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INVESTIGATIVE REPORT

Role of LFA-1/ICAM-1, CLA/E-selectin and VLA-4/VCAM-1 Pathways in Recruiting Leukocytes to the Various Regions of the Chronic Leg Ulcer

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The center, edge and distant regions of the venous leg ulcer differ in inflammatory cell composition, suggesting that these represent different developmental stages. Our goal was to determine which recruitment pathways contribute to the differences in leukocyte composition between the various ulcer regions. The multiple region biopsy approach, which enables to study the different development phases of the ulcer at one time-point, was employed to immunohistochemically identify the vascular adhesion molecules E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and their counter-ligands on extravasated leukocyte cutaneous lymphocyte-associated antigen (CLA), lymphocyte function-associated antigen (LFA-1) and very late activation antigen-4 (VLA-4), respectively. E-selectin expression was highest at the ulcer edge, while ICAM-1 was highest at the ulcer center. VCAM-1 expression was minor at all ulcer regions. CLA stained up to 80% of the epidermal Langerhans' cells, 62% of the T cells, and only 9% of the macrophages. LFA-1 did not stain Langerhans' cells, stained up to 89% of the T cells and up to 11% of the macrophages. VLA-4 stained up to 30% of the T cells and 71% of the macrophages. In conclusion, the results indicate that Langerhans' cells, T cells and macrophage are each recruited by more than one adhesion-molecule pathway to any of the chronic venous leg ulcer regions. ***Key words: adhesion molecule; cutaneous lymphocyte-associated antigen; venous leg ulcer; wound healing.***

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Following coagulation, wound healing proceeds with leukocyte recruitment to the wound site, and is governed through several sequential processes by the continuously changing cellular composition (1, 2). Leukocyte extravasation into inflammatory sites is mediated by adhesion molecules termed "addressins" on the vascular endothelium and "homing receptors" on circulating cells (3). Intercellular adhesion molecule-1 (ICAM-1, CD54) is constitutively expressed by endothelial cells and further up-regulated during inflammation (4). In contrast, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (CD62E) are expressed at low levels or not at all in normal skin, and are up-regulated upon activation (5, 6). E-selectin differs from other addressins in being restricted to vascular endothelium, preferentially in the skin (7). The counter-ligands of ICAM-1 and VCAM-1 on leukocytes are lymphocyte function-associated antigen-1 (LFA-1) and very late activation antigen-4 (VLA-4), respectively. Cutaneous lymphocyte-associated antigen (CLA), which defines a skin-homing memory T-cell population, serves as counter-ligand for E-selectin (8, 9).

Several studies have extended our knowledge of the role of addressins in the pathogenesis of chronic leg ulcers. The vascular endothelium in biopsies from the margin of venous leg ulcers expresses E-selectin and ICAM-1, but not VCAM-1 (10), and demonstrates an increase in the number of T lymphocytes and macrophages in addition to up-regulation of vascular ICAM-1 (11). On the other hand, dermatoliposclerotic skin from patients with chronic venous insufficiency expresses increased levels of vascular ICAM-1 and VCAM-1 (12, 13), but not of E-selectin (12). LFA-1 and VLA-4 have been expressed on 60–70% of the perivascular infiltrate (12).

By employing the multiple region biopsy approach of Wakita et al. (14), which enables study of the different development phases of a lesion at one time-point, we have previously shown that the leukocyte composition differs between the center, edge and 2 cm distant to the edge of venous leg ulcers, and that the distant region is heavily infiltrated by leukocytes (15). The goals of the present study were (i) to identify whether ICAM-1/LFA-1, E-selectin/CLA and VCAM-1/VLA-4 pathways contribute to differences in recruitment of Langerhans' cells, macrophages and T lymphocytes to the different venous leg ulcer regions; and (ii) to further establish the role of the 2 cm distant skin in the pathogenesis of venous leg ulcers.

METHODS

Patients

Nineteen patients (12 men and 7 women; mean age 71 years (range 41–93)) were included in the study after giving written consent (15). Mean duration of the ulcer was 34.1 months (range 3–180) and mean ulcer area was 24 cm^2 (range 1–200). Three patients received topical steroids. None received systemic immunosuppressive therapy. The study was approved by the local ethics committee of Copenhagen, Denmark.

Specimens

Three-millimeter punch biopsies were obtained from the center, the edge and from macroscopically intact skin 2 cm from the edge of the ulcer (15). The "center" was defined as area devoid of epidermis located 2 cm apart from the area covered by epidermis when the

shorter diameter of this area was more than 4 cm. Otherwise, the center was the middle of the area if the longest diameter was 4 cm or less. The "edge" was defined as tissue containing epidermis and dermis immediately adjacent to an area devoid of epidermis. The biopsies were snap-frozen and stored at – 80°C. Serial 3-mm thick sections were cut, air-dried overnight at room temperature, fixed in acetone for 10 min and stored at – 80°C until further processed. The stainings were conducted on the same biopsies that previously served to identify the inflammatory cell composition at the various ulcer regions (15).

Antibodies

The primary monoclonal antibodies are listed in Table I. All the secondary and tertiary antibodies were purchased from DAKO, Glostrup, Denmark.

Single-staining procedure

A three-step immunoperoxidase method was performed as previously described (16). In brief, the specimens were incubated sequentially with unlabelled primary monoclonal antibody, peroxidase-conjugated rabbit anti-mouse IgG (DAKO, Glostrup, Denmark) and swine anti-rabbit IgG (DAKO). Each incubation step was 30 min with 5-min TRIS-buffered saline (TBS) washes between. Peroxidase activity was visualized by incubating the specimens with 3-amino-9-ethyl-carbazole (AEC) (Sigma, St. Louis, MO, USA) for 10 min, giving an orange color. The specimens were then counterstained with hematoxylin and mounted in glycogel (DAKO). Single-staining for E-selectin was performed on endothelial cells.

Double-staining procedure

Double-staining was performed sequentially by three-step immunoperoxidase (16) and immunoalkaline phosphatase, as described previously (17). Briefly, after visualization of the first primary mAb by AEC, a second mAb was applied, followed by incubation with alkaline-phosphatase (AP)-conjugated rabbit anti-mouse IgG (DAKO) and then by mouse immunoalkaline phosphatase complexes (DAKO). Each incubation lasted for 30 min, separated by 5-min TBS washes. AP activity was detected by incubating the specimens for 30 min with both naphthol-AS-MX-phosphate and Fast Blue BB salt (Sigma), giving a blue color. For inhibition of endogenous AP activity, levamisole (Sigma) was added to the developing solution. The labeling combinations are listed in Table II. Factor VIII was used to confine addressins to endothelial cells as previously described (6).

Controls

Tonsil and skin specimens from chronic inflammatory lesions of psoriasis and lichen planus were used as positive controls. Skin specimens incubated without the primary mAb served as negative controls.

Table II. *Labeling combinations*

Cell marker	Double-stained with
CD3 (lymphocyte)	LFA-1, CLA, VLA-4
CD68 (macrophage)	LFA-1, CLA, VLA-4
CD1a (Langerhans' cell)	LFA-1, CLA
Factor VIII (endothelial cell)	ICAM-1, VCAM-1

LFA-1 = lymphocyte function-associated antigen-1, CLA = cutaneous lymphocyte-associated antigen, VLA-4 = very late activation antigen-4, ICAM-1 = intercellular adhesion molecule-1, VCAM-1 = vascular cell adhesion molecule-1.

Quantification

Masked enumeration of cells was done at 400 × magnification with the use of an ocular grid consisting of a simple square lattice of 100 test points. The field of view comprised the stained cells in the epidermis per millimeter length of surface line, and in the dermis per square millimeter of dermis reaching 0.4 mm below the lowest rete ridges. The mean value of counted cell profiles of three sequential horizontal fields gave the relative density of the cells in the epidermis and the upper dermis, respectively. Evaluation of addressin expression on vascular endothelium was conducted by enumeration of positively stained endothelial cells, as previously described (6).

Statistical analysis

The Kruskal-Wallis ANOVA by ranks test was used to investigate differences in the number of cells in biopsies taken from center, edge and 2 cm distance. The Mann-Whitney U test was used to investigate the significance of differences in cell numbers between two of these three groups. In both analyses, the biopsy location was used as independent variable and a p-value < 0.05 was considered statistically significant. Correlations between cells and the addressins were investigated by Spearman rank order correlation test.

RESULTS

E-selectin, ICAM-1 and VCAM-1 expression on vascular endothelium

E-selectin stained the vascular endothelium in all the tested areas of the ulcer (Figs. 1a, 2). The number of endothelial

Table I. *Primary monoclonal antibodies used in the study*

Antibody	Epitope identified	Tissue reactivity	Company	Product no.	Dilution
NA1/34	CD1a	Langerhans' cells	Dako (Glostrup, Denmark)	M721	1:10
UCHT1	CD3	Pan T cells	Dako (Glostrup, Denmark)	M835	1:20
EBM11	CD68	Macrophages-monocytes	Dako (Glostrup, Denmark)	M718	1:80
RSMM	CLA/HECA-452	Leukocytes, HEVs	*	*	1:600
IOT-16	LFA-1a/CD11	Leukocytes	Immunotech (Marseilles, France)	0157	1:70
IOP49D	VLA-4a/CDw49d	Leukocytes, fibronectin	Immunotech (Marseilles, France)	0764	1:100
BBIG-E6	E-selectin	Activated endothelium	Brit. Bio. Tech. (Abingdon, UK)	BBA1	1:600
84H10	ICAM-1/CD54	Endothelium, APC	Immunotech (Marseilles, France)	0753	1:40
BBIG-V1	VCAM-1	Activated endothelium	Brit. Bio. Tech. (Abingdon, UK)	BBA5	1:900
F8/86	Factor VII	Endothelium	Dako (Glostrup, Denmark)	M616	1:100

CD1a = Langerhans' cells, CD3 = T lymphocytes, CD68 = macrophages, LFA-1 = lymphocyte function-associated antigen-1, CLA = cutaneous lymphocyte-associated antigen, VLA-4 = very late activation antigen-4, ICAM-1 = intercellular adhesion molecule-1, VCAM-1 = vascular cell adhesion molecule-1. *Gift of Dr. Robert Rothlein, Boehringer Ingelheim; HEV = high endothelial venules, APC = antigen-presenting cells.

cells (EC) expressing E-selectin was highest at the edge and lowest 2 cm distant from the ulcer ($p < 0.05$) (Fig. 2). No difference in distribution of E-selectin-positive EC was observed between other regions of the ulcer. Double-staining with anti-factor-VIII confined ICAM-1 (Fig. 1b) and VCAM-1 to the EC. The number of ICAM-1$^+$ EC increased towards the center of the ulcer ($p < 0.05$) (Fig. 2). A significant difference was observed when comparing center versus edge ($p < 0.05$) and center versus distant ($p < 0.01$). VCAM-1 was weakly expressed on a minor portion of the blood vessels in less than 30% of the sections, with no difference in distribution between the regions of the ulcer (Fig. 2). The lumen area of blood vessels was packed with leukocytes to less than a third in all sections.

CLA and LFA-1 expression on CD1a$^+$ epidermal Langerhans' cells

The mean number of CLA$^+$CD1a$^+$ cells at the distant area was approximately three times higher than at the edge of the ulcer ($p < 0.01$). Eighty percent of the CD1a$^+$ cells expressed the CLA homing receptor at the edge of the ulcer as compared to 51% at the distant area (Table III, Fig. 1c). Langerhans' cells double-stained for both CD1a and LFA-1 were found in only one biopsy taken 2 cm distant from the ulcer edge.

CLA, LFA-1 and VLA-4 expression on CD3$^+$ lymphocytes and on CD68$^+$ macrophages

A significant difference in distribution of the CLA$^+$CD3$^+$ cell population was observed between the three regions of the ulcer ($p < 0.01$) and between edge and distant areas ($p < 0.01$), but not between the center versus the other two areas (Figs. 1d, 1e, 3a). The highest proportion of lymphocytes expressing the CLA skin-homing receptor was found at the edge of the ulcer (62%), followed by 58% at the center and 50% at 2 cm lateral to the edge ($p < 0.05$) (Table III). The majority of lymphocytes in the ulcer expressed LFA-1 (85–89%) (Table III; Fig. 1f). However, no significant difference was observed in the distribution of LFA-1$^+$CD3$^+$ cells between the different regions of the ulcer. Only 21–30% of the lymphocytes in the ulcer expressed VLA-4 (Table III). Neither the number of VLA-4$^+$CD3$^+$ cells nor the proportion of total CD3$^+$ cell populations demonstrated significant differences.

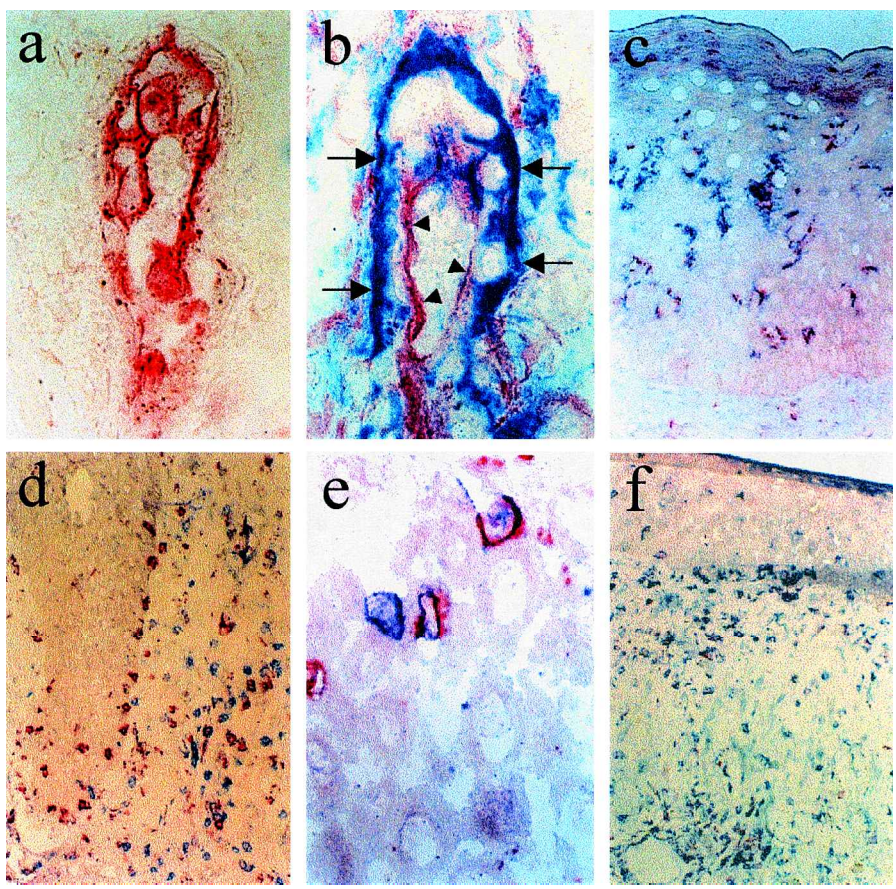

Fig. 1. Expression of E-selectin (a), ICAM-1 (b), LFA-1 (f) and CLA (c–e) in the different regions of the chronic leg ulcer. Stainings: blue = immunoalkaline phosphatase; brown = three-step immunoperoxidase; brown on blue = expression of two receptors on the same cell. (a) E-selectin expression on vascular-endothelial cells, (b) ICAM-1 and Factor VIII expression on vascular-endothelial cells (tailed arrows point at Factor VIII = blue; non-tailed arrows point at ICAM-1 on the luminal surface = brown), (c) CLA and CD1a expression on Langerhans' cells (CD1a = blue, CLA = brown), (d & e) CLA and CD3 expression on lymphocytes (CD3 = blue, CLA = brown), (f) LFA-1 and CD3 expression on lymphocytes (CD3 = blue, LFA-1 = brown). Biopsies (b, c, e and f) were obtained from region 2 cm distant to the margin of the ulcer, and (a) and (d) from the ulcer margin. The original magnification for (d) and (f) is ×200, for (c) ×400, and for (a), (b) and (e) ×1000. For abbreviations, see Table II.

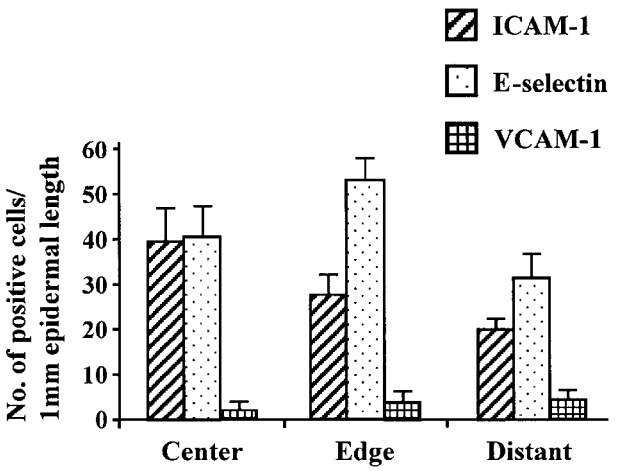

Fig. 2. Number (mean ± SEM) of endothelial cells expressing addressins in various regions of the ulcers ($n = 19$). ICAM-1 = intercellular adhesion molecule-1; VCAM-1 = vascular cell adhesion molecule-1.

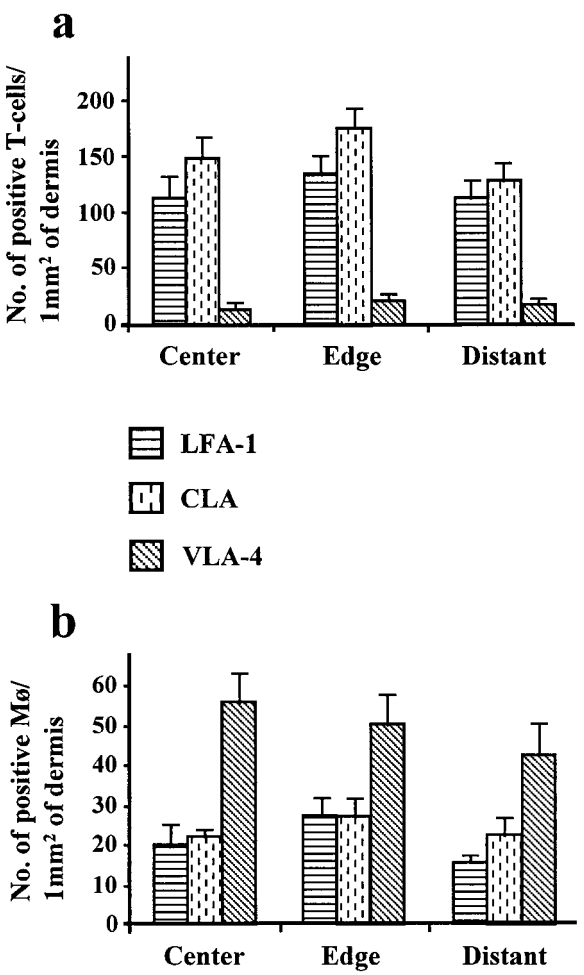

Fig. 3. Number (mean ± SEM) of T lymphocytes (a) and macrophages (Mø; b) expressing homing receptors in various regions of the ulcers ($n = 19$). LFA-1 = lymphocyte function associated antigen-1; CLA = cutaneous lymphocyte associated lymphocyte; VLA-4 = very late activation antigen-4.

The edge and distant areas of the ulcer differed in the mean numbers of $LFA\text{-}1^+CD68^+$, 27.1 and 15.4, respectively ($p < 0.05$). No difference was observed between other regions. Furthermore, no significant difference in the distribution of the CLA^+CD68^+ and $VLA\text{-}4^+CD68^+$ subsets between the various regions of the ulcer was observed. The proportions of macrophages expressing the CLA and LFA-1 homing receptors were small, 7–9% and 8–11%, respectively, while VLA-4 was demonstrated on 39–71% of the macrophages (Fig. 3b; Table III). None of the macrophage fractions displayed any significant difference in distribution between the regions of the ulcer.

DISCUSSION

Previously, we showed that at 2 cm distant from the edge of the venous leg ulcer the epidermis was heavily infiltrated by Langerhans' cells, and the dermis by T lymphocytes and macrophages and to a lesser extent by neutrophils (15). This region is also characterized by a decreased capillary density compared to the ulcer margin (18). In the present study, we found that also E-selectin is up-regulated at the ulcer's distant region compared to its expression level in normal skin (12, 19). This provides further support of the notion that this region is actively involved in recruitment of leukocytes to the ulcer. The increased expression of E-selectin at the ulcer edge supports Veraart et al. (10) and differs from Weyl et al. (12). The discrepancy may reflect differences between active ulcers (10) and dermatoliposclerotic skin (12). The ongoing expression of E-selectin in chronic leg ulcers is in contrast to *in vitro* rapid down-regulation (20). This may reflect its *in vivo* expression of the more stable E-selectin type I form (21), and the high levels of IL-1 alpha and TNF alpha in the chronic leg ulcer fluid (22–24).

The increased CLA^+ Langerhans' cells from 2% in normal epidermis (25) to 51% at the 2 cm distant region and to 80% at the ulcer margin correlated with the increased expression of vascular E-selectin. The lack of LFA-1 expression by Langerhans' cells rules out their recruitment through the ICAM-1/LFA-1 pathway. It is therefore concluded that the E-selectin/CLA pathway participates in the recruitment of more than half of epidermal Langerhans' cells to the venous leg ulcer. The remaining Langerhans' cells may be recruited by other adhesion-molecule pathways, including the sialyl Lewis X/CLA pathway (26).

In view of the neutrophil's limited presence within the chronic leg ulcer, < 7% of the cellular infiltrate, and of its marginal role in wound healing (15, 27), we focused in the present study on dermal recruitment of T lymphocytes and macrophages. The increase in CLA^+ T cells from 41% in normal skin (28) to 50% at the 2 cm distant region, and to 62% at the ulcer margin, correlated with the increased expression of vascular E-selectin. This suggests that the E-selectin/CLA pathway takes part in recruiting CLA^+ T cells to the ulcer. However, the percentage of CLA^+ T cells in the ulcer was lower than the 85% in a range of inflammatory and neoplastic skin diseases (29). The reasons for the relatively lower recruitment of the skin homing CLA^+ T cells to venous leg ulcer are not clear. The ICAM-1/LFA-1 pathway apparently plays a larger part in recruiting T cells to the ulcer, since ICAM-1 was up-regulated at the ulcer's center and edge compared to the distant region, and LFA-1 was expressed on

Table III. *Comparison of LFA-1, CLA- and VLA-4-positive leukocytes between the various regions of the leg ulcer (n = 19 patients)*

			% of adhesion molecule expressing cells, mean ± SEM		
	Cell markers	Adhesion molecules	Center	Edge	Distant
Epidermis	CD1a	LFA-1	NE	0	1[Y]
		CLA	NE	80 ± 8.5	51 ± 3.3
Dermis	CD3	LFA-1	87 ± 5.4	85 ± 4.3	89 ± 4.6
		CLA	58 ± 6.4	62 ± 2.6	50 ± 2.8
		VLA-4	21 ± 2.3	23 ± 2.6	30 ± 2.4
	CD68	LFA-1	8 ± 0.9	11 ± 0.9	9 ± 0.8
		CLA	7 ± 0.2	9 ± 0.7	7 ± 0.6
		VLA-4	71 ± 4.5	56 ± 4.5	39 ± 3.9

NE = no epidermis, Y = four positive cells observed in only one biopsy, CD1a = Langerhans' cells, CD3 = T lymphocytes, CD68 = macrophages, CLA = cutaneous lymphocyte-associated antigen, LFA-1 = lymphocyte function-associated antigen-1, VLA-4 = very late activation antigen-4.

approximately 90% of the T cells. The expression of VLA-4 on 30% of the T cells is in contrast to a previous report of a "massive accumulation of VLA-4$^+$ lymphocytes in venous leg ulcer" (12). However, in that study (12) the VLA-4$^+$ infiltrate was not dissected by dual staining with CD3 and CD68. Therefore, the VLA-4$^+$ cells (12) could also represent macrophages, which we found to be numerous at the venous leg ulcer (15), and up to 70% of the CD68$^+$ cells expressed VLA-4. Taken together, the data suggest that E-selectin/CLA and ICAM-1/LFA-1 pathways participate in recruiting the vast majority of T cells to the venous leg ulcer.

The low expression of CLA and LFA-1 on macrophages at the different regions of the ulcer (7–11%) and the low expression of vascular VCAM-1 do not support a substantial role in macrophage recruitment to the ulcer for any of the three analyzed pathways. VLA-4 can adhere to both VCAM-1 and extracellular matrix (30). Therefore, the high percentage of VLA-4$^+$ macrophages (39–71%) may suggest that the VLA-4 serves to anchor the macrophages to extracellular matrix proteins (31).

The initial drive behind venous leg ulcer propagation is the reduction in capillary density within at least 2 cm of the ulcer margins (18), resulting in local changes that lead to up-regulation of E-selectin and possibly of other addressins. This, along with the constitutively expressed ICAM-1, increase the recruitment of Langerhans' cells, T lymphocytes and macrophages to the distant region (15). The total T-cell population did not increase at the ulcer's margin and center, indicating that T-cell recruitment peaks during the initial phase (15). The following expansion phase is represented at the ulcer margins by further up-regulation of E-selectin and ICAM-1, resulting in an increased recruitment of macrophages and the CLA$^+$ T-cell subset. In non-chronic lesions, the initial and expanding phases are followed at the center region by an involution phase, which is characterized by a decrease in vascular ICAM-1 expression and T-cell population size (14). In contrast, we found an increased vascular ICAM-1 at the ulcer center, with no reduction in either T-cell or macrophage populations (15). These findings are part of the processes that divert leg ulcers from a healing course into a chronic course.

In conclusion, Langerhans' cells, T cells and macrophages are each recruited by more than one adhesion-molecule pathway to any venous leg ulcer region. The region 2 cm distant from the ulcer edge participates in the evolution of the venous leg ulcer by up-regulating E-selectin and recruiting leukocytes. The peaked expression of vascular ICAM-1 and the maintenance of high-density infiltrate of T cells and macrophages at the center of the ulcers characterize the chronic status of the leg ulcers.

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