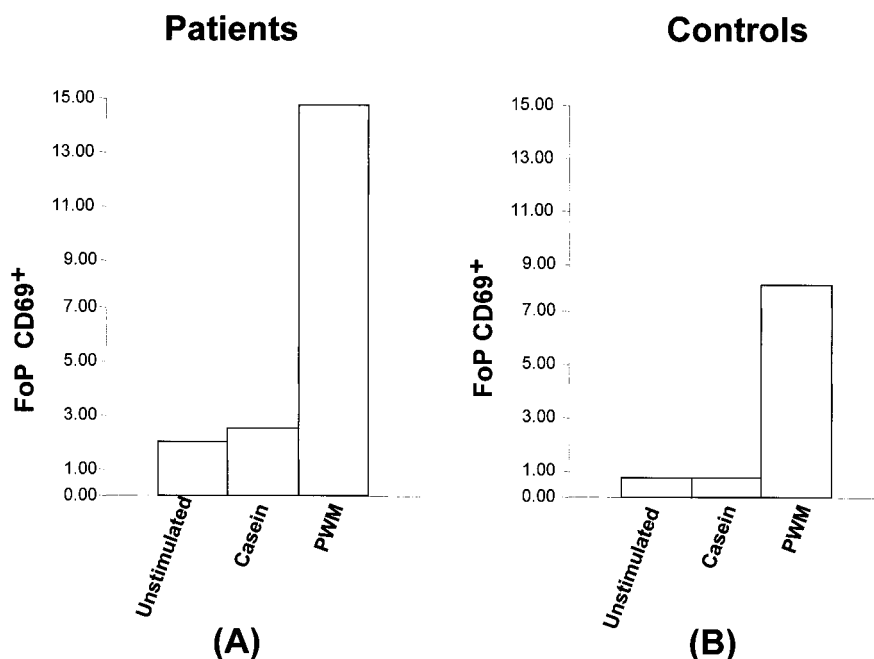


Figure 9. Increase in levels of CD69 with stimulation. FoP indicates frequency of parent; PWM, pokeweed mitogen.

important link between AD with a history of milk hypersensitivity and other associated allergic conditions, such as asthma and allergic rhinitis, which might serve as an important predictor of disease activity in these patients.

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