

Peripheral Lymph Node Addressins are Expressed on Skin Endothelial Cells

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The term “peripheral node addressins” describes a set of several endothelial adhesion molecules, which collectively bind to L-selectin and react with monoclonal antibody MECA-79. They regulate lymphocyte recirculation through peripheral nodes. Their expression is thought to be restricted to a specialized vascular segment within the node, called the high endothelial venule. In certain chronic skin diseases, however, postcapillary venules of the skin may also acquire a high endothelial venule-like morphology. Employing immunohistochemistry on cryostat sections, we found these skin endothelial cells – like peripheral node high endothelial venules – to be reactive with monoclonal antibody MECA-79. Tissue lysates from the same specimens were then analyzed by immunoprecipitation using recombinant human L-selectin Fc-chimeras followed by immunoblotting using monoclonal antibody MECA-79. In contrast to peripheral node

endothelium, which mainly expressed peripheral node addressin moieties of molecular sizes 90–110 kDa and 160 kDa, endothelial cells in cutaneous T cell lymphoma skin lesions expressed an additional and not yet defined 220 kDa peripheral node addressin-like molecule. Most surprisingly, even in normal skin specimens, we found a distinct subset of endothelial cells located around hair follicles constitutively expressing 90–110 kDa peripheral node addressin-like moieties. It is intriguing to speculate that – in analogy to the role of peripheral node addressins in peripheral nodes – the induced expression of peripheral node addressins in chronic T cell mediated skin diseases is responsible for a sustained lymphocyte recruitment. The constitutive expression of peripheral node addressins on perifollicular endothelium may serve for a continuous lymphocyte recirculation through normal skin. *Key words: L-selectin ligands/MECA-79/skin microvasculature. J Invest Dermatol 113:410–414, 1999*

In order to populate and continuously recirculate through peripheral lymph nodes, lymphocytes utilize a distinct cell adhesion molecule, L-selectin, expressed on their cell surface. The biologic importance of L-selectin was demonstrated by experiments showing that L-selectin-deficient lymphocytes are unable to enter peripheral nodes (Arbones *et al*, 1994; Steeber *et al*, 1996; Tang *et al*, 1998). As a functional consequence, lymphocytes cannot “see” antigens presented by antigen-presenting cells within the regional nodes, which results in impaired sensitization (Catalina *et al*, 1996).

L-selectin exerts this function by binding to ligands expressed on a specialized vascular segment within the node, called the high endothelial venule (HEV). Most of the current knowledge about these “node-specific L-selectin ligands” is derived from studies using monoclonal antibody (MoAb) MECA-79. This antibody reacts with sialylated, fucosylated, and sulfated carbohydrates (sulfo-sLex) coupled to a diverse set of glycoproteins and blocks lymphocyte homing to peripheral nodes (Streeter *et al*, 1988; Hemmerich *et al*, 1994). Therefore, these MECA-79-reactive L-selectin ligands expressed on HEV have been collectively called peripheral node

addressins (PNAd). PNAd isolated from human lymph nodes and tonsils encompasses several glycoproteins of molecular weights 50–60, 90–110, 160, and 200 kDa (Berg *et al*, 1991; Suzuki *et al*, 1996; Sasseti *et al*, 1998). CD34 represents a part of the 90–110 kDa component and the 160 kDa molecule is a podocalyxin-like protein, whereas the identities of the remaining proteins of the “PNAd complex” in humans are as yet unknown (Berg *et al*, 1991; Baumhueter *et al*, 1993; Puri *et al*, 1995; Sasseti *et al*, 1998). PNAd has to be separated from MECA-79-nonreactive L-selectin ligands, such as PSGL-1 or ELAM-1, which do not function as lymph node homing receptors and have an altered tissue distribution (Imai *et al*, 1993; Kogelberg and Rutherford, 1994; Tu *et al*, 1996; Sackstein *et al*, 1997; Zöllner *et al*, 1997; Berg *et al*, 1998; Clark *et al*, 1998; Snapp *et al*, 1998).

Based on the observation that postcapillary venules in certain chronic skin diseases such as psoriasis, pseudolymphoma, and T cell lymphoma acquire a HEV-like morphology, similar to that seen in peripheral nodes (Streilein, 1990), we set out to explore, whether these skin endothelial cells also acquire a HEV-like phenotype, namely the expression of L-selectin ligands of the PNAd complex.

MATERIALS AND METHODS

Human tissues Human lymph nodes from the axillary and inguinal region and normal human skin from the thighs, limbs, and trunk were obtained from cadaveric donors through the Department of Pathology, University of Vienna, according to an approved protocol of the University of Vienna Ethical Committee. Biopsies from cutaneous T cell lymphoma skin lesions (plaque and tumor stage of mycosis fungoides) were obtained

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Abbreviations: HEV, high endothelial venules; PNAd, peripheral node addressins.

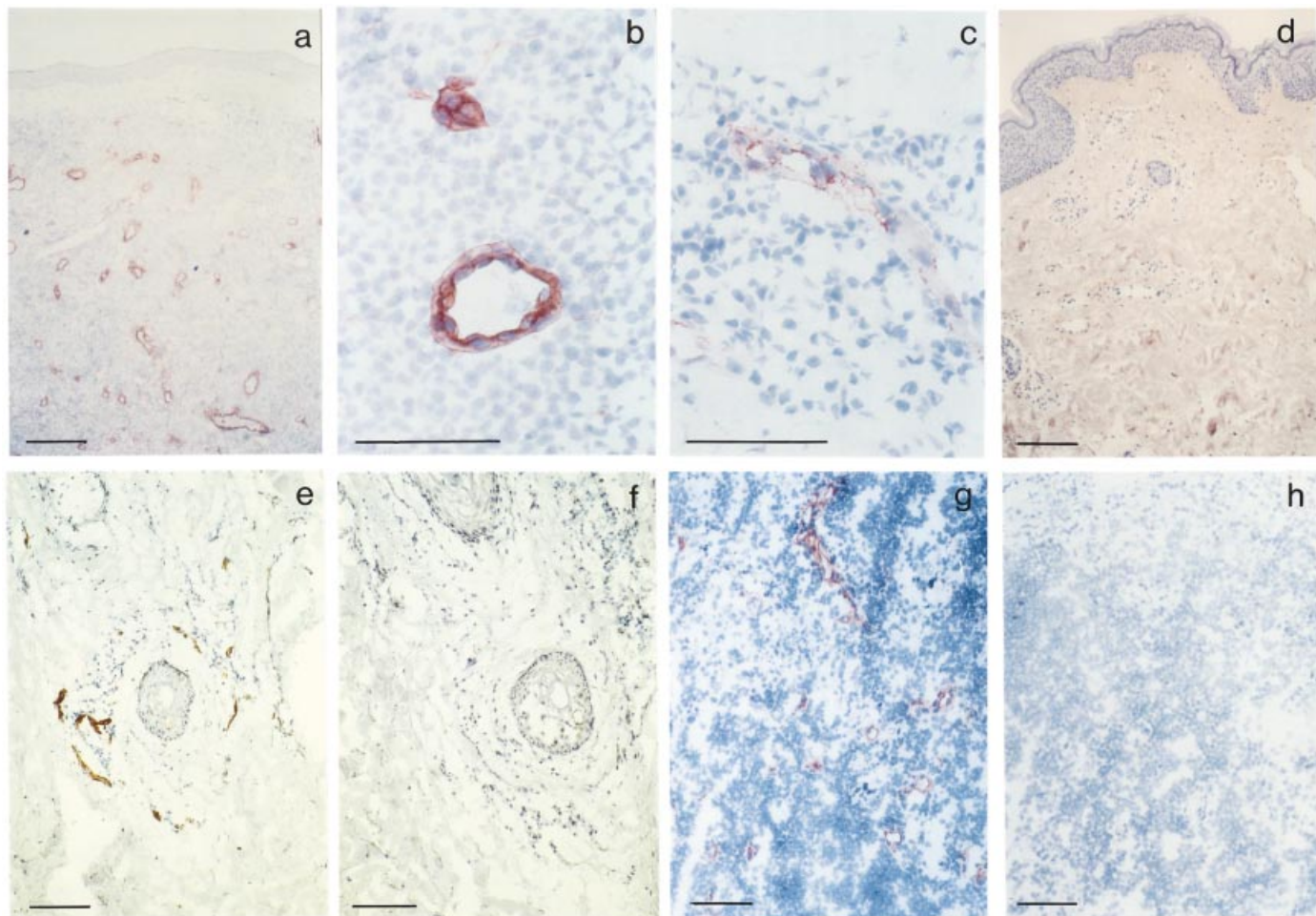


Figure 1. Skin endothelial cells react with MoAb MECA-79. Immunohistochemistry on frozen tissue sections: (a) nodular cutaneous T cell lymphoma lesional; (b, c) HEV-like vessels in cutaneous T cell lymphoma lesions; (d) superficial vascular plexus of normal skin; (e, f) perifollicular vessels of normal skin; (g, h) peripheral lymph node. All images except f and h show MECA-79 staining, f and h are isotype IgM stainings. Scale bar: 0.1 mm

from five different patients after informed consent. After removing the subcutaneous tissue, all samples were immediately snap frozen and stored in liquid nitrogen. For selected experiments, dermal microvascular endothelial cells were isolated from 100 cm² normal cadaveric thigh skin by dispase digestion followed by mechanical expression of cells into phosphate-buffered saline as described previously (Petzelbauer *et al*, 1995). The expressed cells contained approximately 5×10^3 endothelial cells per sample. After pelleting they were immediately snap frozen and stored in liquid nitrogen.

Immunohistochemistry Five micrometer cryostat sections were prepared and three step immunohistochemistry using the avidin-biotin system (peroxidase ABC-Elite kit; Vector Laboratories, Burlingame, CA) was performed as described previously (Kunstfeld *et al*, 1997). As first step reagents, we used MoAb MECA-79 (rat anti-mouse antibody, IgM; Pharmingen, San Diego, CA) or an isotype control antibody diluted 1:1000 in phosphate-buffered saline containing 3% rabbit serum. The second step antibody was a biotin-conjugated rabbit anti-rat IgM from Jackson (West Grove, PA).

Immunoprecipitation Frozen tissues (≈ 200 mg per experiment) were cut into pieces, submersed in 1 ml lysis buffer containing 50 mM Tris, 2 mM CaCl₂, 2 mM MnCl₂, 2 mM MgCl₂, 2% NP-40, and 1 mM phenylmethylsulfonyl fluoride, 10 μ g per ml aprotinin, 15 μ g per ml leupeptin, pH 7.5, and homogenized using the Dispersing-Tool from IKA (Stauffen, Germany). Following 1 h incubation on ice, centrifugation at $13,000 \times g$ for 20 min at 4°C, the supernatants were collected and precleared with protein G Sepharose beads (GammaBind G; Pharmacia, Uppsala, Sweden). For selective experiments, endothelial cells were isolated from cadaveric thigh skin by dispase digestion as described above. Endothelial cells were submersed in lysis buffer as described for full skin samples.

For immunoprecipitation of L-selectin ligands, 50 μ g protein G Sepharose beads (Sigma, St Louis, MO) were loaded with 25 μ g L-selectin-Fc

protein (gift from Ray Camphausen, Genetics Institute, Cambridge, MA). Alternatively, 50 μ g protein G Sepharose beads were loaded with 10 μ g anti-rat IgM antibody (Jackson) followed by incubation with 20 μ g MECA-79. As a negative control, beads were loaded with 25 μ g L-selectin-Fc chimeric protein in the presence of 10 M ethylenediamine tetraacetic acid, as it has been shown previously that the L-selectin/L-selectin ligand interaction is Ca²⁺ sensitive (Sassetti *et al*, 1998). To control for the precipitation with MoAb MECA-79, the specific MoAb was replaced by an irrelevant rat IgM. The respective beads were incubated with lysates derived from ≈ 200 mg tissue for 2 h at 4°C under continuous rotation. Beads were then pelleted by centrifugation at $3000 \times g$, washed in 50 mM Tris buffer, pH 7.5, containing 2 mM CaCl₂, 2 mM MnCl₂, 2 mM MgCl₂, and immunoprecipitates were eluted using a 2 \times sample buffer (62.5 mM Tris/HCl, pH 6.8, 10% glycerol, 1% sodium dodecyl sulfate, 10 μ g bromophenol blue per ml, 2% β -mercaptoethanol) for 5 min at 95°C.

Immunoblotting Precipitated proteins were separated by polyacrylamide gel electrophoresis under reducing conditions according to the method described by Laemmli (1970). After electrotransfer on to nitrocellulose membranes (BioRad, Richmond, CA), incubation with MECA-79 (5 μ g per ml) in 50 mM Tris-buffered saline, pH 7.5, containing 1% low-fat milk, rinsing, incubation with a horseradish peroxidase-conjugated second step antibody (Pierce, Rockford, IL), peroxidase was visualized by chemiluminescence (Amersham Corporation, Buckinghamshire, U.K.) and recorded on film. Appropriate isotype controls were run in parallel.

RESULTS AND DISCUSSION

Tissue distribution of MECA-79-reactive epitopes in T cell lymphoma skin lesions and in normal skin Screening lesional skin from chronic eczema (n = 3), psoriasis (n = 4), parapsoriasis (n = 8), and cutaneous T cell lymphoma (n = 5) by immunohistochemistry for the expression of MECA-79-reactive epitopes

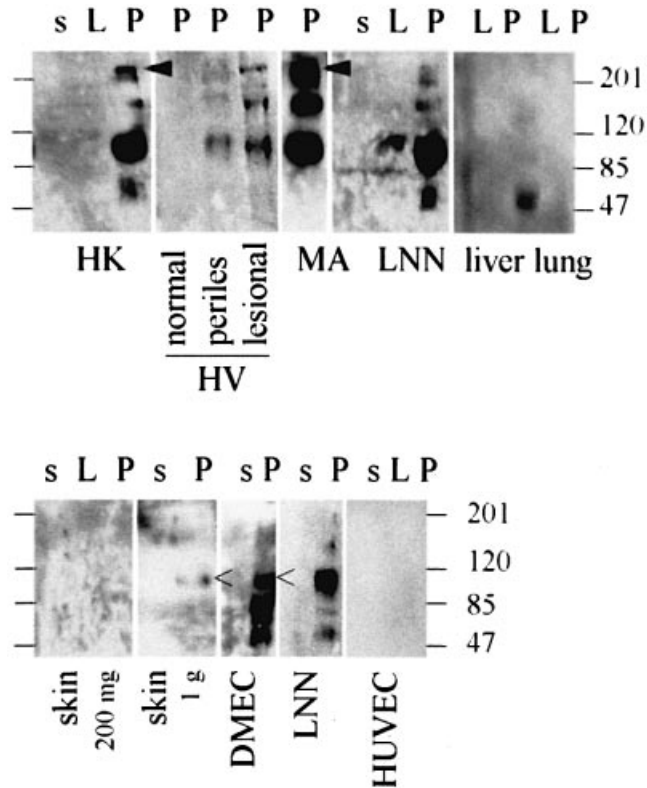


Figure 2. PNAd on skin endothelial cells. Immunoprecipitation with recombinant human L-selectin-Fc chimeric protein; precipitated proteins were electrophoresed, immunoblots were performed using MoAb MECA-79 for detection. The respective lanes were loaded with full lysates (L), with the supernatants after removing L-selectin ligands by precipitation(s) and with L-selectin precipitates (P). HK, MA, and HV are three different individuals with cutaneous T cell lymphoma skin lesions used for the respective experiment. LNN, lymph node; DMEC, dermal microvascular endothelial cells freshly isolated from normal human skin; HUVEC, human umbilical vein endothelial cells. *Closed arrows* point to the 220 kDa bands, *open arrows* to the 110 kDa bands.

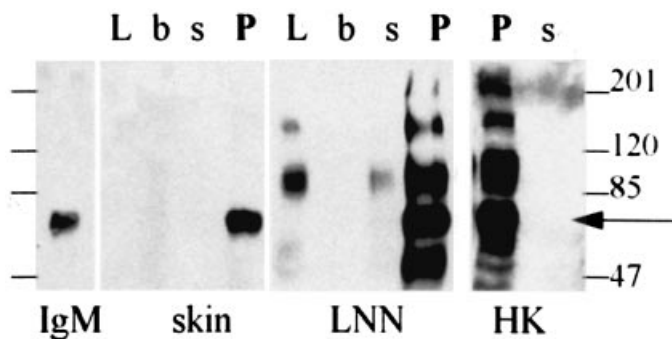


Figure 3. MECA-79 is specific for L-selectin ligands. Immunoprecipitation with MoAb MECA-79, precipitated proteins were electrophoresed, immunoblots were performed using MoAb MECA-79 as the detection antibody. The respective lanes were loaded with full lysates (L), with the material precipitated with empty beads (b), with the supernatants after removing L-selectin ligands by precipitation (s) and with L-selectin precipitates (P). Please note, that detected proteins match by size with those precipitated with L-selectin and detected with MECA-79, see **Fig 2**. The *arrow* points to the IgM antibody used for precipitation.

revealed that both, the numbers of positively stained vessels within the superficial vascular plexus as well as their relative staining intensity was most abundant in patch and tumor stages of cutaneous T cell lymphoma lesions (data shown for T cell lymphoma only; **Fig 1a-c**). This has been reported previously (Mackay *et al*, 1992; Michie *et al*, 1993), but we wish to draw attention to the fact

that all endothelial cells reactive with MECA-79 had acquired morphologic features of HEV, being of a cuboidal shape with large and prominent nuclei (**Fig 1b, c**). In normal skin samples, endothelial cells within the superficial vascular plexus were reproducibly negative for MECA-79 (**Fig 1d**). Most interestingly, screening normal skin samples from 10 different donors by serial sectioning revealed 20%–60% of hair follicles being surrounded by MECA-79-positive vessels (**Fig 1e**). It should be noted that endothelial cells from the perifollicular vascular segment differ by morphology from the superficial vascular plexus endothelium by having bridged fenestrations (Braverman and Keh, 1981).

HEV in peripheral nodes, investigated as a positive control, showed a staining pattern with MoAb MECA-79 very similar to that observed in tumor stages of cutaneous T cell lymphoma lesions (**Fig 1**). Moreover, immunohistochemistry revealed a striking specificity of MoAb MECA-79 for endothelium, hematopoietic cells always being nonreactive for MECA-79 (**Fig 1**). As an additional control for the specificity of MECA-79, peripheral blood nuclear cells were tested for MECA-79 expression by fluorescence-activated cell sorter and this also resulted in a negative staining (data not shown).

PNAd in cutaneous T cell lymphoma lesions We next addressed the question whether these MECA-79-reactive epitopes found on skin endothelium are indeed L-selectin ligands or even resemble PNAd molecules. For these experiments we chose cutaneous T cell lymphoma lesions, because of the abundance of endothelial MECA-79 reactivity. Detergent lysates of lesional skin from five different patients were analyzed. Lysates from four patients gave reproducible results in at least two independent experiments each, material from the fifth patient was not sufficient for an independent repeat and was therefore excluded from this study. From each of the four lysates, L-selectin precipitated a pattern of MECA-79-reactive proteins of molecular weights of 90–110 kDa, 160 kDa, 200 kDa, and 220 kDa (**Fig 2**). Fifty to 60 kDa proteins were only found in one patient (HK; **Fig 2**). With the exception of the 220 kDa component, the pattern of these skin-derived MECA-79-reactive L-selectin ligands exactly matched the PNAd complex isolated from peripheral nodes, which were examined as a positive control (**Fig 2**). In peripheral nodes, the 220 kDa component was either absent or not clearly separable from the 200 kDa protein (**Fig 2**). As a negative control, precipitation with the L-selectin-Fc chimeric protein in the presence of Ca^{2+} was performed, the subsequent immunoblot of these precipitates did not reveal any MECA-79-reactive proteins (data not shown).

To control for the possibility that the MECA-79-reactive L-selectin ligands detected in our skin samples were expressed by infiltrating white blood cells, peripheral blood nuclear cells were analyzed for comparison. In these cells, L-selectin precipitated the 240 kDa dimeric protein PSGL-1, a MECA-79 nonreactive L-selectin ligand expressed on lymphocytes, but no MECA-79-reactive proteins (data not shown). This experiment confirmed our immunohistochemical observation that MoAb MECA-79 reacts exclusively with endothelial epitopes and not with leukocytes.

We next subjected skin lysates to MECA-79 immunoprecipitation followed by immunoblotting using MoAb MECA-79 for detection. The molecular sizes of proteins detected by this procedure matched those precipitated by L-selectin and detected by MECA-79 immunoblotting (**Fig 3**). These experiments do not prove but strongly suggest that virtually all MECA-79-reactive epitopes detected in our skin samples stand for L-selectin ligands “of the PNAd type”.

PNAd in normal skin samples In seeming contrast with our immunohistochemical observation that MoAb MECA-79 reacted with perifollicular endothelial cells, we were unable to precipitate L-selectin ligands from normal skin samples when lysing 200 mg of tissue for one experiment (**Fig 2**). We attributed this to the fact that L-selectin ligands were below the detection limit and tried to enrich for L-selectin ligands. For this purpose, L-selectin-Fc-protein G Sepharose beads were incubated with lysates from normal

skin, centrifuged and reincubated with another fresh lysate. This procedure was repeated up to five times. In the following blot, MoAb MECA-79 now detected a faint band of ≈ 110 kDa (Fig 2). As this result was still unsatisfactory, we isolated microvascular endothelial cells by dispase digestion, a technique, which is routinely used for generating microvascular endothelial cell cultures. The resulting cell suspension was lysed and subjected to precipitation with L-selectin, electrophoresed, and blotted. Now MoAb MECA-79 clearly detected a 110 kDa protein band and two other low molecular weight bands (most likely artifacts due to dispase treatment). We next analyzed MECA-79 expression on dermal microvascular endothelial cells placed in cell culture and passaged as described previously (Petzelbauer *et al*, 1995). Unfortunately, neither cultured human foreskin-derived nor cultured adult human thigh skin-derived endothelium reacted with MoAb MECA-79 (data not shown). This indicated that cell culture either selected for MECA-79 negative cells or resulted in a downregulation of these moieties, which prevented further characterization of the nature of the 110 kDa L-selectin ligand expressed on perifollicular endothelium seen *in situ*. For comparison, human umbilical vein endothelial cells were also subjected to the same procedure and did not reveal any MECA-79 reactivity.

CONCLUSIONS

These experiments show that L-selectin ligands of the "PNAd type" are not a specific feature of peripheral node HEV, but are also expressed on skin endothelial cells. By size, these MECA-79-reactive L-selectin ligands detected in T cell lymphoma skin largely corresponded to proteins of the PNAd complex expressed on peripheral lymph node HEV. We want to emphasize, however, that skin endothelial cells expressed an additional PNAd-like 220 kDa protein, which has yet not been identified and extends the list of proteins capable of holding PNAd moieties. This 220 kDa protein appears to be preferentially expressed by skin endothelium and is absent or expressed in very low levels on peripheral node HEV. It should be noted that Sasseti *et al* (1998) were able to identify a >200 kDa protein in human tonsils, which they postulated to represent a multimer of the 160 kDa podocalyxin-like protein. Whether the herein described 220 kDa protein represents a podocalyxin multimer remains to be determined. For obvious reasons, the availability of sufficiently large samples of diseased human skin is limited and we thus have not yet succeeded in characterizing the protein backbone of this 220 kDa L-selectin ligand.

Yet, the role of PNAd-like L-selectin ligands within the skin remains speculative. The 110 kDa PNAd-like molecule constitutively expressed on perifollicular endothelium might be part of the continuous lymphocyte recirculation pathway through the skin used for regional sensitization or tolerance development (Enk and Katz, 1995; Alfernik *et al*, 1998). The situation appears different with regard to the induced expression of PNAd-like molecules on superficial vascular plexus endothelium in T cell mediated diseases. In the early phase of inflammation, superficial vascular plexus endothelium expresses molecules such as P-selectin, E-selectin, and/or VCAM-1, whereas MECA-79 reactivity is absent (Whyte *et al*, 1996). In line with this immunohistochemical observation, employing L-selectin(-/-) mice, Catalina *et al* (1999) demonstrated that during the first 20 h after skin challenge, P-selectin and E-selectin but not L-selectin were crucial for the development of both, irritant-induced as well as allergen-induced skin inflammation.

In the late phase of skin inflammation and, most abundant, in chronic T cell mediated skin diseases such as psoriasis and cutaneous T cell lymphoma, the superficial vascular plexus endothelium acquires robust MECA-79 reactivity (Michie *et al*, 1993; Whyte *et al*, 1996). In a sheep model analyzing tuberculin reactions within the skin, MECA-79 expression did not appear before day 3 after antigenic challenge (Mackay *et al*, 1992). In a mouse model, skin graft rejection was delayed and numbers of infiltrating T lymphocytes reduced in L-selectin(-/-) mice as compared with

L-selectin(+/+) littermates, despite the fact that cytotoxic T cell responses in L-selectin(-/-) mice equaled those seen in L-selectin(+/+) controls, indicating an impaired migration of effector cells rather than an impaired cytotoxic T cell response (Tang *et al*, 1997). An apparent difference in graft rejection between L-selectin(-/-) and (+/+) mice was not observable before day 4. Taken together, these data indicate that L-selectin does not play a part in skin inflammation before days 3-4 after initiation of the inflammatory reaction.

Whether the switch to PNAd-like adhesion molecule moieties in chronic skin diseases is an attempt of the organism to change patterns of recruited white blood cells, remains to be elucidated. Anyway, such a concept would be in line with the fact that acute inflammatory infiltrates mainly consist of CD45RO memory type cells (Sterry *et al*, 1990), whereas chronic skin diseases, such as chronic cutaneous lupus (Rijlaarsdam *et al*, 1990) and cutaneous T cell lymphoma lesions (unpublished observation) contain considerable numbers of CD45RA positive T cells. This assumption is further supported by Mackay's sheep model where the authors found an association between MECA-79 expression on skin endothelium and the appearance of naïve T cells within the draining lymphatic vessel (Mackay *et al*, 1992).

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