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Up-regulation of the chemokine CCL21 in the skin of subjects exposed to irritants

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Abstract

Background: Expression of murine CCL21 by dermal lymphatic endothelial cells (LEC) has been demonstrated to be one of the most important steps in Langerhans cell emigration from skin. Previously, our group and others have found that this chemokine is up-regulated in different human inflammatory skin diseases mediated by diverse specific immune responses. This study was carried out to investigate the involvement of CCL21 in human skin after challenge with irritant agents responsible for inducing Irritant Contact Dermatitis (ICD).

Results: Eleven normal individuals were challenged with different chemical or physical irritants. Two patients with Allergic Contact Dermatitis (ACD) were also challenged with the relevant antigen in order to have a positive control for CCL21 expression. Macroscopic as well as microscopic responses were evaluated. We observed typical ICD responses with mostly mononuclear cells in perivascular areas, but a predominance of polymorphonuclear cells away from the inflamed blood vessels and in the epidermis at 24 hours. Immunohistochemical studies showed up-regulation of CCL21 by lymphatic endothelial cells in all the biopsies taken from ICD and ACD lesions compared to normal skin. Kinetic study at 10, 48, 96 and 168 hours after contact with a classical irritant (sodium lauryl sulphate) showed that the expression of CCL21 was increased in lymphatic vessels at 10 hours, peaked at 48 hours, and then gradually declined. There was a strong correlation between CCL21 expression and the macroscopic response ($r = 0.69$; $p = 0.0008$), but not between CCL21 and the number of infiltrating cells in the lesions.

Conclusions: These results provide new evidence for the role of CCL21 in inflammatory processes. Since the up-regulation of this chemokine was observed in ICD and ACD, it is tempting to speculate that this mechanism operates independently of the type of dermal insult, facilitating the emigration of CCR7+ cells.

Background

Skin derived Dendritic cells (DC) such as Langerhans cells (LC) and interstitial DC play vital roles in immune surveillance because they have efficient uptake and antigen

presentation mechanisms. Antigens are captured and processed, and few hours later DC reach peripheral lymph nodes (PLN) via afferent lymphatics [1].

The molecular mechanisms that regulate DC emigration are not fully understood. It seems to depend on the selective responsiveness of DC to special sets of signals. Cytokines such as IL-1β, TNF-α, IL-18 [2], CCL19 [3], and other molecules like Osteopontin [4], MMPs [5], prostaglandins [6,7] leukotriene C4 [8] and, Lactoferrin [9], are proved to regulate DC migration by several mechanisms. One of the most important mechanisms is the up-regulation of CCR7 in the emigrating DC, which seems to be crucial for these cells to reach PLN through lymphatics [10]. Only some subsets of DC and lymphocytes express CCR7, allowing them to be attracted by the specific ligands CCL21 and CCL19 [11].

CCL21 has been reported as the major chemokine responsible for DC emigration from peripheral tissues [1,12]. This chemokine is expressed in murine and human high endothelial venules (HEV) within secondary lymphoid organs. Saeki et al demonstrated that mice lymphatic endothelial cells (LEC) produce CCL21 [1], and more recently a similar observation has been reported in human normal skin [13]. Since it was discovered, CCL21 was classified as a constitutive chemokine. However our group and others have clearly demonstrated up-regulation of this chemokine by LEC and blood endothelial cells (BEC) in different inflammatory skin diseases mediated by specific immune response [13-15]. The selective expression of CCR7 on immune cells, and the constitutive and inflammatory CCL21 production would suggest two different roles for this chemokine.

We conducted this study in order to investigate this chemokine in human irritant contact dermatitis (ICD). We found a weak expression of CCL21 in dermal LEC from normal skin; however after contact with several irritant agents, CCL21 was up-regulated.

Results

As shown in Table 1 all irritant patch test reactions induced by chemical agents were macroscopically classified as moderate (grade 2) or strong (grade 3) and physical agents induced weak (grade 1) or strong (grade 3) macroscopic responses. Skin biopsies taken from Allergic Contact Dermatitis (ACD) patients at 48 hours after contact with specific antigens were classified as grade 3, whereas normal skin biopsies from all individuals and a skin biopsy from a patient with an empty patch were grade 0 (no irritation) and 1, respectively.

Table 1: Individuals, contact agents and macroscopic responses

Subjects	Contact agents	Concentration (w/v)	Time of exposition	Macroscopic response#
Control skin				
	Normal skin from all patients	-	-	0
GN	Patch alone	-	24 h	1
Specific antigens				
PF	Nickel	5%	48 h	3
GL	Peru Balsam	25%	48 h	3
Chemical agents				
RL	Aqueous Sodium Lauryl Sulphate	10%	24 h	2
EY	Toluene	undiluted	24 h	2
AR	Trichloroacetic acid	35%	24 h	3
CL	Sodium Hypochlorite	undiluted	24 h	2
SD	Salicylic Acid	30%	24 h	2
CM	Sodium Hydroxide	10%	24 h	3
PC	Glycolic Acid	70%	24 h	2
KM	Benzalkonium chloride	5%	24 h	2
Phisical agents				
KR	Tape striping	-	15 min	1
EE	Liquid Nitrogen	-	2 cycles of 15 sec	3
MA	Ruda tea + UVA	-	5 min	1

Table 1: Individuals, contact agents and macroscopic responses *(Continued)*

			Kinetic response	
AJ	SLS	10 %	-	0
AJ	SLS	10 %	10 h	1
AJ	SLS	10 %	48 h	2
AJ	SLS	10 %	96 h	1
AJ	SLS	10 %	168 h	1

Macroscopic response grading scale: no irritation = 0, weak = 1, moderate = 2, strong = 3

Table 2: Histological and Immunohistochemical studies in Contact Dermatitis

Subjects	Contact agents	Total number of inflammatory cells in dermis (mm^2)		DERMIS				
				Number of leukocytes (%)				CCL21 expression in LEC*
		Normal skin	Inflamed skin	Perivascular infiltrate		Non-perivascular infiltrate		
				MC	PC	MC	PC	
Control skin								
Normal skin from all patients		-	-	ND	ND	ND	ND	**1**
GN	Patch alone	436	468	224 (**47.9**)	96 (**20.5**)	76 (**16.2**)	72 (**15.4**)	**1**
Haptens								
PF	Nickel	117	652	512 (**78.5**)	16 (**2.4**)	104 (**16.0**)	20 (**3.1**)	**3**
GL	Peru Balsam	44	1104	496 (**44.9**)	60 (**5.4**)	448 (**40.6**)	100 (**9.1**)	**3**
Chemical irritants								
RL	SLS 10%	292	988	152 (**15.4**)	392 (**39.7**)	43 (**4.4**)	404 (**40.9**)	**2**
EY	Toluene	568	1200	468 (**39.0**)	168 (**14.0**)	168 (**14.0**)	396 (**33.0**)	**3**
AR	Trichloroacetic acid	432	952	300 (**31.5**)	132 (**13.9**)	48 (**5.0**)	472 (**49.6**)	**2**
CL	Sodium Hypochlorite	540	948	108 (**11.4**)	160 (**16.9**)	96 (**10.1**)	584 (**61.6**)	**3**
SD	Salicylic Acid	324	608	276 (**45.4**)	116 (**19.1**)	32 (**5.3**)	184 (**30.3**)	**2**
CM	Sodium Hydroxide	616	1108	40 (**3.6**)	236 (**21.3**)	60 (**5.4**)	776 (**70.0**)	**2**
PC	Glycolic Acid	688	1768	191 (**10.8**)	1093 (**61.8**)	19 (**1.1**)	463 (**26.2**)	**2**
KM	Benzalkonium chloride	488	1268	416 (**32.8**)	634 (**5.0**)	112 (**8.8**)	676 (**53.3**)	**3**
Physical irritants								
KR	Tape striping	352	988	268 (**27.1**)	232 (**23.5**)	108 (**10.9**)	380 (**38.5**)	**1**
EE	Liquid Nitrogen	192	1824	111 (**6.1**)	396 (**21.7**)	77 (**4.2**)	1240 (**68.0**)	**3**
MA	Rue + UVA	304	344	184 (**53.5**)	48 (**13.9**)	8 (**2.3**)	104 (**30.2**)	**3**
Statistical differences caused by exposure to irritant agents (p values)		**p < 0.05**		p > 0.05		**p < 0.05**		
KINETIC STUDY								
AJ-10 h	SLS 10%	204	372	132 (**35.5**)	80 (**21.5**)	44 (**11.8**)	120 (**32.2**)	**2**
AJ-48 h	SLS 10%	204	1032	548 (**53.1**)	124 (**12.0**)	124 (**12.0**)	236 (**22.9**)	**3**
AJ-96 h	SLS 10%	204	308	80 (**62.5**)	4 (**3.1**)	36 (**28.1**)	8 (**6.2**)	**1**
AJ-168 h	SLS 10%	204	456	512 (**78.5**)	16 (**2.4**)	104 (**15.9**)	20 (**3.1**)	**1**

*CCL21 expression was semi-quantified as 0 = negative; 1 = weak; 2 = moderate; 3 = strong. Abbreviations: SLS, sodium lauryl sulphate; MC, mononuclear cells; PC, polymorphonuclear cells; ND, not done.

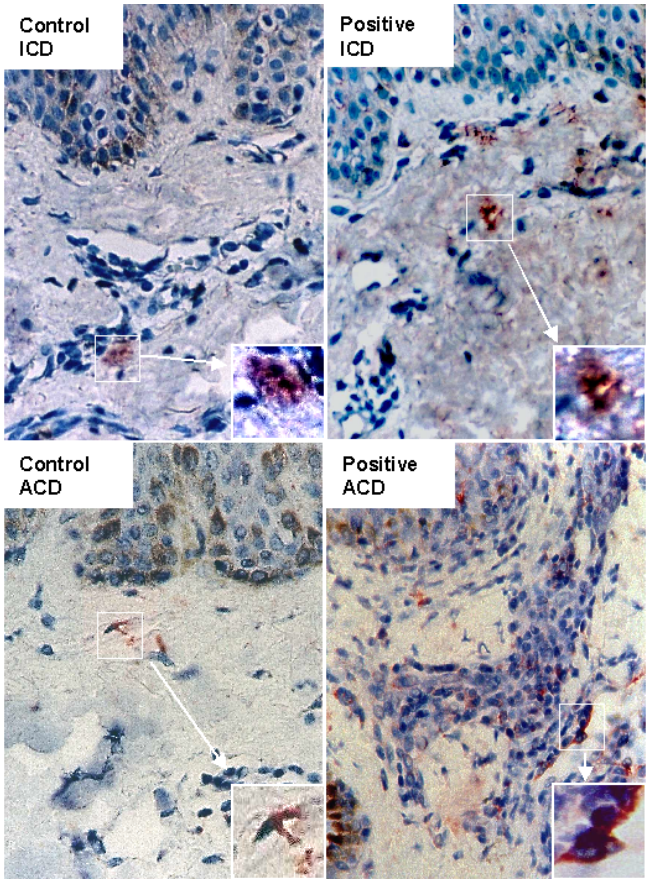

Figure 1
CCL21 expression in Contact Dermatitis Immunohistochemistry for CCL21 in biopsies taken from: normal skin (Control ICD); skin challenged with 10 % aqueous sodium lauryl sulphate at 24 hours (Positive ICD); non-challenged skin (Control ACD); and skin challenged with 5% Nickel at 48 hours (Positive ACD). Insets shows the marked areas amplified in order to reveal the $CCL21^+$ lymphatic vessels. 400 × (original magnifications).

The histopathological study of dermal blood vessels at 24 hours in ICD lesions showed a moderate hypertrophy and swelling of endothelial cells with an important infiltration of leukocytes when compared with normal skin ($p < 0.05$, Table 2). When perivascular mononuclear cells (MC) or polymorphonuclear cells (PC) cells were studied and compared with infiltrating cells located away from the blood vessels (non-perivascular) we found mostly PC ($p < 0.05$) only in ICD. The inflammatory cells found out in the epidermis were principally PC in most of the individuals with ICD (9/11), but MC in patients with ACD (2/2) (Table 2).

The kinetic study performed with 10% Sodium Lauryl Sulphate (SLS) showed at 10 hours a small number of inflammatory cells being mostly PC in non-perivascular areas when compared with perivascular areas (Table 2). The total number of infiltrating cells in irritated skin peaked at 48 hours and decreased significantly at 96 hours and 168 hours.

In order to investigate CCL21 expression we performed immunohistochemical studies with an amplifier system to improve the sensitivity. Weak CCL21 expression was found in all normal skin biopsies, but this expression increased to moderate or intense in skin biopsies taken at 24 hours after contact with any of the irritant agents and at 48 hours in the ACD patients (Figure 1 and Table 2). In normal and damaged skin, CCL21 was found in small sub-epidermal lymphatic vessels but in ICD and ACD lesions it was also seen in lymphatic vessels next to blood vessels surrounded by inflammatory cells (data not shown). These findings were obtained in all biopsies independently of the irritant agent applied.

When we investigated levels of CCL21 expression in the skin biopsies obtained at different times from one ICD patient we observed moderate levels of this chemokine at 10 hours, which further increased at 48 hours and declined thereafter (Figure 2).

In some individuals with ICD we perform immunohistochemistry in serial sections of several biopsies using anti CCL21 and anti Prox-1 (specific marker for lymphatic vessels). Vessels expressing CCL21 were also positive for Prox-1, however we could not get good quality pictures with this antibody (data not shown).

Positive correlation was found when we compared the macroscopic response with CCL21 expression ($r = 0.69$; $p = 0.0008$; CI = 0.34–0.87) but there was no correlation between the number of infiltrating cells and CCL21 expression in all the patients studied ($r = 0.29$; $p = 0.2155$; CI = -0.19–0.66).

Discussion

Irritant contact dermatitis (ICD) is a multifactor disease, which involves a combination of endogenous and exogenous factors. The exact mechanisms involved in ICD responses are not fully understood. ICD can only be distinguished from allergic contact dermatitis (ACD) by patch tests as histological examination of skin biopsies from both entities shows MC infiltration at 48 hours [16]. In this study we observed in ICD patients a predominance of PC in areas away from blood vessels and the epidermis at 24 hours after contact with several irritants, whereas in patients with ACD we previously found mostly MC in the

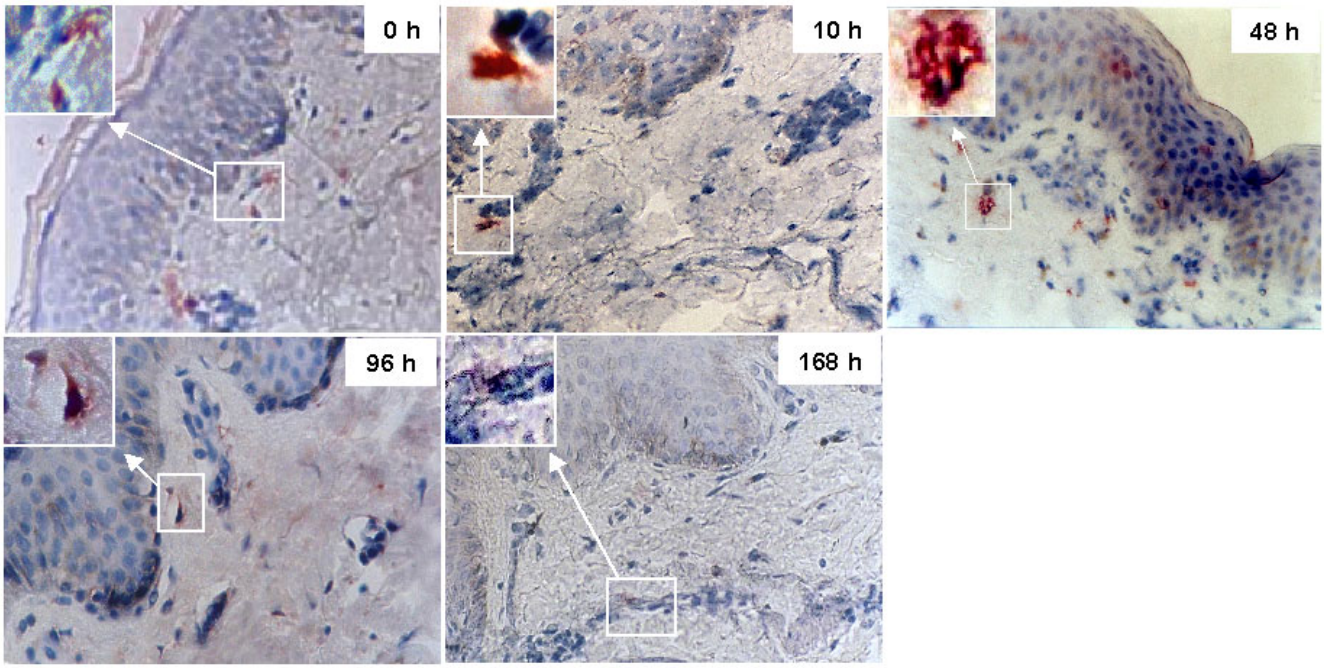

Figure 2
Kinetics of CCL21 production during Irritant Contact Dermatitis Expression of CCL21 studied by immunohistochemical assay in skin biopsies taken before (0 hours) and after contact with 10 % aqueous sodium lauryl sulphate at 10, 48, 96 and 168 hours. Insets shows the marked areas amplified in order to reveal the CCL21$^+$ lymphatic vessels. 400 × (original magnifications).

dermis and epidermis as early as 10 h after challenge with the relevant antigen [17].

These differences are probably due to the nature of the immune responses in each entity; ICD is a non-immunological local reversible inflammatory reaction induced by contact with irritants [18] whereas ACD is a specific immune response mediated by Th1/Tc1 lymphocytes [19]. In ICD, PC are attracted to the inflammatory area at a very early stage followed by bystander lymphocytes. By contrast, specific lymphocytes attracted to the site of hapten contact in ACD and are responsible for the inflammatory response.

In 1997 Hedrick et al.[20] and Nagira et al. [21], identified a novel human CC chemokine mapped at chromosome 9p13. Using Northern blot analysis they found human CCL21 expression restricted to lymphoid organs, while murine CCL21 showed a broader tissue distribution with the highest mRNA levels found in spleen and lung. Later on, Gunn et al found CCL21 expression HEV from secondary lymphoid organs and lymphatics from several murine tissues [22].

CCL21 expression by HEV has been reported to be involved in CCR7$^+$ naive lymphocytes recruitment in secondary lymphoid organs in human and mice [22-24]. Around the same time Saeki et al demonstrated that CCL21 production by LEC is crucial for the emigration of murine CCR7$^+$ mature LC from the dermal compartment [1].

A couple of years ago it was demonstrated by *in vitro* experiments that human LEC constitutively secrete CCL21 at their basal side [25], and more recently Weninger et al. found expression of this chemokine by LEC from normal human skin using immunofluorescence staining [13]. Our previous studies and other investigations did not find CCL21 expression by LEC in normal human skin. Since these could be attributed to the low sensitivity of the assay used, in the present study, we run an immunohistochemical technique with a catalyzed amplification signal system to increase the level of detection of this chemokine. We reported here that all normal skin biopsies express low levels of CCL21 in LEC.

Recent reports have provided evidence that CCL21 plays a role during inflammatory processes. Up-regulation of this

chemokine by human BEC was demonstrated by Christopherson et al using conventional immunohistochemistry on skin biopsies taken from patients with inflammatory skin diseases such as atopic dermatitis, lichen planus and graft-versus-host [26]. Weninger et al performing immunofluorescence staining found CCL21 expression by BEC from rheumatoid arthritis and ulcerative colitis biopsies, but not psoriasis where CCL21$^+$ vessels were rare [13]. They suggested that this could be related to the significant number of CCR7$^+$ T cells in biopsies from rheumatoid arthritis and ulcerative colitis patients, but not in psoriasis, supporting the idea that CCL21 was only recruiting naive T lymphocytes to rheumatoid arthritis and ulcerative colitis tissues. These data identify CCL21 expression by BEC as an important determinant for naive T cell migration to tertiary lymphoid tissues, and suggest the CCL21/CCR7 pathway as a therapeutic target in diseases associated with naive T cell recruitment.

On the other hand, our group and others have demonstrated that up-regulation of CCL21 could also take place in dermal LEC from patients with other diseases. In this regard, Katou et al. have shown by RT-PCR and immunohistochemistry CCL21 expression only in LEC from chronically inflamed skin infected with *Candida albicans*. Moreover, the lymphocytes that infiltrate this inflamed tissue were mostly CCR7$^-$ memory T cells [15].

Using *in situ* hybridization and immunohistochemistry, we have previously found low levels of CCL21 in lymphatic vessels at 2 hours, which further increased at 10 and 48 hours in positive patch tests from patients with ACD. Very low expression of this chemokine was observed at 48 hours in skin biopsies from the sites challenged with an irrelevant antigen [14]. In this study we showed that CCL21 up-regulation was not related with an increase of CCR7$^+$ infiltrating lymphocytes but it was associated with the disappearance of Langerin+ cells from the epidermis. Since Langerin is a marker of immature LC [27], we speculate that under this inflammatory condition the increased production of CCL21 could facilitate the emigration of CCR7$^+$ cells through lymphatic vessels. Based on these evidences we have proposed that CCL21 is probably much more than a constitutive chemokine [28].

In this paper we found that CCL21 is also up-regulated by LEC during ICD by all of the irritant agents used. This up-regulation was found as early as 10 hours and reached a maximum level at 48 hours after contact with irritant agents, and it correlated with the macroscopic response. It is worth mentioning that Cumberbatch et al [29] have shown that skin application of 10 % SLS in mice resulted in a significant elevation of DC numbers in PLN, which could also be mediated by up-regulation of this chemokine by dermal lymphatics in those animals.

In spite of the fact that cytokines and chemokines involvement in ICD and ACD responses has been broadly studied [30-32], the molecular mechanisms responsible for up-regulation of CCL21 remind to be elucidated. We are currently investigating this unanswered question.

Conclusions

Based upon these results, we concluded that two different types of stress signals affecting the skin, antigen specific or non-specific immune responses are capable of inducing CCL21 up-regulation by LEC. This may enhance the steady state emigration of CCR7$^+$ LC.

Methods

Subjects

Thirteen healthy Caucasian volunteers, 7 males and 6 females were recruited from the Dermatological Clinic at the Hospital Privado (Córdoba, Argentina). Their ages ranged from 21 to 60 years (average = 35 years) and they did not have any skin allergies or other illnesses. Two 46 years old women diagnosed as having ACD were also studied. The Institutional Ethics Committee approved the experimental protocols and all the subjects gave informed consent. Individuals were not taking oral or topical medication during at least one month before the beginning of this study.

Contact agents

In order to get objective ICD [33] individuals were exposed to different irritants (8 chemicals and 3 physicals) as shown in table 1. Chemical irritant agents were: 10% Aqueous Sodium Lauryl Sulphate (SLS), undiluted Toluene, 35% Trichloroacetic acid, undiluted Sodium Hypochlorite, 30% Salicylic Acid, 10% Sodium Hydroxide, 70% Glycolic Acid 5% Benzalkonium chloride; and 30 μl of each irritant were applied at previous determined concentrations in order to achieve a similar clinical response at 24 hours (grade 2 or 3) [34]. The physical agents used were: tape stripping, liquid nitrogen and contact with rue (*Ruta graveolens*) followed by ultraviolet A radiation (Waldmann Lichtechnik UV 7001 K) which produce a phytophototoxic reaction [35]. None of these irritants caused the irreversible damage of the skin.

Specific antigens (30 μl of 5% Nickel or 25% Peru Balsam) were applied in non-irritant concentrations in order to get efferent responses in two patients already diagnosed with ACD.

Chemical and contact agents were applied under 8-mm Finn chambers on Scanpor tape (patches) at 5 cm of the spine.

Study design and samples collection

The normal individuals and the two patients with ACD were challenged with one of the irritant agents and their relevant antigens respectively, as previously described. One control individual was in contact with a patch without any irritant (control patch). Macroscopic responses were measured at 24 hours in the normal individuals and at 48 hours in the ACD patients, by evaluating in a blinded way skin erythema, dryness, edema and eschar formation using the following arbitrary units (0 = no reaction, 1 = weakly positive reaction, 2 = moderately positive reaction, 3 = strongly positive reaction) [30]. At the time of the macroscopic read out, skin biopsies from normal skin and patch sites from all these individuals were taken after local anesthesia (1 ml of 2% xylocain).

In order to perform a kinetic study, four patches with 10 % (w/v) aqueous SLS were applied in the back of another volunteer and macroscopic responses and biopsies were taken from the irritated site at 10, 48, 96 and 168 hours. A control biopsy from normal skin was also taken.

All tissue biopsies were immediately fixed in 4% buffered formalin, pH 7.4 and then embedded in low-temperature paraffin wax. Serial sections (5 μm) were cut from biopsies, mounted on 0.1% poly-L-lysine-coated slides, dried overnight and stored at room temperature until use.

Histology and immunohistochemistry

One skin section from each biopsy was processed for histological study and stained with haematoxylin/eosin. Two pathologists examined all the slides blindly and made a semiquantitative assessment of dermis infiltration, congested or dilated vessels, vesicles, spongiosis and levels of expression of CCL21.

The sections were counted in duplicate on 16 adjacent fields at × 400 magnification blinded to the patient's clinical status. The number of mononuclear cells and polymorphonuclear cells were counted per mm^2.

For immunohistochemistry skin sections (6 mm) 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 (Catalyzed Signal Amplification System (CSA), DAKO Corporation, USA) for 5 min and subsequently washed thoroughly with PBS. To block unspecific sites, tissue samples were incubated with Blocking solution (CSA, DAKO Corporation, USA) in PBS for 10 min, incubated for 1 hours with goat anti human CCL21 (SC-5808, Santa Cruz Biotechnologies) at 0.067 μg/ml or rabbit anti human Prox-1 (kindly provided by Dr. Guillermo Oliver, Department of Genetics, St Judes Children's Hospital, Memphis, USA) at 1/1000. After washing, sections were incubated for 30 minutes with anti-goat IgG-Biotin at 0.25 μg/ml (sc-2042, Santa Cruz Biotechnologies). Isotype-matched immunoglobulins were used as negative controls. After washing with PBS, the slides were incubated with the amplification and streptavidin solutions (CSA, DAKO Corporation, USA) according to the manufacturer's instruction. All incubations were performed at room temperature in a humid chamber, the reaction cascade was visualized by incubation with 3-amino-9-ethylcarbazole (AEC, Dako, USA) as a substrate and the slides were counterstained with haematoxylin.

Positive CCL21 staining was only found on lymph vessels which were identified based on their characteristic histological appearance (irregular morphology, very thin walls, absence of red blood cells, light projections toward the light that are interpreted as valves), as well as their localization: distant from the infiltrating cells. The expression of $CCL21^+$ in lymphatics was semi-quantified using the following criteria (0 = negative; 1 = weak; 2 = moderate; 3 = strong staining).

Statistical analysis

Variability of the parameters studied was analyzed using Friedman's test and correlation coefficient was obtained by Spearman's method with correction for tied values. For all tests, $p \leq 0,05$ was considered significant.

Authors' contributions

EY carried out and analyzed the histological and immunohistochemical studies, and drafted the manuscript, OS participated in the histological and immunohistochemical studies, RLA participated in the design of the study as the chief of the Dermatological Clinic, KR recruited the individuals, performed the skin biopsies and evaluated the macroscopic responses, SHM conceived the study, participated in its design and coordination. All authors read and approved the final manuscript.

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