

Expression of intercellular adhesion molecules on circulating lymphocytes in relation to different manifestations of cow's milk allergy

P. Österlund*, T. Smedberg*, J. Schröder† and K.-M. Järvinen*

*Skin and Allergy Hospital, Department of Dermatology, Helsinki University Central Hospital, Helsinki, Finland and †Department of Biosciences, Division of Genetics, University of Helsinki, Helsinki, Finland

Summary

Background The complex interactions between immune cells are partly mediated by different adhesion molecules, but little is known about their role in the systemic immunoinflammatory process following sensitization to food antigens in early infancy.

Objective The aim of this study was to investigate the expression of intercellular adhesion molecule-1 (ICAM-1 or CD54) and the α subunits of its ligands' lymphocyte function-associated antigen-1 (LFA-1) (α^L subunit or CD11a) and Mac-1 (α^M subunit or CD11b) on peripheral blood leucocytes in infants with cow's milk allergy (CMA) and in healthy controls.

Methods Thirty-nine breastfed infants, aged from 0.6 to 8.3 months, and their lactating mothers were included in the study from delivery onwards. During follow-up, 25 infants developed CMA and 14 remained healthy. Expressions of CD54 and CD11b on peripheral blood leucocytes were evaluated by flow cytometry. In addition, the expression of CD11a on peripheral blood leucocytes was analysed by immunocytochemistry. Mothers' milk samples were collected and their leucocyte content was evaluated using a light microscope.

Results The frequency of ICAM-1 expressing peripheral blood lymphocytes was significantly higher in patients with CMA than in healthy infants ($P = 0.03$, Mann–Whitney U -test). Furthermore, the high proportion of ICAM-1-expressing cells was associated with gastrointestinal and multiorgan symptoms in the CMA infants. There was no significant difference in the expression of Mac-1 α^M on lymphocytes in our study groups, but the LFA-1 α^L expression seemed to be higher in the IgE-mediated CMA.

Conclusion We suggest that the high expression of ICAM-1 on peripheral blood lymphocytes may reflect enhanced stimulation of T cells *in vivo* and their migration to the effector tissues in an early-phase of developing CMA. Furthermore, high ICAM-1 expression may be associated with the presence of multiorgan manifestations of CMA, whereas high LFA-1 expression may reflect the IgE-mediated disease.

Keywords adhesion molecules, cow's milk allergy, flow cytometry, immunocytochemistry, food allergy, gastrointestinal symptoms, ICAM-1, infants, LFA-1, Mac-1

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Introduction

Cow's milk allergy (CMA) is frequently the first manifestation of the 'allergic march' in infancy [1]. CMA is an example of a disturbance in the development or breakdown of oral tolerance to ingested milk proteins. The symptoms of CMA commonly appear during the first months of life, within days or weeks after commencing feeding with a cow's milk-based formula, or even during exclusive breastfeeding [2, 3]. Immune mechanisms behind the development of CMA are still incompletely understood, especially in infants with delayed-type hypersensitivity [4]. There is some evidence

supporting a delayed maturation of Th1 cells in CMA and allergic diseases [5–7].

Interactions between cells are mediated by a magnitude of molecules. Different adhesion molecules, the most important of which are selectins, the leucocyte-specific β_2 -integrins and the intercellular adhesion molecules (ICAMs), play a central role in the interaction of cells of the immune system [8]. The β_2 -integrins, lymphocyte function-associated antigen-1 (LFA-1) and Mac-1, consist of the same β subunit (CD18) and of a specific α subunit, α^L (CD11a) in LFA-1 and α^M (CD11b) in Mac-1. ICAM-1 is constitutively expressed at low levels on the surface of a wide variety of cell types and is up-regulated in response to several inflammatory mediators [9]. ICAM-1 interactions with the β_2 -integrins, such as LFA-1 and Mac-1 on the surface of leucocytes, are important for their transendothelial migration to the sites of inflammation and necessary for their function as co-stimulatory molecules for

Correspondence: P. Österlund, MSc, Skin and Allergy Hospital, Department of Dermatology, Helsinki University Central Hospital, PO Box 160 FIN-00029 HUS, Helsinki, Finland. E-mail: pamela.osterlund@helsinki.fi

T cell activation [9, 10]. In atopic diseases, the previous studies on adhesion molecules mostly deal with the homing of leucocytes to the inflammation site either in the skin lesions or in the mucosa of the small intestine [11–13]. Little is known about their role in the systemic immunoinflammatory process following sensitization to the food antigens in early infancy.

Human milk is believed to impart specific immune advantages to the neonate through enhancement or induction of the still-developing neonatal immune system [14]. We have previously shown that the immunological composition of human milk differs significantly in mothers with an infant allergic to cow's milk from that of mothers with a healthy infant, potentially influencing the health status of the breastfed infant [15–17]. However, the association of specific immune factors in mothers' milk with markers of systemic immune activation in breastfed babies has not been investigated previously.

The purpose of this study was to evaluate the proportions of compatible co-stimulatory adhesion molecules (e.g. ICAM-1 and LFA-1/Mac-1) expressing peripheral blood lymphocytes from CMA infants and healthy controls as markers of immune activation. We also sought the association of mother milk leucocyte subsets with adhesion molecule expression of the breastfed infant.

Materials and methods

Subjects and study protocol

The study population comprised 39 breastfed infants, aged from 0.6 to 8.3 months, and their 38 lactating mothers. The infants were followed prospectively from delivery: one group ($n = 31$) because of the infant's high risk for atopy/allergy (atopic or food-allergic sibling(s) and/or parent(s)) and the other group ($n = 8$) because of the infant's low risk for atopy/allergy (no signs of atopy in first-degree relatives). Of the breastfeeding mothers, 19 (50%) had atopic constitution (hayfever, atopic dermatitis or asthma) and 19 were non-atopic. The mothers and their infants were examined and blood and human milk samples were collected frequently (0, 1, 3, 6, 12 and 24 months) to detect the development of CMA. Infants showing symptoms suggestive of CMA (urticaria, atopic eczema, diarrhoea, vomiting, abdominal pain/infantile colic) were tested for cow's milk-specific IgE and underwent a cow's milk elimination-challenge procedure to confirm the diagnosis. All the infants were born at term and had no other chronic diseases or infections at the time of the study. The parents of these infants gave written informed consent to the study. Mothers with clinical mastitis during the preceding 4 weeks were excluded. The Ethical Committee of the Skin and Allergy Hospital of the Helsinki University Central Hospital and the City of Helsinki approved the study protocol.

Oral cow's milk challenge

The diagnosis of CMA was confirmed by open cow's milk challenge, which gives appropriate accuracy in infants [18]. After elimination of cow's milk for 2–4 weeks, patients were admitted for a cow's milk challenge. The challenge was started with a drop of cow's milk on the skin or lips.

Thereafter, cow's milk was given in increasing doses at half-hour intervals: on day 1: 1, 10, 50 and 100 mL and on day 2: the normal milk intake appropriate to the infant's age was commenced. The challenge was stopped immediately when any adverse reaction was noticed. The patients were followed over a 1-week period of challenge. Reactions occurring within an hour from the last dose on the first day were defined as immediate ones and the reactions occurring thereafter were considered to be delayed ones. All the elimination diets as well as cow's milk challenges were carried out based on clinical indications, and not for research purposes.

Skin prick tests (SPTs) and RAST

SPTs were performed on the volar side of the forearm using a commercial cow's milk extract (ALK, Hørsholm, Denmark). Histamine dihydrochloride 10 mg/mL (ALK) was used as a positive control and at least a 5 mm diameter of the reaction was taken as an adequate histamine reaction. Reactions were read at 15 min and at least a half (3 mm) of the size of histamine was taken as a positive reaction. The commercial RAST method (Pharmacia Diagnostics, Uppsala, Sweden) was used to measure cow's milk-specific IgE antibodies in patients' sera.

Heparin and EDTA blood samples

Blood was drawn both in heparin and in EDTA. Peripheral blood mononuclear cells (PBMC) consisting mainly of lymphocytes were isolated from heparinized blood by Ficoll-Hypaque gradient centrifugation at 400g for 30 min at 20 °C. The cells were resuspended in RPMI-1640 containing 5% fetal calf serum (FCS, Sigma, Steinheim, Germany), antibiotics (penicillin 100 IU/mL and streptomycin 100 µg/mL) and 2 mM L-glutamine (Sigma, Irvine, UK). After washing three times, the cells were visually counted in Bürker chambers with a light microscope, and were used for flow cytometry and immunocytochemistry as follows. EDTA blood was used for flow cytometry as described below.

Flow cytometry

Fifty microlitres of blood-EDTA samples and 2×10^5 PBMC in 0.2 mL were taken per FACS sample. The blood-EDTA samples were treated at room temperature (RT) to improve the effects of the FACS lysing solution (Becton Dickinson (BD), San Jose, CA, USA) and the PBMC samples at 4 °C to better the cell survival. The samples were incubated for 15 min with an appropriate concentration of fluorescent monoclonal antibodies against CD11b (Mac-1 α^M subunit), CD54 (ICAM-1), CD4, CD8, CD19, CD23, CD16⁺CD56, CD45 or CD14 (BD). The blood-EDTA samples were incubated with the FACS lysing solution (BD) for 5 min at RT to lyse the erythrocytes. After washing, the EDTA and PBMC samples were analysed with a flow cytometer (FACScan, BD).

Immunocytochemistry

Cytospin preparations of PBMC were obtained with a sample application of 1×10^5 cells per slide (Cytospin3, Shandon, Life Sciences International (Europe) Ltd, Astmoor, Runcorn,

Cheshire, UK). The cytosin slides were air-dried and stored as frozen. The slides were slowly thawed and then fixed with acetone for 10 min at RT. After protein blocking with BSA solution, the slides were stained with primary antibodies to CD11a, which recognize the α^L subunit of the LFA-1 (Novocastra Laboratories Ltd, Newcastle, UK), at a dilution of 1:400. Incubation with anti-CD11a was performed at RT in a humidity chamber for 30 min. The antigen–antibody complex was visualized using a commercial AEC kit (K3461, DAKO Corporation, Carpinteria, CA, USA) in accordance with the manufacturer's instructions. Slides were covered with Mountex (Histolab, Göteborg, Sweden). Immunostained cell samples were examined microscopically and the results were expressed as percentages of positive cells. Immunolabelling was carried out in a single series to minimize batch-to-batch variation.

Human breast milk samples

The breast was washed with warm water and fresh human milk was collected by manual expression or with a breast pump, always at the same time in the morning. The volume of each milk sample was measured and the samples were immediately centrifuged (400 g at RT for 15 min) to remove the lipids and the whey. The leucocytes were then collected and resuspended in RPMI-1640 containing 5% FCS, antibiotics (penicillin 100 IU/mL and streptomycin 100 µg/mL) and 2 mM L-glutamine. After four washes the cells were visually counted in Bürker chambers with a light microscope.

Light microscopy

Cytospin preparations of milk cells were obtained with a sample application of 1×10^5 cells. The slides were treated with Vectapond™ (Vector Laboratories Inc., Burlingame, CA, USA) before sample application to improve the fixation of breast milk cells. The numbers of macrophages, monocytes, lymphocytes, neutrophils and eosinophils were evaluated on slides stained with application of Wright (Merck, Darmstadt, Germany) combined with May-Grünwald-Giemsa (MGG, Oy Reagenia Ltd, Kuopio, Finland).

Statistical analysis

Expressions of surface antigens of leucocytes were compared in patients and controls by non-parametric tests (Mann–Whitney *U*-test and Kruskal–Wallis test) because of the small sample counts in the study groups. The χ^2 test was applied to determine differences in proportions. We sought associations between data with Spearman's rank correlation test: results are given as correlation coefficient ρ and *P*-value. The analyses were carried out with NCSS2000 software (Kaysville, UT, USA).

Results

Clinical characteristics of CMA infants and controls

By the end of the follow-up, 25 infants had challenge-proven CMA and 14 infants remained healthy. We used only one sample from each of the subjects in the analyses, which was

chosen from the time point of early CMA (all the infants either showed symptoms suggestive of CMA or had recently been diagnosed with CMA), and their age-matched controls. At that time, the median age of the infants with CMA was 3.7 months (range: 0.6–7.5 months) and that of control infants was 3.5 months (range: 0.9–8.3 months). A positive family history of atopy was detected in 88% of CMA infants and in 64% of control infants, $P = 0.08$ (χ^2). In the 25 CMA infants, the symptoms suggestive of CMA were as follows: urticarial or eczematous skin eruptions ($n = 6$), or gastrointestinal symptoms ($n = 4$), or both skin and gastrointestinal symptoms ($n = 12$), and multiorgan symptoms (skin, gastrointestinal and respiratory, $n = 3$). Of these 25 infants with CMA, 11 had symptoms at the time of the analyses and 14 were free of symptoms due to a successful elimination diet. Four out of the 25 infants with CMA and one out of the 14 healthy infants had developed asthma.

CMA was diagnosed at the median age of 4.8 months (range: 2–16 months). In the clinical cow's milk challenge, four patients manifested urticaria immediately after the introduction of a drop of cow's milk on their skin or lips. The patients challenged perorally manifested as either urticarial or eczematous skin eruptions ($n = 5$), and reacted mainly in an early phase of the challenge, mean 1.75 h (range: 0.5–3 h), after beginning the challenge in response to low volumes of milk, median total dose of 50 mL (range: 22–52 mL). Those with gastrointestinal symptoms or slowly developing eczema ($n = 16$) frequently showed delayed reactions after a mean of 50 h (range: 1–144 h), in response to larger amounts of milk, median 137 mL (range: 12–3300 mL). Of these CMA infants, 32% (8/25) had positive SPT or RAST to cow's milk.

Expression of ICAM-1 and Mac-1

The frequency of ICAM-1 expressing peripheral blood lymphocytes, measured by flow cytometry, was significantly higher in the infants with CMA than in the healthy ones ($P = 0.03$, Mann–Whitney *U*-test) (Fig. 1). Moreover, higher

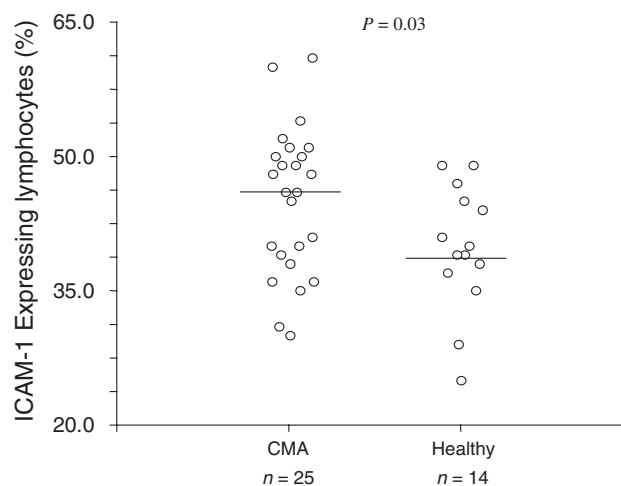


Fig. 1. Intercellular adhesion molecule-1 (ICAM-1) expressing peripheral blood lymphocytes in infants with cow's milk allergy (CMA) and in healthy controls. The horizontal lines denote the median values, 46% (range: 30–61%) and 39.5% (range: 25–49%), respectively. *P*-value calculated with the Mann–Whitney *U*-test.

Table 1. ICAM-1, LFA-1 and Mac-1 expressing peripheral blood lymphocytes (%) in cow's milk-allergic (CMA) infants with different clinical features and in healthy controls

	n	ICAM-1	n	LFA-1	n	Mac-1
Symptoms of CMA						
Skin	6	38	5	21	3	21
Gastrointestinal (GI)	4	47	3	19	1	13
Skin and GI	12	50	11	21	9	19
Skin, GI and respiratory	3	49	3	42	2	14
Reaction type in challenge						
Drop on skin	4	50	4	29	3	15
Immediate	5	36	4	28	3	20
Delayed	16	46	14	19	9	20
Positive skin prick test or RAST						
Yes	8	47	7	31	5	21
No	17	45	15	19	10	19
Symptoms at the time of test						
Acute symptoms	11	46	10	25	6	17
Symptom-free	14	47	12	19	9	20
Healthy infants	14	39	14	22	6	18

Results are expressed as medians.

Statistics with Kruskal–Wallis or Mann–Whitney *U*-test (*significant at $P < 0.05$).

ICAM-1 expression was found in the infants with CMA who manifested with skin and gastrointestinal or multiorgan symptoms than in those with skin symptoms only or healthy controls ($P = 0.005$, Kruskal–Wallis test) (Table 1). The ICAM-1 expression was not associated with the positive SPT or RAST or with the presence of active symptoms of CMA at the time of the sample collection (Table 1). The ICAM-1 expression was lower in the CMA infants reacting immediately in the challenge, frequently manifesting with skin symptoms, than in those with delayed reactions (Table 1). The expression of the α^M subunit of Mac-1 (CD11b) was comparable on mononuclear cells (Table 1) and total blood leucocytes in our two study groups (data not shown).

Expression of LFA-1

LFA-1 expression was assessed by measuring the expression of its α^L subunit (CD11a) from the peripheral blood lymphocytes by immunocytochemistry. The proportions of LFA-1 α^L expressing lymphocytes were comparable in both CMA and control infants, median 21% (range: 14–59%) and 21.5% (range: 3–35%), respectively ($P = 0.53$, Mann–Whitney *U*-test). However, the expression of LFA-1 was higher in the CMA infants reacting immediately at the challenge than in those with delayed reactions or healthy controls (Table 1). Furthermore, LFA-1 expression was higher in the infants having positive SPT or RAST than in those with negative SPT or RAST (Table 1).

Proportions of mononuclear cell types

We analysed the proportions of different mononuclear cell subtypes by flow cytometry. Both CMA infants and healthy

controls had comparable proportions of T cells ($CD4^+$ and $CD8^+$), B cells ($CD19^+$ and $CD23^+$), NK cells ($CD16^+CD56^+$ but $CD3^-$) and monocytes ($CD14^+$) (data not shown).

Cellular composition of mother's milk

The proportions of different leucocytes in mother's milk were analysed with a light microscope. As reported in our earlier study [15], the percentage of lymphocytes in the milk of atopic mothers was higher than in that of non-atopic mothers ($P = 0.03$, Mann–Whitney *U*-test). There were no differences in mother's milk leucocyte contents between the CMA and healthy breastfed infants (data not shown). However, the proportion of mother's milk neutrophils positively correlated with the proportion of ICAM-1 expressing peripheral blood lymphocytes in breastfed infants ($P = 0.04$, $r = 0.32$, Spearman correlation test). Furthermore, the mother's milk macrophages tended to correlate negatively with the proportion of ICAM-1 expressing lymphocytes of breastfed infants ($P = 0.1$, $r = -0.25$).

Discussion

In the present study, we have for the first time evaluated the expression of two integrins, LFA-1 and Mac-1, and their complementary ligand ICAM-1 on peripheral blood leucocytes of CMA and healthy infants. We found significantly higher proportions of ICAM-1 expressing lymphocytes in CMA infants having gastrointestinal or multiorgan symptoms when compared with those manifesting with only cutaneous symptoms or healthy controls. At the same time, the proportions of Mac-1 α^M expressing cells were comparable between our study groups, but the LFA-1 α^L expression seemed to be higher in the IgE-mediated subgroup of CMA infants.

Food allergy is a disease manifested in multiple effector organs, some distant from the initial sensitization site in the gut. This suggests that migrating cells may play a major role not only in the pathogenesis of the disease but also in the regulation of the immune response [19] and determining the sites of involvement in tissue-directed allergic responses. Milk-specific lymphocytes were found to express more cutaneous lymphocyte antigen (CLA) in milk-allergic patients with atopic dermatitis, suggesting preferential homing to the skin of circulating milk-specific T cells from these patients [20]. $\alpha_4\beta_7$ -integrin is described as an integrin expressed in activated T cells with homing properties to the gut, and demonstrated to be highly up-regulated in β -lactoglobulin (BLG)-specific T cells from patients with CMA [21]. In allergic conjunctivitis in mice, antibodies against ICAM-1 and LFA-1 inhibited the clinical and histological signs of the disease, suggesting these molecules to be critical for the homing and migration of leucocytes into the inflamed tissues in this disease [22]. Interactions of ICAM-1 with LFA-1 and Mac-1 on the surface of leucocytes are important for their transendothelial migration [9]. The high proportion of circulating ICAM-1 expressing lymphocytes in CMA infants reported in the present study could reflect their high activation stage for migrating to the site of inflammation.

In our study, CMA patients who had high numbers of ICAM-1 expressing lymphocytes presented with gastrointestinal, skin and gastrointestinal or multiorgan symptoms, whereas those who manifested with only skin symptoms had ICAM-1 expression similar to the controls. Although the numbers of subjects in these subgroups of CMA are quite small, these results suggest that high ICAM-1 expression may be associated with the presence of gastrointestinal or multiorgan manifestations of CMA. Furthermore, the infants who manifested with immediate-type reactions expressed lower levels of ICAM-1⁺ lymphocytes than those with delayed reactions. Inconsistently, the infants who showed urticaria at cutaneous challenge expressed high levels of ICAM-1⁺ cells. However, it is impossible to speculate whether their oral challenges, if performed, would have shown immediate- or delayed-type reactions.

Interactions of ICAM-1 with LFA-1 and Mac-1 are also important for their function as co-stimulatory molecules in T cell activation [9, 10]. Because most leucocyte functions depend on cell-cell contact, they are strictly controlled both at the level of specificity and strength of interaction. Thereby, several molecular systems are involved beginning from the selectin-induced weak contact between the cells, followed by the firm adhesion through integrin intercellular adhesion molecular binding, which allows the stimulating antigen presentation to occur [8]. In a previous study, it has been demonstrated that anti-CD54 (anti-ICAM-1) antibodies effectively suppressed the proliferative responses of PBMC both in patients with atopic dermatitis and in healthy controls [23]. Consistently, a high proportion of ICAM-1 expressing lymphocytes found in the present study in infants with CMA might reflect a high activation and proliferation of the circulating lymphocytes in these patients. In atopic dermatitis, a decrease in the level of serum ICAM-1 was correlated with the improvement of the disease, suggesting this to be a useful inflammatory marker in monitoring disease activity [24]. All our study patients with a high expression of ICAM-1 already had symptoms suggestive of CMA or had recently been diagnosed with CMA reflecting enhanced immune activation in infants who are in the early phase of developing CMA. This being the case, it is surprising that ICAM-1 expression was not increased in patients with cutaneous symptoms only. Furthermore, these patients showed active atopic eczema at the time of analyses, further arguing against a role for ICAM-1 solely as an inflammation marker.

In this study, the high expression of LFA-1 on lymphocytes was associated with the IgE-mediated CMA. LFA-1 has been reported to up-regulate IgE synthesis in B cells [25]. Also, in mast cells usage of anti-LFA-1 and anti-ICAM-1 antibodies inhibited the adhesion-induced mast cell degranulation [26]. Moreover, eosinophils from atopic individuals have shown to express more LFA-1 than non-atopic controls, but similar levels of Mac-1 [27]. The high LFA-1 expression seen in our patients with a positive SPT or infants reacting immediately at cutaneous challenge could reflect the IgE-mediated mast cell activation and, furthermore, eosinophil activation.

Because all the infants in the present study were still being breastfed at the time of symptoms suggestive of CMA, we sought to investigate the relationship between breast milk leucocyte composition and adhesion molecule expression in infants. Surprisingly, we found the breast milk neutrophils to

be positively correlated with the ICAM-1 expression in the infant. Although the precise effect of immunological components of human milk on breastfed infants is unknown, it is believed that they gain access to the infant's immune system with a contributing effect [14]. In asthma and nasal inflammatory diseases, a positive correlation of ICAM-1 expression and number of neutrophils at the epithelium and mucosa of the inflamed tissue has been reported [28, 29]. Moreover, Altman et al. [30] have demonstrated that eosinophil granule products caused significant up-regulation of ICAM-1 on human nasal epithelial cells *in vitro*. We have previously found increased levels of eosinophil cationic protein (ECP) in the milk of mothers with food-allergic infants [31]. These increased numbers of neutrophils found in mother's milk might hypothetically serve as storage of ECP because it is reported that ECP is actively taken up by neutrophils and stored in the granules of these cells [32]. Hypothetically, after being released ECP could induce ICAM-1 expression on infants' lymphocytes in the gut mucosa. Although hypothetical, this could provide an explanation for the detected increased numbers of milk neutrophils and high ICAM-1 expression in breastfed infants.

In conclusion, we found an increased number of ICAM-1 expressing lymphocytes in CMA infants with gastrointestinal or multiorgan symptoms. This may suggest that the high expression of ICAM-1 on peripheral blood lymphocytes may reflect inflammation and enhanced stimulation of circulating T cells. Alternatively, ICAM-1 expression may play a role in the migration of T cells to the effector tissues in infants in the early phase of developing CMA and the multiorgan manifestations of CMA. In addition, high LFA-1 expression may reflect the IgE-mediated disease.

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