

Expression of lymphatic markers and lymphatic growth factors in psoriasis before and after anti-TNF treatment*

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Abstract: BACKGROUND: Angiogenesis is an early stage of psoriatic lesion development, but less is known about lymphangiogenesis and its role in the development of psoriasis.

OBJECTIVE: To examine the expression of specific lymphatic markers and lymphatic growth factors in untreated psoriatic skin, in the unaffected skin of patients and skin of healthy volunteers, as well as their alteration after treatment with an anti-TNF agent.

METHODS: Immunohistochemistry for the lymphatic markers D2-40 and LYVE-1, in addition to the VEGF-C and VEGF-D growth factors, was performed in the skin biopsies of psoriatic lesions and adjacent non-psoriatic skin of 19 patients before and after treatment with etanercept, as well as in the skin biopsies of 10 healthy volunteers.

RESULTS: The expressions of D2-40, VEGF-C and VEGF-D on lymphatic vessels underwent statistically significant increases in untreated psoriatic skin compared with non-lesional skin, in contrast to LYVE-1, which did not involve significant increase in expression in psoriatic skin. VEGF-C expression on lymphatic vessels diminished after treatment with etanercept. Moreover VEGF-C and VEGF-D staining on fibroblasts presented with higher expression in lesional skin than in non-lesional adjacent skin.

CONCLUSION: Remodeling of lymphatic vessels possibly occurs during psoriatic lesion development, parallel to blood vessel formation. The exact role of this alteration is not yet clear and more studies are necessary to confirm these results.

Keywords: Lymphangiogenesis; Lymphatic vessels; Vascular endothelial growth factor receptor-1

INTRODUCTION

Psoriasis is an immune-mediated inflammatory disease of the skin characterized by thickening of the epidermis due to keratinocyte proliferation, inflammatory cell infiltration and increased dermal vascularity.¹ Angiogenesis is an early stage of psoriasis, occurring before the development of psoriatic lesions and manifesting with increased tortuosity, permeability and elongation of dermal papillary capillaries.² Vascular endothelial growth factor A (VEGF-A) seems to be the main angiogenic factor contributing to vascular expansion.³ VEGF-A induces blood endothelial cell (EC) proliferation and increases vascular permeability by stimulating two tyrosine kinase receptors, VEGFR-1 and VEGFR-2 on EC surface. Experimental studies have shown that VEGF-A levels are high in psoriatic skin of patients and is correlated with disease severity, while increased secretion of VEGF-A has also been identified in uninvolved skin of psoriatic

patients.⁴ Additionally, levels of serum VEGF-A, as well as soluble VEGFR-1, are elevated in the sera of psoriatic patients and correlated with disease severity.^{4,5,6} Morphological changes in blood vessels occur early in the development of psoriatic lesions, in parallel to overexpression of specific angiogenic markers like VEGF - A 121, 165, 189, VEGFR-2, NRP-1, PIGF-2.⁷ Further, molecular changes have been observed in non-lesional skin of psoriatic patients, such as a specific isoform of VEGF-A, VEGF-121 and neuropilin-1 (NRP-1), a co-receptor for VEGF-A are overexpressed compared with healthy volunteers' skin.⁸

In the last decades, new members of the VEGF family, with potent lymphangiogenic action have been discovered, namely VEGF-C and VEGF-D. Both these growth factors stimulate the tyrosine kinase receptor VEGFR-3, which is restricted to lymphatic endothelium in adult skin.⁹ Lymphatic vessel (LV) endothelium

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hyaluronic acid (HA) receptor (LYVE-1) and D2-40 are two other lymphatic markers. LYVE-1, which is a restricted lymphatic marker, was initially described as an HA receptor and homologue of CD44 by Benerji S et al. in 1999.¹⁰ It is co-expressed with vascular endothelium growth factor receptor 3 (VEGFR-3) but not the blood vessel markers CD34 or the von Willebrand factor.¹¹ D2-40 or podoplanin is a mucin-type protein, initially described in vivo by Wetterwald et al., expressed in small lymphatics, but not the large ones with smooth muscle presence.¹²

In this study we investigated LV density in psoriatic lesions versus the adjacent normal skin of the same patients, before and after treatment with an anti-TNF agent (etanercept). We also compared the density of LVs in normal patients' skin and skin from healthy individuals, evaluating the expression of LYVE-1, D2-40, VEGF-C and VEGF-D.

MATERIALS AND METHODS

Patients: punch biopsies of 3mm were sampled from 19 patients (12 men, 7 women) with plaque type psoriasis from lesional and uninvolved skin, and a distance of at least 3cm from the psoriatic plaque. All patients suffered from moderate to severe psoriasis and were monitored at the Psoriatic Clinic of the A. Sygros Hospital. Their mean age was 49.11 years (sd: 13.6). None of them had received systemic treatment during the preceding three months or topical treatment in the preceding four weeks. Skin biopsies were repeated in the same body area after 12 weeks of treatment with etanercept 50mg twice per week sc. Skin biopsies were also collected from 10 healthy volunteers (7 men, 3 women) with a mean age 49.9 (sd: 14.4) – (Table 1). Punch biopsies were performed for all subjects in the same anatomical area (more specifically the upper thigh). All the participants signed an informed consent form and the study was approved by the Ethics Committee of the “A. Sygros” Hospital.

TABLE 1: National Cholesterol Education Program's Adults Treatment Panel III (NCEP - ATPIII)

	Patients		Volunteers	
	n	(%)	n	(%)
Male	12	(63)	7	(70)
Female	7	(37)	3	(30)
	Mean (SD)		Mean (SD)	
Age in years	49.11	(13.6)	49.9	(14.4)
PASI				
Initial median PASI (min, max)			13.2	(5.7, 42.9)
Median PASI after treatment (min, max)			4.4	(0.9, 11.1)

Immunohistochemistry: a three-step Avidin –Biotin –Peroxidase (ABC) method was applied on 4µm-thick paraffin sections from formalin-fixed, paraffin-embedded skin biopsies. Briefly, the sections were dewaxed, rehydrated, and incubated with 0.3% hydrogen peroxide (H₂O₂) for 30 minutes to block endogenous peroxidase activity. To enhance antigen retrieval, sections were microwave-treated in 0.01M citrate buffer pH 6.0 at 750 W for 10 minutes (D2-40 and VEGF-D); and in EDTA pH 8.0 (Trilogy, Cell Marque, Rocklin, CA) at 750 W for 15 minutes (LYVE-1 and VEGF-C). After rinsing with Tris-Buffered Saline (TBS), normal horse serum was applied for 30 minutes to block non-specific antibody binding. Subsequently, sections were incubated overnight at 4° C with the primary antibodies. The following antibodies were used: 1) mouse monoclonal against human D2-40 (Sig 3730-1000, Signet Laboratories, Dedham, MA) at a dilution of 1:40; 2) goat polyclonal against human LYVE-1 (AF2089 R&D System, Minneapolis, MN) at a dilution of 1:80; 3) goat polyclonal against human VEGF-C (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100; and 4) mouse monoclonal against human VEGF-D (clone 78923, R&D Systems, Minneapolis, MN) at a dilution of 1:160. After additional rinsing with TBS, sections were incubated with biotinylated secondary antibodies (Vector Labs, Burlingame, CA) for 30 minutes at room temperature and then incubated with avidin-biotinylated peroxidase complex (Vectastain Elite ABC kit, Vector Labs) for 30 minutes. Diaminobenzidine was used as chromogen substrate and finally sections were counterstained with haematoxylin.

Lymphatic vessels were identified as structures with positive intense linear staining for D2-40 and LYVE-1, and measured as numbers of LVs per mm², counted by visual inspection. We also measured LVs using VEGF-C and VEGF-D staining. We detected LVs as the capillaries with distinct morphological features of lymphatics (wider and more irregular lumen than blood vessels, not encircled by pericytes), showing intensively granular staining.¹³ We also evaluated VEGF-C and VEGF-D staining on fibroblasts. After taking into consideration the intensity of staining and the percentage of stained fibroblast of VEGF-C and VEGF-D, we used the following evaluation score: weak (score 1+), moderate (score 2+) and strong (score 3+) staining. Two pathologists (PA, ST) performed the immunohistochemical evaluation without knowing patients' clinical information and worked together in cases of disagreement.

Statistical analysis was performed using the Statistical Software STATA 11.2. Statistical significance was set at two-sided $p < 0.05$. Simple tabulations were

made for sociodemographic data, and mean or medians (ranges) were calculated for the continuous variables, as appropriate. Non-parametric tests were used (Wilcoxon signed-rank test and two-sample Wilcoxon rank-sum (Mann-Whitney)). Non-parametric tests were used (Wilcoxon **matched-pairs** signed rank test and two **independent** sample Wilcoxon ranksum (MannWhitney)).

RESULTS

The initial median PASI of patients was 13.2 (range: 5.7-42.9). After 12 weeks of treatment, their median PASI was 4.4 (range: 0.9-11.1), presenting an improvement of 69%.

LV density regarding D2-40 expression in untreated psoriatic skin was higher than LV density in the adjacent normal skin (P=0.008). After treatment, LV density did not reveal a statistically significant difference between psoriatic skin and normal skin (P=0.099). No difference in D2-40 expression was

observed in the psoriatic skin before and after treatment (P=0.643). Also, we did not find any difference in LV density between skin of healthy volunteers and uninvolved skin of untreated patients (P=0.094) – (Table 2 and Figure 1).

There was no significant difference in LYVE-1 expression between psoriatic skin and non-psoriatic skin, before treatment (P=0.080) and after treatment (P=0.126). Further, there was no difference between psoriatic skin before and after treatment (P=0.494). No statistically significant difference was confirmed between normal skin of untreated psoriatic patients and healthy volunteers (P=0.198) – (Table 2 and Figure 2).

The evaluation of LVs according to VEGF-C staining revealed a statistically increased number of LVs in psoriatic skin compared with non-psoriatic adjacent skin of patients (P=0.001). This difference remained after treatment with etanercept (P=0.011). We also observed a decrease in LVs expressing VEGF-C in psoriatic skin after treatment (P=0.045).

TABLE 2: Median number of lymphatics/mm² according to the expression of D2-40, LYVE-1, VEGF-C and VEGF-D in psoriatic patients and healthy volunteers. The density of LVs appeared a statistically significant difference between L.S. and N.L.S. before treatment, according to D2-40 (P=0.008), VEGF-C (P=0.001) and VEGF-D (P<0.001) staining. LVs tended to decrease with treatment in psoriatic skin, according to VEGF-C staining (P=0.045), while the difference between L.S and N.L.S. remained after treatment (P=0.011). Moreover, VEGF-C expression revealed more LVs in N.L.S of untreated patients than in healthy volunteers (P=0.004)

		L.S. (min, max)	N.L.S. (min, max)	p-value	Healthy volunteers (min, max)	p-value*
D2-40	Before treatment	9.53 (3.81. 33.04)	6.35 (4.24. 14.61)	0.008	5.08 (3.81. 15.25)	0.094
	After treatment	8.47 (4.52. 38.63)	5.85 (2.54. 21.60)	0.099		
	p-value	0.643	0.643			
LYVE-1	Before treatment	7.31 (4.45. 33.76)	5.72 (2.96. 13.34)	0.080	4.34 (3.18. 12.71)	0.198
	After treatment	7.06 (3.56. 3.56)	5.51 (2.80. 13.98)	0.126		
	p-value	0.494	0.841			
VEGF-C	Before treatment	10.01 (6.04. 29.23)	6.17 (1.09. 11.44)	0.001	1.59 (0.85. 7.62)	0.004
	After treatment	7.62 (2.54. 12.71)	5.24 (0.85. 11.12)	0.011		
	p-value	0.045	0.268			
VEGF-D	Before treatment	3.93 (1.91. 6.99)	1.91 (0.85. 4.83)	0.001	2.33 (1.27. 5.51)	0.216
	After treatment	2.97 (1.27. 7.31)	2.90 (1.02. 7.62)	0.365		
	p-value	0.028	0.080			

L.S.: lesional skin, N.L.S.: non-lesional skin.

*The p value in this column represents the contrast between skin of healthy volunteers and non-lesional skin of patients.

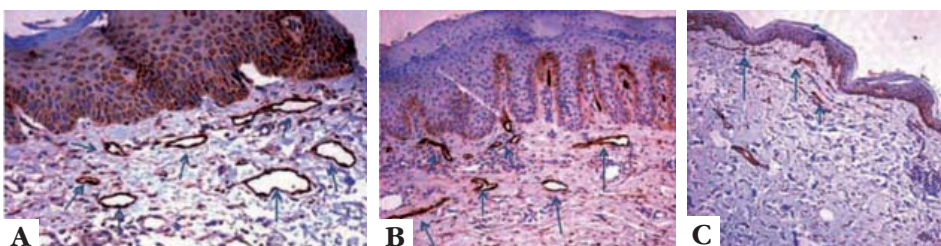


FIGURE 1: Psoriatic skin before treatment **A**, after treatment **B** and normal skin of patient **C** Linear D2-40 staining on LVs. Arrows indicate LVs. Original magnification x200

Comparing LV density in non-lesional skin of untreated patients with that of healthy volunteers' skin, we found significantly more LVs expressing VEGF-C in non-lesional skin of patients ($P=0.004$) - (Table 2). VEGF-C staining in fibroblasts increased considerably in psoriatic skin compared with non-psoriatic skin before treatment ($P<0.001$) and after treatment ($P<0.001$) - (Figure 3). Interestingly, we observed increased expression of VEGF-C in the fibroblast of normal adjacent skin of psoriatic patients before treatment, compared with normal skin after treatment ($P=0.031$). Moreover, VEGF-C expression was higher in fibroblasts for normal skin of untreated patients than in healthy individuals ($P=0.047$).

The evaluation of LVs according to VEGF-D staining revealed a statistically significantly increased LV density, expressing VEGF-D in psoriatic skin compared with non-psoriatic skin of patients before treatment ($P<0.001$). This difference did not remain after treatment ($P=0.365$). In addition, treatment did not alter the number of lymphatics in psoriatic skin ($P=0.384$), as per VEGF-D staining. Moreover, we did not find any difference between LVs in non-lesional skin of untreated patients and those in skin of healthy volunteers ($P=0.216$) - (Table 2). VEGF-D staining in

fibroblasts was higher in psoriatic skin than non-psoriatic skin before treatment ($P=0.004$) and after treatment ($P=0.011$), though there was no difference between psoriatic skin before and after treatment ($P=0.813$) and no difference between non-lesional skin before treatment and the skin of healthy volunteers ($P=0.132$) - (Figure 4).

DISCUSSION

Although the initial stage of psoriasis development remains obscure and is still under debate, angiogenesis is considered a crucial factor in the pathogenesis of the disease.¹⁴ Blood vessels in the papillary dermis become elongated, dilated and hyperpermeable; these changes precede clinical abnormalities.¹⁵

Less is known about lymphatic vessel formation and participation in the development of psoriasis. The initial absence of special markers for LVs made their observation difficult. Before the discovery of special markers for LVs, there was evidence showing that dysregulation of lymphangiogenesis can occur along with blood vessels changes in the psoriatic plaque.¹⁶ Staberg et al. suggested that an increased lymphatic network in psoriasis is mandatory to drain the increased extravasation of protein at the inflam-

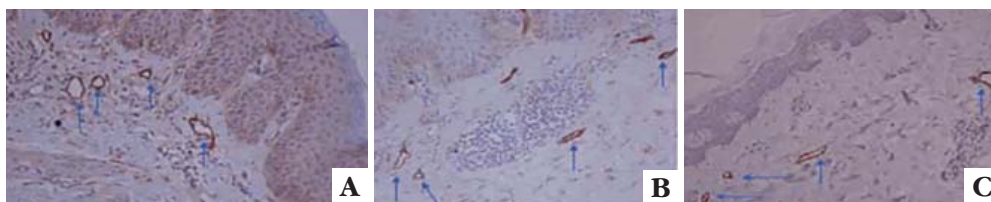


FIGURE 2: Psoriatic skin before treatment **A:** after treatment **B:** and normal skin of patient **C:** Linear LYVE-1 staining on LVs. Arrows indicate LVs. Original magnification x200

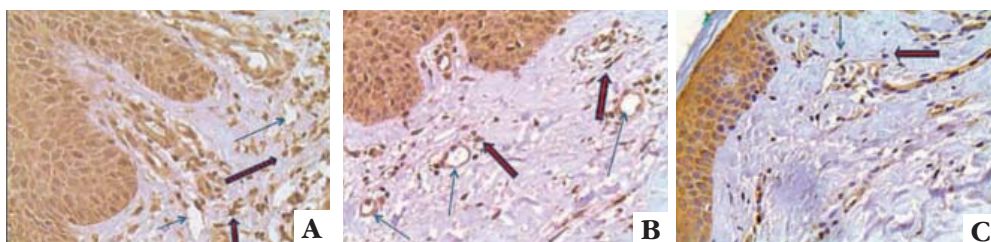


FIGURE 3: Granular staining of VEGF-C on LV wall (blue arrows). Intense VEGF-C staining in fibroblasts was observed mostly on psoriatic skin (red arrows). **A:** psoriatic skin before treatment, **B:** psoriatic skin after treatment, **C:** normal skin of patient

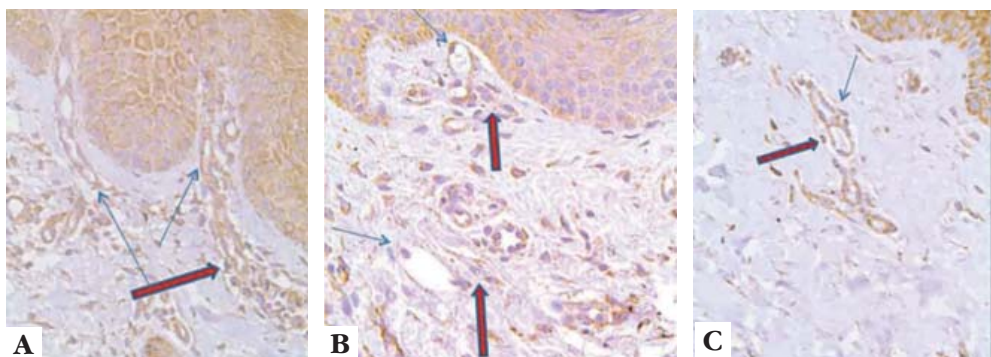


FIGURE 4: Granular VEGF-D staining on LV wall (blue arrows). VEGF-D is also expressed in fibroblasts with intensity on psoriatic skin (red arrows). **A:** psoriatic skin before treatment, **B:** psoriatic skin after treatment, **C:** normal skin of patient. Original magnification x200

matory site.¹⁷ According to other investigators, LVs in psoriasis do not increase in the plaque but increase significantly in the perilesional area, suggesting that edema in psoriatic skin may be due to ineffective lymph drainage.¹⁸

The discovery of new specific molecules for detecting lymphatic vessels allowed for closer study of the lymphatic network. D2-40 or podoplanin is a mucin-type transmembrane glycoprotein, which is expressed on lymphatic endothelial cells and positive for VEGFR-3 LVs, but not on blood vessels. Its function is not clear, though it may regulate the permeability of LVs and their integrity.¹⁹ Henno et al. studied the expression of D2-40 by immunohistochemistry in psoriatic skin and uninvolved skin of patients, finding a larger area in the superficial dermis of psoriatic skin to be occupied by LVs, compared with non-psoriatic skin. The same investigators described a gradual increase in number and size of LVs between uninvolved skin and psoriatic skin.^{7,8} Fielder et al. also described a higher number of LV sections in the upper dermal plexus of psoriatic skin than in non-psoriatic skin.²⁰ In accordance with these findings, we observed a significant increase in LV density in psoriasis using D2-40 marker, indicating that there is an induction of lymphangiogenesis in psoriasis. LYVE-1, another specific lymphatic marker, which has been used in studies of tumour lymphangiogenesis and localizes across lymphatic EC, may have a role in transporting hyaluronic acid from tissues to lymph.¹¹ Our immunohistochemical analysis for LYVE-1 did not reveal significant changes in the expression of this molecule between psoriatic and non-psoriatic skin.

We also estimated the density of LVs in skin biopsies before and after treatment, with an anti-TNF alpha agent, etanercept. We did not confirm a significant difference in the number of LVs in psoriatic skin after 12 weeks of treatment, according to the expression of D2-40 and LYVE-1. Etanercept is a fusion protein and soluble TNF type II receptor that binds to TNF trimer and lymphotoxin a (LTa), inhibiting partially the action of TNF, because of a fast association and dissociation rate with TNF.^{21,22} Its favorable effect in psoriasis is due to the decrease in inflammatory infiltration from the first month of treatment and even before histological and clinical changes.²³ There are data supporting a secondary angiogenic effect of TNF by recruitment of inflammatory cells in psoriatic plaques and a significant improvement of psoriatic microangiopathy after TNF inhibition.^{24,25} In another study by Avramidis et al., reduction in blood vessels and significant reduction in CD31 and VEGF expression was observed in psoriatic skin, after 10 weeks of treatment with etanercept. Moreover, etanercept was found to minimize the expression of anti-apoptotic

proteins of endothelial cells (Kf-kB, Bcl-2, Bcl-xL) and upregulate thrombospondin-1 (TSP-1) expression, which is an inhibitor of angiogenesis.²⁶ Considering the parallel expansion of blood vessels and LVs in normal skin and psoriasis, we would expect to see a decrease in LVs density after anti-TNF therapy. Our results did not confirm a decrease in LV density after treatment, following the use of specific endothelial lymphatic markers (D2-40, LYVE-1). This possibly indicates a delay in lymphatic remodeling, similar to what occurs during psoriatic lesion development, where LV formation follows blood vessel formation.⁷ In the case of LYVE-1, there is *in vitro* experimental evidence showing that this marker is downregulated after TNF-alpha exposure.^{27,28} In a recent study of gene expression in psoriasis, investigators described a 21% improvement in LYVE-1 expression, after 12 weeks of etanercept treatment, possibly indicating a delay in lymphatic remodeling or incomplete reversal of some molecular markers after treatment.²⁹ An additional interpretation of our findings could be that skin biopsies after treatment were taken from lesional skin, which was not completely but only partially resolved.

VEGF-C and VEGF-D are members of the family of VEGF growth factors, acting on VEGFR-3 with high affinity, which has been shown to regulate the growth of the lymphatic system. VEGF-C is considered a mitogenic molecule for lymphatic EC, inducing the migration of EC and increasing vascular permeability, as a primary mediator of lymphatic growth.^{19,30} VEGF-D is also a mitogenic and lymphangiogenic factor, which, moreover, binds to VEGFR-2, suggesting a possible angiogenic effect.¹⁹ Measurement of VEGF-C using PCR-RNA revealed overexpression of this marker in psoriatic skin compared with uninvolved skin, with a parallel increase in VEGFR-3 and NRP-2a expression, indicating that lymphangiogenesis contributes to the development of psoriasis.⁷ Evaluating VEGF-C and VEGF-D staining in LVs, we observed a higher number of LVs expressing VEGF-C and VEGF-D in psoriatic skin than in non-psoriatic skin. Following VEGF-C staining, LVs were significantly less numerous after treatment with etanercept. We also found higher VEGF-C and VEGF-D expression in fibroblasts of psoriatic skin than in non-psoriatic skin of affected patients. Cells that express VEGF-C and VEGF-D include mesenchymal cells, inflammatory cells (macrophages and dendritic), skeletal muscle and smooth muscle cells from arteries.^{13,27,31} Dermal fibroblasts strongly express VEGF-C and a higher expression is induced by inflammatory cytokines like TNF- α .¹³ VEGF-C and VEGF-D amounts may be elevated in an inflammatory skin disease and this is consonant with our findings. Reduction in VEGF-C expression

after anti-TNF- α blockage is also expected, given the induction effect of TNF on VEGF-C production.¹³

Interestingly, we found a higher expression of VEGF-C in LVs and fibroblasts of uninvolved patients' skin compared with that of healthy volunteers. Pre-psoriatic phenotype has been described previously in non-lesional skin from patients. A higher expression of adhesion molecules like ICAM-1 and E-selectin on EC, and LFA-1 on Langerhans cells, has been found in the non-lesional skin of patients, than in normal, healthy individuals.³² Metabolic markers, like GAPDH, angiogenesis-related marker (VEGF-121) and lymphangiogenesis marker VEGFR-3, were overexpressed in non-lesional skin of patients compared with healthy volunteers.⁸ VEGF-C overexpression in uninvolved skin of patients may be part of the prepsoriatic phenotype.

VEGF-C and VEGF-D are both growth factors that are expressed by several cells and diffused through the tissue. This may represent a limitation and cause difficulty in evaluating these stains on lymphatic vessels. To reduce this inconvenience, two pathologists evaluated the skin biopsies independently. LVs detected the capillaries bearing distinct histological features of lymphatics (wide and irregular lumen, not encircled by pericytes), with intensive granular staining. Pathologists worked together only in cases of disagreement. In addition to previous limitations, we have to consider that the agent and treatment duration possibly reflect specific results. Longer treatment or different agents may reveal more intensive changes.

CONCLUSION

In conclusion, the present study shows that lymphatic growth factors (VEGF-C and VEGF-D) and the specific lymphatic marker D2-40 are overexpressed in psoriatic skin, indicating that remodeling of the lymphatic vascular system occurs in psoriasis, possibly following the expansion of blood vessels. The actual molecular mechanism of this vascular development, as well as the role of vascular remodeling, is not yet clear. Treatment with etanercept has proved to decrease VEGF-C and VEGF-D expression, while D2-40 and LYVE-1 did not reveal a statistically significant alteration. It remains unclear whether the impact of treatment is a direct or indirect effect and if this effect may interfere with disease development and disease recession. As little is known about the lymphatic network in psoriasis, further experimental studies are necessary to elucidate the structure and function of this system, and to clarify the effect of anti-TNF treatment on it. □

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