

Expression of CS-1 fibronectin precedes monocyte chemoattractant protein-1 production during elicitation of allergic contact dermatitis

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Summary

Background Leucocyte migration within inflammatory skin compartments in allergic contact dermatitis (ACD) is the result of a sophisticated multi-step event where multiple molecules are involved.

Objective Since non-antigen-specific mechanisms have been described as an early participant in elicitation of ACD, we investigated the kinetics of the expression of monocyte chemoattractant protein-1 (MCP-1/CCL2) and the type of infiltrating cells. We compared the time course production of MCP-1/CCL2 with connecting segment-1 (CS-1) fibronectin and thymus and activation-regulated chemokine (TARC/CCL17) expression.

Methods Biopsies from 10 individuals challenged in their back with the antigen responsible for their contact dermatitis and an irrelevant antigen were taken at different times and histology, immunohistochemistry for CS-1 fibronectin, TARC/CCL17, CD3, CD68, CXCR3, CCR4 and *in situ* hybridization for MCP-1/CCL2 were performed.

Results At positive antigen stimulated sites expression of MCP-1/CCL2 by basal keratinocytes and isolated cells in dermis started at 10 h. CS-1 fibronectin and TARC/CCL17 expression by blood endothelial cells was found at 2 and 10 h, respectively. This was followed by dermal accumulation of mononuclear cells with a significant increase of CD3⁺ and CD68⁺ cells. At 48 h, approximately 58% of infiltrating cells were CXCR3⁺, and 35% CCR4⁺.

Conclusions We showed evidence of the fact that CS-1 fibronectin expression precedes the production of MCP-1/CCL2 and TARC/CCL17 in the skin of patients with ACD, suggesting that these molecules participate in the early complex process of migrating mononuclear cells during elicitation of ACD.

Keywords Allergic contact dermatitis, CS-1 fibronectin, TARC/CCL17, MCP-1/CCL2

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Introduction

Allergic contact dermatitis (ACD) is one of the most common inflammatory diseases of the skin with unknown genetic basis, which is often an occupationally related disorder in industrialized countries with an important socio-medical impact [1, 2]. The clinical aspects of this disease have been reviewed elsewhere [3].

ACD has been one of the most intensively investigated *in vivo* immune responses by immunologists and clinicians and it has been considered a model for antigen-specific T-lymphocyte-mediated immune response with induction, effector and resolution phases [4]. The asymptomatic sensitization phase

begins when the Langerhans cells (LCs), exposed to hapten and stress signals, down-modulate Langerin, the majority of inflammatory chemokine receptors (CR) and CCR6 in order to exit this environment [5]. These maturing cells become DC-LAMP⁺ as well as CCR7⁺, which is a very important receptor in the traffic to the draining lymph node (LN) where they encounter specific T cells that exit the blood using high endothelial venules (HEVs) and generate adaptative immune responses [6, 7].

During the clinically visible elicitation phase of ACD, it has been shown that some contact allergens have irritative properties that promote inflammatory skin reactions via induction of cytokines, adhesion molecules and chemoattractants before the action of the antigen-specific response [8, 9]. The inflammatory reaction involved in ACD has been studied extensively in the murine system [10, 11], but only in recent years chemokines have emerged as very important players in the complex network of interactions that cause recruitment of mononuclear cells in elicitation of human ACD [12, 13].

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The effector mechanisms involved in this disease are primarily mediated by a type 1 immune response [14–16], although the participation of NKT cells, B-1 cells and TCR $\gamma\delta$ T cells have also been demonstrated to contribute to keratinocyte apoptosis, which is the key event in the pathogenesis of ACD [17, 18]. Expression of IFN- γ -inducible protein-10 (IP-10/CXCL10), monokine induced by IFN- γ (Mig/CXCL9), regulated on activation, normal T expressed and secreted (RANTES/CCL5) and pulmonary and activation-regulated chemokine (PARC/CCL18) mRNAs begins 12 h after hapten application and parallels the heavy lymphocyte infiltration [19]. I-309 mRNA, which is produced later on by dendritic cells (DCs), keratinocytes and activated T cells, potentially attracts T regulatory lymphocytes that may contribute relevantly to the termination of ACD [20].

At present, treatments include avoidance of contact with the sensitizer or the use of different drugs such as corticosteroids, cyclosporine and vitamin D₃, but an increased knowledge of the immunopathogenic mechanisms will help in the future to develop new therapeutic strategies to target molecules involved early in this inflammatory process, and thereby diminish the aberrant immune response in a more effective way and with less undesirable effects.

There was little information about endothelial cell molecules mediating the accumulation of immune cells to challenge sites during provoked human ACD until we found an early expression of connecting segment-1 (CS-1) fibronectin, followed by thymus and activation-regulated chemokine (TARC/CCL17) expression in blood endothelial cells (BECs) [21, 22]. In this study, we compared the time course expression of these molecules to monocyte chemoattractant protein-1 (MCP-1/CCL2) and infiltrating cells expressing CCR4 and CXCR3 during elicitation of ACD.

Materials and methods

Human subjects

Subjects already diagnosed with ACD ($n = 10$) were recruited from the Allergy and Dermatology Clinic at the Hospital Privado (Córdoba, Argentina). Inclusion criteria were as follows: (1) age between 25 and 79 years, (2) history of ACD, (3) absence of any other illness and (4) positive skin patch tests at 48 h to any antigen from the Patchkit Standard (FDA Allergenic Ltda., Rio de Janeiro, Brazil). Patients gave informed consent and the Institutional Ethics Committee approved the protocol. Patients were not taking oral or topical medication during at least 1 month prior to the beginning of this study.

Study design and processing specimens

Non-irritating concentrations of antigens were applied under 8-mm Finn chambers on Scanpor tape in the scapular area at 5 cm of the spine. Using this method, three chambers containing the antigen responsible for the ACD (positive antigen) and one chamber with a non-relevant antigen (negative control) were used in each patient. Macroscopic responses were measured at 2, 10 and 48 h by evaluating skin erythema, papule and vesicle using the following arbitrary

units (0 = absent, + = mild, ++ = moderate and +++ = intense).

A 3-mm disposable biopsy punch was used to take a biopsy from the centre of each reaction at 2, 10 and 48 h after using 1% lidocaine for local anaesthesia. The control site (negative antigen) was biopsied only at 48 h. In this way, each patient served as his/her own control. Tissue biopsies were immediately fixed in 4% buffered formalin, pH 7.4 and were then embedded in low-temperature paraffin wax. Serial sections (6 μ m) were cut from biopsies, mounted on 0.1% poly-L-lysine-coated slides, dried overnight at 37 °C and stored until use.

Histology

One skin section from each biopsy was processed for histologic conventional study and stained with haematoxylin/eosin. The mononuclear cell infiltration in the dermis was classified as follows: scattered (+/–), mild (+), moderate (++) and intense (+++).

Immunohistochemistry

Skin sections (6 μ m) were dewaxed, rehydrated and then boiled in Vector antigen unmasking solution (catalog H-3300, Vector Lab., Inc., Burlingame, CA, USA) in an 850-W microwave oven for 10 min. The sections were then washed in phosphate buffered saline (PBS pH 7.6) for 20 min and the endogenous peroxidase activity in the skin was blocked with 3% hydrogen peroxide (Sigma Chemical Co., St Louis, MO, USA) in PBS for 5 min and subsequently washed thoroughly with PBS. To block unspecific sites, tissue samples were incubated with 3 bovine serum albumin (Sigma Chemical Co.) in PBS for 20 min, incubated overnight at room temperature with anti-CD3 at 1:50 or anti-CD68 at 1:100 (Dako, Glostrup, Denmark) or incubated for 2 h with one of the following mAbs: mouse anti-human CXCR3 (clone 1C6), anti-CCR4 (clone 1G1), anti-TARC/CCL17 (clone 2D8) at 10 μ g/mL, or anti-CS1 fibronectin at 1:60 (clone 90.45, CYTEL Corporation, San Diego, CA, USA). LeukoSite Inc. (Cambridge, MA, USA) kindly provided all mAbs against chemokine and chemokine receptors. Isotype-matched immunoglobulins were used as negative controls. After washing with PBS, the slides were incubated with the labelled streptavidin–biotin (LSAB) method according to the manufacturer's instruction (Dako, Carpinteria, CA, USA). All incubations were performed at room temperature in a humid chamber, the reaction cascade was visualized by incubation with 3-amino-9-ethylcarbazole (AEC) as a substrate (Dako) and the slides were counterstained with haematoxylin. The sections were counted in duplicate on 14–17 adjacent fields at $\times 1000$ magnification blinded to the patient's clinical status. The number of mononuclear cells expressing a particular antigen were informed per mm², whereas the expression of CS-1 fibronectin and TARC/CCL17 was measured using the following criteria (– = negative, + = less than 40% of blood vessels, ++ = between 40% and 70% of blood vessels and +++ = more than 70% of blood vessels).

In situ hybridization

³⁵S-labelled MCP-1/CCL2 mRNA probe was generated by *in vitro* transcription (Boehringer Mannheim GmbH, Mannheim, Germany) and *in situ* hybridization was performed as described previously [23].

Statistical analysis

Variability of the parameters studied was analysed using Friedman's test, followed by two-by-two comparisons between time-points using the Wilcoxon signed rank test. For all tests, $P < 0.05$ was considered to be significant.

Results

As can be seen in Table 1 all the subjects exhibited a specific antigen-induced clinical cutaneous response at 48 h and a moderate to intense infiltration of mononuclear cells distributed mainly around blood vessels. The histopathologic study of dermal vessels from positive patch tests at 48 h showed a significant hypertrophy and swelling of endothelial cells compared to negative patch tests. These changes appeared at 2 h when the vessels showed an incipient but significant swelling (data not shown).

To assess the time course and the expression pattern of MCP-1/CCL2, biopsies were hybridized with sense or anti-sense probes of this chemokine. Negative results were

obtained in all samples when the sense probe was used. Cells expressing mRNA for MCP-1/CCL2 were not present at 2 h but they were found at 10 and 48 h in every skin biopsy from the positive challenged sites, and they correspond to basal keratinocytes and isolated cells in the dermis. It is worth noting that the appearance of MCP-1/CCL2⁺ dermal cells correlates with the disappearance of Langerin⁺ cells in the dermis (data not shown). A low level of mRNA for MCP-1/CCL2 was seen in the negative antigen-challenged sites at 48 h (Fig. 1).

Immunohistochemistry studies demonstrated CS-1 fibronectin expression in the lumen of endothelial cells from all positive patch test samples at 2, 10 and 48 h with a maximum level at 10 h. Different results were seen with regard to TARC/CCL17, since positive expression was observed in positive patch tests after 10 h (Table 2). Non-immunoreactivity for both markers was found in biopsies from the negative control challenge sites at 48 h.

It should be emphasized that positive reactions for MCP-1/CCL2, CS-1 fibronectin and TARC/CCL17 were observed in the positive challenged sites regardless of the antigen applied.

Although the macroscopic response to the positive antigens was negative at 10 h, skin biopsies of these sites showed a mild infiltration of mononuclear cells. At 48 h, the sham-challenged sites showed the cell infiltrate comparable to the positive antigen sites at 2 h (Table 1). As expected, there were significant increases in CD3⁺ T cells and CD68⁺ macrophages at positive antigen-challenged sites at all time-points

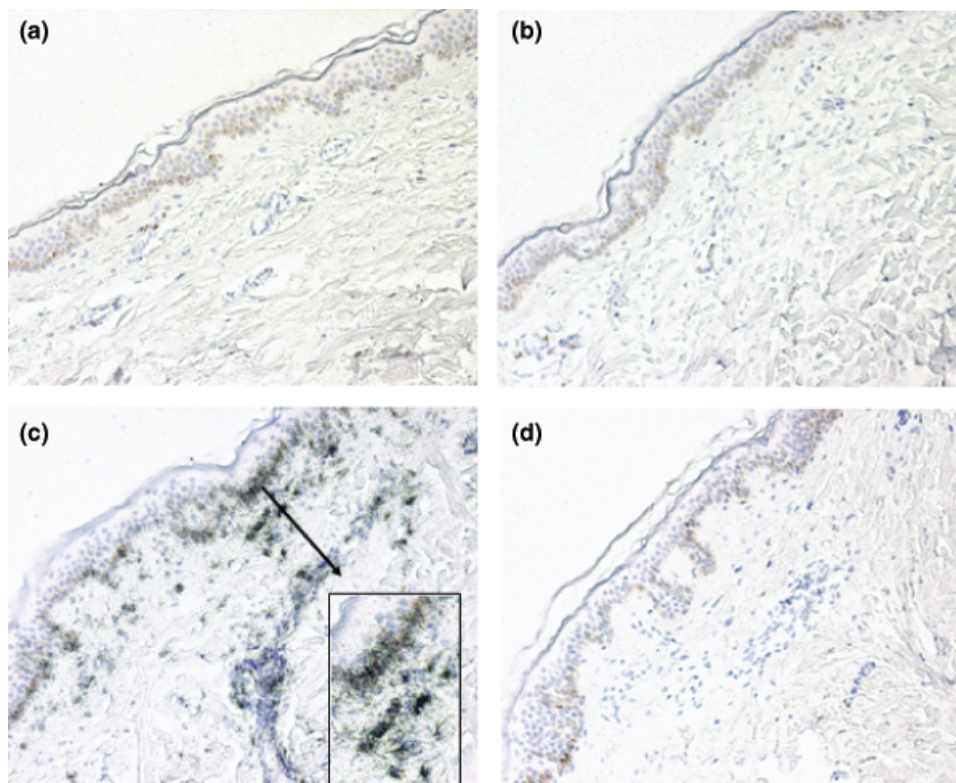


Fig. 1. *In situ* expression of MCP-1/CCL2. Anti-sense hybridization is shown in skin sections of positive antigen sites at 2 h (a), 10 h (b), 48 h (c) and negative antigen site at 48 h (d) from one representative patient with ACD. Expression is observed in basal keratinocytes and in discrete areas in the dermis at 10 and 48 h. Original magnifications $\times 10$. The insets on the right-hand side of each image show enlarged views of cells from the same tissue section at higher magnification.

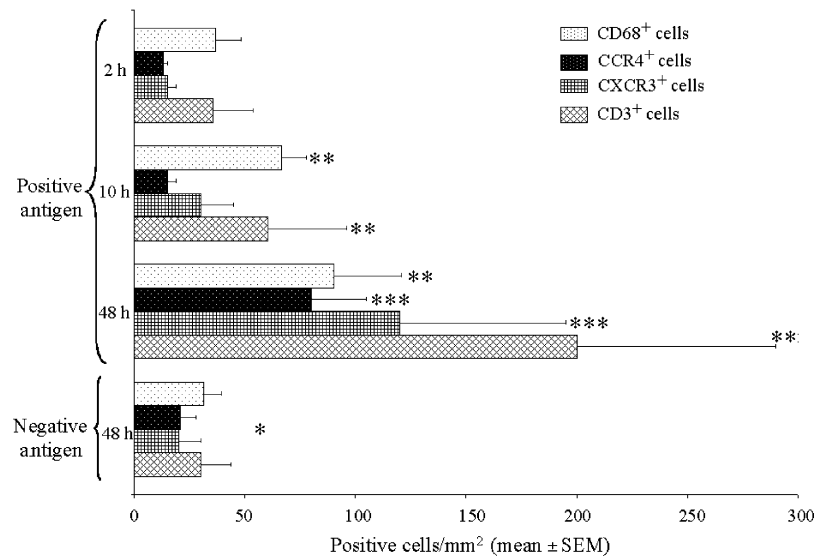


Fig. 2. Time course of increases in CD3⁺, CD68⁺, CXCR3⁺, and CCR4⁺ infiltrating mononuclear cells into antigen-challenged skin sites in 10 patients with allergic contact dermatitis. Negative antigen-challenged sites (Ag⁻) at 48 h were used as control. Results are shown as the numbers of positive cells (mean ± SEM) per square millimetre of biopsy. Significant differences (Ag⁻, and 2-, 10- and 48-h positive Ag⁺ challenge time-points, *, Friedman's test) were observed for CD3⁺ T lymphocytes ($P < 0.003$), CD68⁺ macrophages ($P < 0.003$), CXCR3⁺ cells ($P < 0.003$) and CCR4⁺ cells ($P < 0.003$). The Wilcoxon signed rank test was used to analyse the differences between time-points (** $P < 0.004$, CD3⁺ and CD68⁺ cells, 10 h and 48 h vs. 2 h and 48 h vs. 10 h). (***) $P < 0.007$, CXCR3⁺ and CCR4⁺ cells, 48 h vs. 10 h).

Table 1. Clinical and histological characteristics of ACD patients

Patients	Sex/age (years)	Skin patch test reactivity		Macroscopic response*				Histologic grade†			
		Positive antigen (concentration)	Negative antigen (concentration)	Positive antigen			Negative antigen	Positive antigen			Negative antigen
				2 h	10 h	48 h		2 h	10 h	48 h	
MO	F/58	Neomycin (20%)	Antraquinone (2%)	—	—	+++	—	+/-	+/-	+++	+/-
MR	M/65	Thimerosal (0.1%)	Antraquinone (2%)	—	—	+++	—	+	++	+++	+/-
AS	F/46	Neomycin (20%)	Antraquinone (2%)	—	—	+++	—	+/-	+	++	+/-
ER	M/49	<i>p</i> -Phenylenediamine (1%)	Antraquinone (2%)	—	—	+++	—	+/-	++	+++	+/-
IL	F/49	Quaternium 15 (1%)	Antraquinone (2%)	—	—	+++	—	+/-	+	+	+/-
AC	F/49	Quinolina mix (6%)	Antraquinone (2%)	—	—	+++	—	+/-	++	+++	+/-
LG	F/46	Balsamo Peru (25%)	Antraquinone (2%)	—	—	+++	—	+/-	+	+++	+/-
MG	F/29	Nickel (5%)	Antraquinone (2%)	—	—	+++	—	+/-	+	++	+/-
SA	F/64	Irgasan (1%)	Antraquinone (2%)	—	—	+++	—	+/-	+	++	+/-
TV	F/23	Nickel (5%)	Antraquinone (2%)	—	—	+++	—	+/-	+	+++	+/-

*Skin erythema, papule and vesicle: — (absent); + (mild); ++ (moderate); +++ (intense).

†Mononuclear cell infiltration: +/- (scattered); + (mild); ++ (moderate); +++ (intense). See Materials and Methods.

compared with negative antigen-challenged sites ($P < 0.0001$). The numbers of CD3⁺ and CD68⁺ cells were maximal 48 h after the specific antigen challenge. T lymphocytes were observed mainly around blood vessels, and CD68⁺ macrophages were distributed throughout the dermis and the numbers of these infiltrating cells at 48 and 10 h were significantly higher than those at 10 and 2 h, respectively ($P < 0.004$). The numbers of CXCR3⁺ and CCR4⁺ infiltrating cells at the specific antigen challenge sites increased significantly between 10 and 48 h ($P < 0.007$), and at 48 h approximately 58% and 35% of mononuclear infiltrating cells expressed CXCR3 and CCR4, respectively (Fig. 2).

Discussion

We found that the expression of MCP-1/CCL2, which is preceded by the production of CS-1 fibronectin, clearly anticipated the infiltration of CXCR3⁺ and CCR4⁺ mononuclear cells during elicitation of ACD.

Efficient recruitment into inflamed skin requires exceptional adhesive interactions between mononuclear cells and the inflamed endothelial cell lining of draining blood vessels and subsequent migration through the dermis and the epidermis. In this complex trafficking process, different molecules such as selectins, CS-1 fibronectin, chemokines, and increased

Table 2. CS-1 fibronectin and TARC expression in biopsies from ACD patients

Patients	Sex/age	CS-1 expression*				TARC expression*			
		Positive antigen		Negative antigen		Positive antigen		Negative antigen	
		2 h	10 h	48 h	48 h	2 h	10 h	48 h	48 h
MO	F/58	+	+++	+	–	–	++	+++	–
MR	M/65	++	+++	++	–	–	+	+++	–
S	F/46	++	+++	++	–	–	++	+++	–
ER	M/49	+	+++	+	–	–	+	+++	–
IL	F/49	++	+++	+	–	–	+	+++	–
AC	F/49	+	+++	+	–	–	++	+++	–
LG	F/46	++	++	+	–	–	++	+++	–
MG	F/29	+	+++	++	–	–	+	+++	–
SA	F/64	++	+++	++	–	–	+	+++	–
TV	F/23	++	+++	+	–	–	++	+++	–

* – = Negative, + = less than 40% of blood vessels, ++ = between 40% and 70% of blood vessels and +++ = more than 70% of blood vessels). See Materials and Methods.

numbers of integrin ligands have been described as playing an important role [12, 21, 22, 24–26].

Becuase Campbell et al. [27] elegantly showed that TARC/CCL17 was implicated in lymphocyte–endothelial interactions during cell recruitment to normal and inflamed cutaneous sites, there have been growing evidences of the importance of this chemokine in the selective recruitment of Th2 cells to inflammatory sites in atopic dermatitis (AD) [28–30]. Although it has been found that keratinocytes produce TARC/CCL17 in patients with AD [31], and that IL-10 is able to augment the TARC/CCL17 inducing effects of TNF- α and IFN- γ [32], we have mainly detected the expression of TARC/CCL17 in dermal BECs, which was preceded by CS-1 fibronectin.

Another important chemokine involved in the recruitment of inflammatory cells is MCP-1/CCL2 that can be produced by fibroblasts, endothelial cells, macrophages and DC after exposure to stimuli such as IL-1, TNF- α or Th2 cytokines [33]. It was first characterized as a monocyte-chemoattracting protein, but its receptor, CCR2, is also expressed on activated and memory T cells, including Th1, Th2 as well as T-regulatory lymphocytes [20, 34].

In different animal models and human diseases, MCP-1/CCL2 and CCR2 have been shown to be functionally relevant in the genesis of inflammatory reactions mediated by monocytes and T cells [35–37], and in protective cell-mediated immune response to *Leishmania major* [38].

MCP molecules also influence T cell differentiation because they are able to reduce IL-12 production by activated human monocytes in the lymphoid T zone [39]. More recently, Palframan et al. [40] have presented clear data of skin-derived MCP-1/CCL2 transported via the lymph to the luminal surface of HEVs, thus projecting its local profile to draining LNs in this manner.

Goebeler et al. [12] have shown that MCP-1/CCL2 is the first chemokine encounter by mononuclear cells during elicitation of ACD. Our results of the initial production of this molecule by basal keratinocytes at 10 h followed by further expression in isolated areas in the dermis at 48 h are in agreement with the above finding. The positive cells found in

the dermis could represent the emigrating LCs capable of producing this chemokine [41].

T lymphocytes with polarized cytokine production (Th1 and Th2) show a different distribution of receptors for inflammatory chemokines. Although CXCR3 and CCR5 transcripts are markedly enhanced in Th1 cells, increased amounts of transcripts for CCR3, CCR4 and CCR8 are found in Th2 cells [42, 43]. In our kinetic study over a 48-h course of provoked human ACD, we found a gradual accumulation of CXCR3⁺ cells, which was directly associated with CD3⁺ T cells.

Production of Mig/CXCL9, IP-10/CXCL10 and interferon-inducible T cell alpha chemoattractant in the allergic patch test lesions was first described by Flier et al. [44]. More recently, the microanatomical location and longitudinal profile of these chemokines were studied in ACD and found to be preferentially expressed at basal/suprabasal layers of the epidermis and subepidermal regions, reaching maximum levels at 72 h [12]. Our results of a dense infiltration of CXCR3⁺ lymphocytes in the dermis are also in agreement with the finding of Albanesi et al. [16], who have shown that the majority of infiltrating cells in ACD are recruited by CXCR3 agonistic chemokines.

The lower number of CCR4⁺ cells compared to CXCR3⁺ cells found in our study is similar to the reported over-expression of CXCR3 in lesional skin from patients with psoriasis [45].

Our data provide further evidence that the inflammatory cells in ACD lesions are heterogeneous populations and that they encounter different signals in a sophisticated temporal pattern. Based on the pattern of expression of different molecules, we have proposed that the CS-1 fibronectin may work alone in the beginning and together with TARC/CCL17 and cutaneous T cell-attracting chemokine (CTACK/CCL27) later on, inducing the adhesion of passing cutaneous $\alpha\beta 1^+$ mononuclear cells under shear. The production of MCP-1/CCL2 by basal keratinocytes will further allow these infiltrating cells to move toward the epidermis. The early expressions of these molecules clearly precede the infiltration of mononuclear cells, indicating an initial involvement of a

non-antigen-specific mechanism in the elicitation of ACD. The levels found at 48 h probably reflect the positive influence of antigen-specific Th1 cells, since very low or negative expressions of these molecules were detected from the irrelevant antigen-challenged sites at that time.

So, a useful therapeutic strategy may be to block $\alpha 4\beta 1$ and CR interactions with its ligands on inflamed endothelial cell surfaces, by specific antibodies or antagonists. In this regard, CS-1 analogue peptide has been efficiently used to diminish the efferent phases of Th2- and Th1-mediated inflammatory responses in animal models [46, 47], (Martín et al., in preparation).

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