# Regulation of fucosyltransferase-VII expression in peripheral lymph node high endothelial venules

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Binding of L-selectin to the highly glycosylated peripheral lymph node addressins (PNAd) plays a central role in the normal recirculation of lymphocytes between the bloodstream and the lymph node. This interaction requires correct fucosylation of the PNAd, mediated by the recently identified fucosyltransferase-VII (Fuc-TVII). Here we show that during ontogeny Fuc-TVII is absent at the day of birth, barely detectable on day 1, and clearly present from day 2 onwards. PNAd expression as detected by the MECA-79 antibody precedes the expression of Fuc-TVII. Furthermore, we demonstrate that in adult mice antigenic stimulation of peripheral lymph nodes leads to a temporary disappearance of Fuc-TVII at days 2 and 3 after stimulation, followed by a complete reappearance by day 4, while expression of MECA-79 is never completely absent during this period. Finally, occlusion of afferent lymphatics to peripheral lymph nodes resulted in a decreased expression of Fuc-TVII in the high endothelial venules by day 5, and complete disappearance within 8 days. We conclude that the activity of Fuc-TVII in cells of high endothelial venules is directly affected by afferent lymph and activation processes that occur in the lymph node after antigenic stimulation. The expression of Fuc-TVII is therefore yet another level at which the function of high endothelial venules, and thus lymphocyte trafficking, can be regulated.

**Key words:** Fucosyltransferase / Homing / Lymph node / Cell-to-cell interaction

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## 1 Introduction

During lymphocyte recirculation, blood-borne lymphocytes continuously extravasate from the blood into secondary peripheral lymphoid organs. L-selectin, the peripheral lymph node (PLN) homing receptor, is present on the surface of circulating lymphocytes and plays an essential role in the initial interaction between the lymphocyte and specialized cells of the post-capillary high endothelial venules (HEV). L-selectin interacts with carbohydrate-based ligands of peripheral lymph node addressins (PNAd). To date three different PNAd designated GlyCAM-1, CD34 and Sgp200, have been identified on the HEV of PLN. These PNAd display an oligosaccharide epitope that is recognized by the function-blocking mAb MECA-79 [1–3]. All PNAd are mucins showing a high degree of O-glycosylation. The glycosylation of GlyCAM-1 is best characterized. The major capping group of GlyCAM-1 has been determined as 6'-sulfo-Lewis$^x$ [4, 5], in which sialic acid and sulfate are critical components for binding of L-selectin [6]. The sialomucin CD34 is a type I transmembrane protein, which is expressed on hematopoietic stem cells and endothelium. It can be recognized by L-selectin, but only when it is expressed as a particular HEV-glycoform [7]. The most recently described ligand for L-selectin, Sgp200, has not been characterized at the carbohydrate level. However, it can be envisaged that carbohydrate structures resembling the ones on GlyCAM-1 can also be found on this protein.

[I 17595]

**Abbreviations: HEV:** High endothelial venule **PNAd:** Peripheral lymph node addressins **MLN:** Mesenteric lymph node **PLN:** Peripheral lymph node **Fuc-TVII:** Fucosyltransferase-VII

In *in vitro* studies concerning the biosynthetic analyses of GlcCAM-1, it has been shown that on most N-

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acetyllactosamine-containing sugar chains, fucose is present in an $\alpha$ (1 $\rightarrow$ 3) linkage to the N-acetylglucosamine residue in Gal$\beta$1 $\rightarrow$ 4GlcNAc units [8]. It has been suggested that fucosylation might be the final step in the glycosylation of glycoproteins in LN, thereby making them functionally active [8, 9].

Recently, a novel $\alpha$ (1 $\rightarrow$ 3)fucosyltransferase has been identified, designated Fuc-TVII, which is exclusively expressed on the HEV of PLN and mesenteric lymph nodes (MLN) and in Peyer's patches [10]. Using Fuc-TVII knockout mice, an essential role for Fuc-TVII in the biosynthesis of functional HEV-associated L-selectin ligands in LN has been demonstrated: no L-selectin-dependent binding could be observed in Fuc-TVII-deficient mice, and PLN are therefore severely decreased in size [11].

The expression of PNAd is subject to several regulatory influences as can be seen after acute antigenic stimulation in the LN or after disruption of afferent lymphatics [12, 13].

To establish whether there is a relation between Fuc-TVII expression and the appearance of PNAd on HEV, we studied the presence of Fuc-TVII during ontogeny, antigenic stimulation of LN, and after ligation of the afferent lymphatics.

## 2 Results

### 2.1 Expression of Fuc-TVII and PNAd during ontogeny

Ligands for L-selectin are only functionally active in attracting lymphocytes when they are properly fucosylated. The appearance of Fuc-TVII in HEV during ontogeny will therefore be a hallmark of active L-selectin-dependent lymphocyte migration to peripheral LN. Expression of this enzyme was analyzed at several time points after birth. In addition, we studied the appearance of PNAd recognized by MECA-79 and the HEV-specific epitope MECA-325. It was found that the MECA-79 epitope, identifying PNAd, is already expressed at low levels on the abluminal side of the vessels at the day of birth (Table 1). However, in serial sections of the same LN we could not observe any Fuc-TVII expression (Table 1, Fig. 1). At 1 day after birth, when MECA-79 is expressed on the luminal aspect of all HEV, Fuc-TVII can be detected in some HEV. Only at 2 days after birth all HEV show expression of Fuc-TVII (Fig. 1). MECA-325 expression could be detected starting on day 1 after birth and onwards (Table 1).

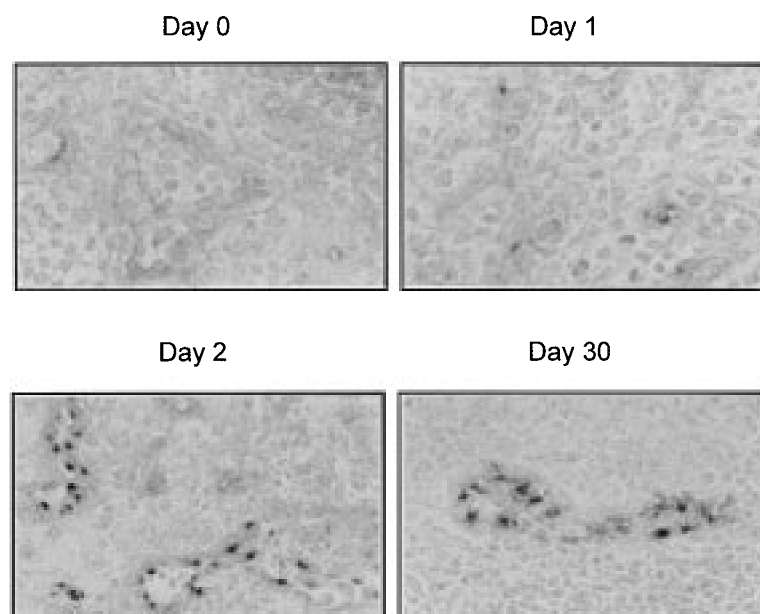


*Figure 1.* Expression of Fuc-TVII during ontogeny. Histochemical staining of PLN using anti-Fuc-TVII polyclonal antibody. Mice were killed at day of birth, and at 1, 2 and 30 days after birth. It is shown that at the day of birth no Fuc-TVII is present on HEV. At day 1 after birth some HEV show slight expression of Fuc-TVII, whereas Fuc-TVII expression is obvious on all 2-day-old HEV, and resembled the expression in adult mice.

## 2.2 Expression of Fuc-TVII in deafferentiated popliteal LN

Previously we have shown that interruption of afferent lymph to PLN results in a strongly reduced expression of the MECA-79 epitope [12]. In addition, we have shown that interruption of lymphatic flow results in complete loss of mRNA for GlyCAM-1 and loss of L-selectin-mediated binding to HEV [14]. To determine if incoming lymph could also affect the enzyme that is responsible for the formation of functional L-selectin ligands, we analyzed the expression of Fuc-TVII on HEV at different time points after interruption of lymphatic vessels. It was found that Fuc-TVII expression on high endothelial cells started to decrease at 5 days after deafferentiation, and was completely absent 8 days after occlusion (Fig. 2). Expression of the pan-endothelial marker MECA-32 did not change after deafferentiation (not shown), as found previously [14]. Expression of the MECA-79 epitope diminished, but never completely disappeared from the abluminal sides of the vessels at the time points studied, in accordance with our earlier findings [12].

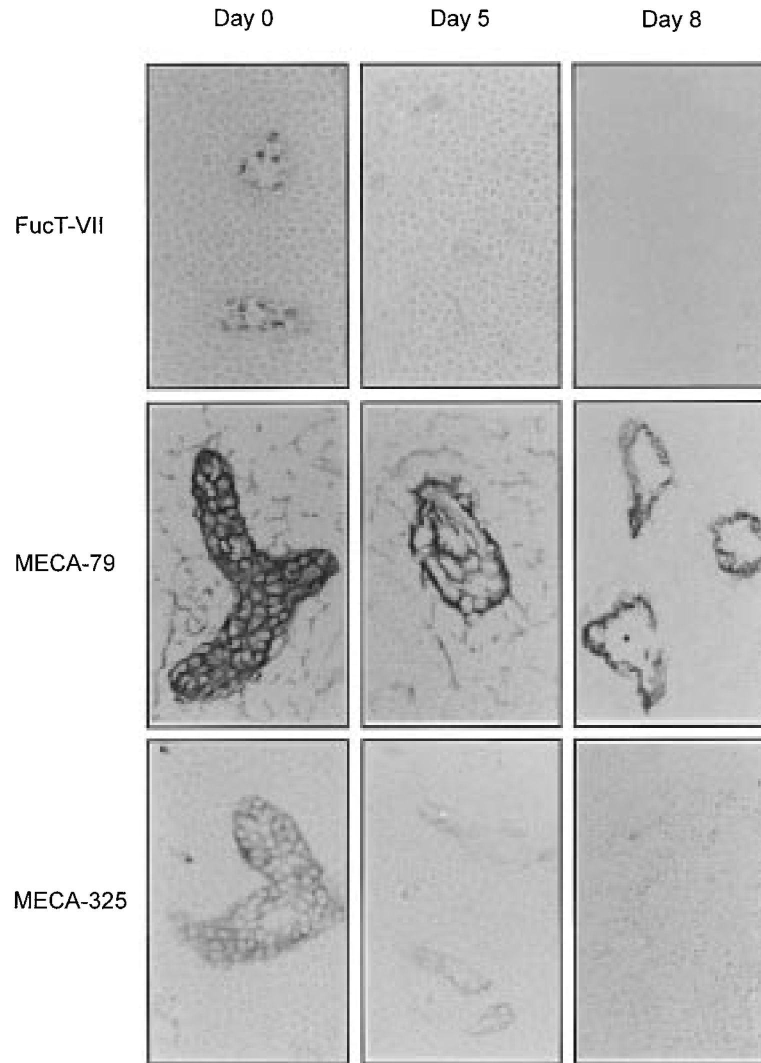


*Figure 2.* Expression of Fuc-TVII on deafferentiated popliteal LN. Histochemical staining of MECA-79-epitope, MECA-325-epitope and Fuc-TVII. Staining was performed on PLN of control mice and at day 5 or day 8 after occlusion of the afferent lymph, as described in Sect. 4.1. It is clear that at day 5 after interruption of lymphatics expression of both Fuc-TVII and MECA-325 is dramatically decreased, and is absent at day 8 after deafferentiation. MECA-79 staining remained on the HEV at all time points tested. However, the localization of expression changes from the luminal side in control HEV to abluminal in deafferentiated HEV.

**Table 1.** Expression levels of Fuc-TVII, MECA-79 and MECA-325 on the HEV of PLN during ontogeny. The levels of expression are indicated as follows: –: no expression; ±: weak expression; +: high expression.

| | Day of birth | Day 1 | Day 2 | Day 30 |
|---|---|---|---|---|
| Fuc-TVII | – | ± | + | + |
| MECA-79 | ± | + | + | + |
| MECA-325 | – | ± | + | + |

Immunohistological examination of serial sections revealed that the MECA-325 epitope was also diminished at day 5 after deafferentiation and absent on the HEV of LN at day 8 after deafferentiation, as described earlier [12].

## 2.3 Expression of Fuc-TVII following antigenic stimulation

It has been shown previously that during a primary immune response the expression and secretion of two L-selectin ligands, GlyCAM-1 and Sgp200, are strongly reduced, while cell-associated forms of CD34 and Sgp200 are unaffected [13]. These studies showed that the functions of HEV are tightly regulated during an immune response. To examine whether antigenic stimulation would also affect Fuc-TVII, we carried out a histochemical analysis of the LN from animals primed with oxazolone. The 1st day after priming, immunofluorescence double staining of LN using MECA-79 and Fuc-TVII revealed that all HEV, as defined by MECA-79 expression, showed Fuc-TVII expression, while no staining was observed in the LN parenchyma (Fig. 3). Similar staining patterns were observed when Fuc-TVII and MECA-325 were compared (not shown). Antigenic stimulation led to complete disappearance of both Fuc-TVII and MECA-325 at 2 and 3 days after priming. In contrast, MECA-79 staining remained unchanged (Fig. 3). The changes in Fuc-TVII and MECA-325 expression were transient and at day 4 after stimulation the expression of both markers was again at control level.

To evaluate at what level the expression of Fuc-TVII is regulated, we performed *in situ* hybridization with a Fuc-TVII antisense probe to detect Fuc-TVII transcripts.

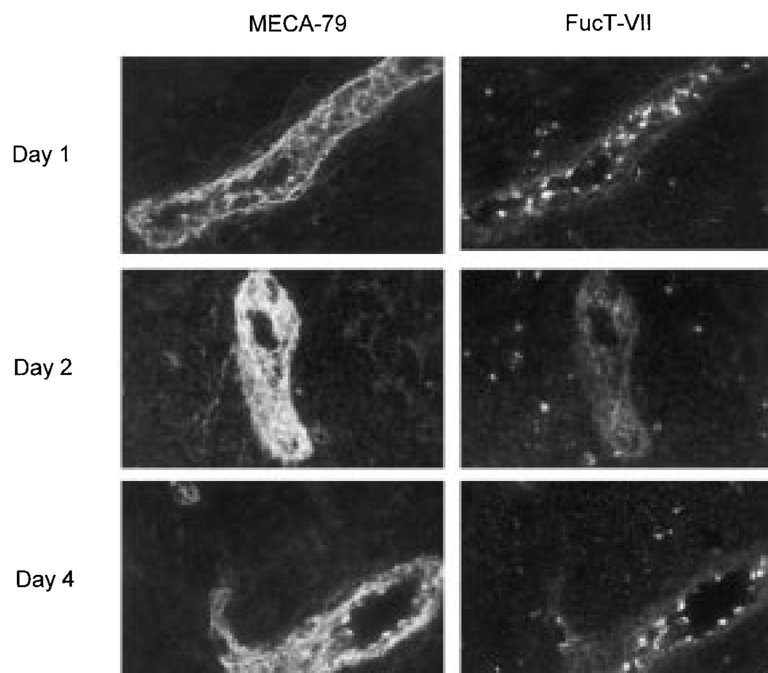


*Figure 3.* Fuc-TVII expression following antigenic stimulation. PLN were isolated at several time points after antigenic stimulation with oxazolone. Immunofluorescence double staining of PLN with MECA-79 (TRITC, left panel) and Fuc-TVII (FITC, right panel) reveals that at day 1 after application of oxazolone, Fuc-TVII is normally expressed in vesicles at the apical site of the HEV. The clear apical staining of Fuc-TVII was lost starting at day 2 and remained absent on day 3 after priming. The observed diffuse outline of the HEV (day 2, right panel) results from incomplete blockade of the bright MECA-79 TRITC label by the FITC filter. From day 4 and onwards, apical staining of Fuc-TVII was as in control HEV.

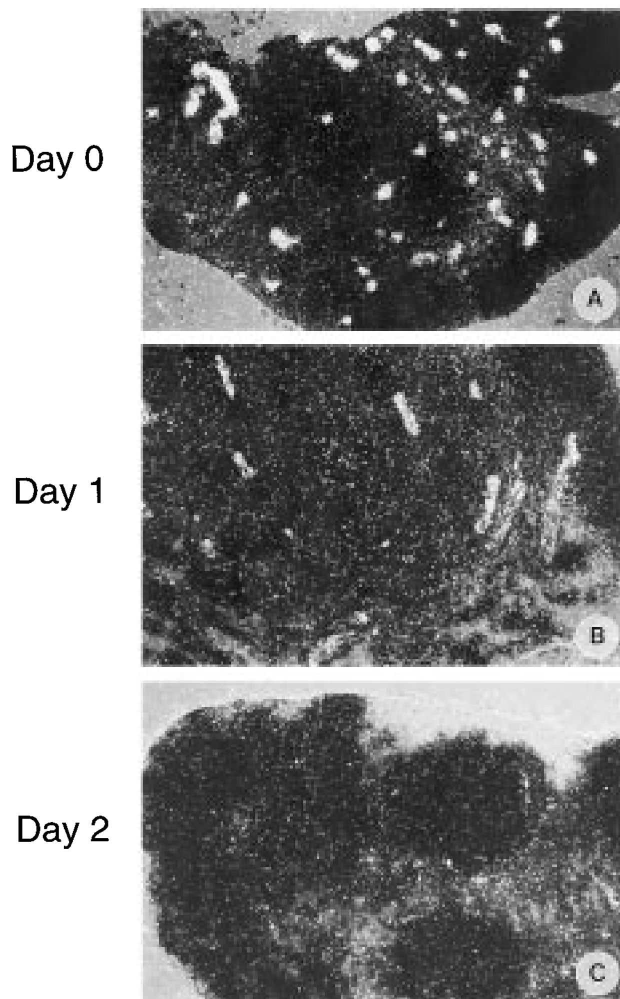


*Figure 4. In situ* hybridization analysis of Fuc-TVII transcripts in PLN following antigenic stimulation. PLN were isolated at several time points after antigenic stimulation with oxazolone and processed for *in situ* hybridization. Sections were photographed using dark field illumination. The binding of Fuc-TVII antisense probe clearly identifies the transcripts localized in HEV in unstimulated LN (A). At day 1 these transcripts are reduced (B), and 2 days after antigenic stimulation they are completely absent (C).

Fig. 4 clearly shows that the reduced expression of the fucosyltransferase is regulated at the transcriptional level. While Fuc-TVII transcripts are abundantly present in control, unstimulated LN, they are rapidly down-regulated and completely absent at 2 days after antigen administration. No hybridization above background levels could be observed with the Fuc-TVII sense probe.

### 2.4 Does the MECA-325 antibody recognize Fuc-TVII?

Considering the fact that similar expression patterns of MECA-325 and Fuc-TVII were observed both after deafferentiation and during antigenic stimulation, we wanted to know whether the mAb MECA-325 would recognize Fuc-TVII. We therefore stained PLN from Fuc-TVII-deficient mice [11] with MECA-325 to see if expression was affected. We could observe normal expression of the antigen recognized by MECA-325 on HEV in LN derived from Fuc-TVII-deficient mice (data not shown).

## 3 Discussion

In this study we focused on the enzyme that is imperative for the production of functional ligands for L-selectin on HEV in LN. We show that during ontogeny, the Fuc-TVII protein can be detected starting 1 day after birth, that Fuc-TVII expression in HEV is down-regulated after interruption of afferent lymph, and that Fuc-TVII expression is temporarily down-regulated after antigenic stimulation of LN. Our data show that in addition to the regulation of PNAd expression, fucosylation of these PNAd by Fuc-TVII is yet another level at which the activity of HEV can be regulated.

During ontogeny, it was shown that expression of the MECA-79 epitope preceded the expression of Fuc-TVII. In addition we showed that before birth and until 0–24 h after birth, no GlyCAM-1 is present, and mucosal addressin cell adhesion molecule (MAdCAM)-1 is the only primary adhesion molecule expressed on PLN HEV via which the first colonization occurs [14, 15]. MAdCAM-1 is normally expressed on the HEV of MLN and Peyer's patches only, and interacts with integrin $\alpha 4\beta 7$ in a glycosylation-independent way. Our finding that Fuc-TVII is absent at the day of birth, together with earlier results that show little L-selectin-dependent binding to HEV at this time in ontogeny [15], supports our hypothesis that no functional L-selectin ligands can be made when Fuc-TVII is absent.

Our experiments showed that an ongoing immune response also could regulate the expression of Fuc-TVII. At 2 and 3 days after antigenic stimulation, mRNA for Fuc-TVII was readily lost from HEV and the enzyme was no longer detectable. This reduction in Fuc-TVII transcripts shows that upon antigenic stimulation the activity of the enzyme is controlled at the transcriptional level.

The fact that MECA-79 staining could still be observed, even when Fuc-TVII was absent, confirms data of Malý et al. [11] in Fuc-TVII-deficient mice. They concluded that

the function-blocking mAb MECA-79 can recognize a carbohydrate moiety on PNAd in the absence of fucose, while fucose is crucial for binding of L-selectin to PNAd. This might implicate that the blocking function of MECA-79 is due to steric hindrance of the interaction between L-selectin and PNAd.

Previous findings already showed that primary immune responses could induce changes within the PLN comprising a loss of GlyCAM-1 expression on HEV, whereas expression of membrane-bound CD34 and Sgp200, both recognized by MECA-79, remains unaffected at the HEV [13]. We would argue that the observed lack of Fuc-TVII expression implicates that the remaining addressins CD34 and Sgp200, which are still actively sulfated during the immune response, as shown by Hoke et al. [13], cannot be fucosylated properly, suggesting that they are therefore not functional in attracting lymphocytes via L-selectin. However, Hoke et al. [13] describe that some L-selectin-dependent entrance of leukocytes can still occur. This might be due to slow turnover of functionally glycosylated CD34 and Sgp200, synthesized before down-regulation of the Fuc-TVII enzyme. In addition, they observed that L-selectin-IgG chimera binding to HEV of antigen-stimulated PLN regains control levels only at day 7 after antigenic stimulation [13], while Fuc-TVII expression is already at control levels at day 4. This might indicate that there is a delay of a few days between reappearance of the Fuc-TVII enzyme and functional expression of properly glycosylated PNAd.

Several studies have been performed in an attempt to identify possible factors involved in the regulation of PNAd expression on the HEV. A role for factors or cells in the incoming lymph has been shown: upon interruption of afferent lymph to PLN, a decrease in binding of lymphocytes to HEV could be observed [15]. Further studies showed that deafferentiation caused a complete loss of mRNA encoding for GlyCAM-1 and lack of L-selectin binding to HEV in PLN [14, 16]. In contrast, CD34 remained on the HEV of deafferentiated PLN; however, this CD34 was not able to interact with L-selectin [17]. This is in agreement with our observations, which show that the Fuc-TVII expression, necessary for functional fucosylation of the PNAd, disappears from the HEV 5 days after deafferentiation of the LN. The rapid changes in HEV are consistent with the intriguing concept of a morphological unit connecting the subcapsular sinus and HEV, through which signals can be delivered instantly to the perimeter of the HEV, *e.g.* in response to an immunological stimulation [18, 19].

Interestingly, MECA-325 expression also changed both after antigenic stimulation and during deafferentiation, indicating that this HEV-specific ligand, to which no function in the LN has been assigned so far, is regulated by a factor in afferent lymph during an immune response. In fact, the Fuc-TVII and MECA-325 expression followed identical patterns, suggesting that MECA-325 mAb could potentially recognize Fuc-TVII. However, positive staining of HEV in PLN from Fuc-TVII knockout mice with mAb MECA-325 showed that the antigen recognized by MECA-325 is not Fuc-TVII.

In this particular study we investigated the involvement of a specific fucosyltransferase in the occurrence of functional PNAd on HEV of PLN, and we showed that this enzyme was susceptible to regulation when changes in LN composition occurred. Other glycosyltransferases such as sialyl- and sulfotransferase also play an essential role in the glycosylation of the PNAd. However, these glycosyltransferases may be essential in other glycosylation processes which are not allowed to be switched off, and therefore might not be susceptible to regulation during immunological responses in the LN, in contrast to Fuc-TVII. It still remains to be determined which factor(s) regulate the expression of Fuc-TVII in order to obtain functionally glycosylated PNAd, and whether the PNAd themselves are regulated concomitantly with Fuc-TVII by the same factor(s) present in afferent lymph.

## 4 Materials and methods

### 4.1 Animals and occlusion of afferent lymphatics

BALB/c mice were purchased from Harlan-CPB Zeist, The Netherlands, and were kept under routine laboratory conditions. For ligation of the lymphatics, mice were anesthetized by i.m. injection of 1 μl/g body weight of a 4:3 mixture of Aescoket (Aesculaap N.V., Gent, Belgium) and Rompun (Bayer, Leverkusen, Germany). The operative procedure was performed as described by Mebius et al. [12]. Briefly, popliteal LN were exteriorized and afferent lymphatic vessels were severed. Efferent lymph vessels and blood vessels emerging from the hilus were left intact. Mice were killed at different time points after occlusion of the afferent lymphatics. Before excision of the operated LN, 0.05 ml of a 10 % India ink solution was injected into the footpad to determine the completeness of the operation procedure. LN with intact afferent lymphatics, as demonstrated by the uptake of ink, were not used. The contralateral popliteal LN served as unoperated control tissue.

### 4.2 Antigenic stimulation of PLN using oxazolone

Oxazolone (Sigma) was dissolved in acetone, and as a 3 % solution applied onto the skin of the axillary, brachial and inguinal regions of the mouse (50 μl per region). At several time points after application of oxazolone, mice were killed.

PLN were removed and frozen in liquid nitrogen before storage at −20 °C.

### 4.3 mAb and immunohistochemistry

MECA-325 (HEV differentiation antigen; [20]) and MECA-79 (PNAd; [2]) were used in a standard two-step immunoperoxidase staining on frozen sections. Briefly, 5 μm cryostat sections were air-dried overnight and fixed in acetone for 10 min. The sections were incubated for 60 min at room temperature with MECA-79 or with MECA-325 diluted 1:10 in PBS/0.1 % BSA. After washing thoroughly in PBS, sections were treated with horseradish peroxidase-conjugated goat anti-rat IgG/IgM (Jackson Immuno Research, San Francisco, CA) diluted 1:150 in PBS/0.1 % BSA containing 1 % normal mouse serum (NMS). Thereafter, the slides were incubated in 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) in Tris-HCl, pH 7.4, containing 0.01 % $H_2O_2$ for 10 min, washed, and the staining was intensified by treating the slides for 10 min with $CuSO_4$ solution (10 % copper(II)sulfate pentahydrate in physiological salt solution). Afterwards, slides were dehydrated and embedded in Entellan neu (Merck).

Anti-Fuc-TVII (antigen-affinity-purified rabbit polyclonal antibody directed against murine Fuc-TVII) was generated by Smith et al. [10]. Briefly, PCR was used to amplify a segment of the murine Fuc-TVII gene corresponding to the enzyme's stem and catalytic domains, using PCR primers directed to base pairs 2194–2224 and 3053–3085. The PCR product was subsequently cloned into an *E. coli* expression vector (pET-3b), and the recombinant protein was isolated from the bacteria by freezing and thawing the bacterial suspension. Recombinant Fuc-TVII protein was fractionated by SDS-PAGE, and segments of the gel containing Fuc-TVII were excised and used subsequently as antigen for rabbit immunizations. Antisera were harvested 10 days following the last of a total of six secondary immunizations. The Fuc-TVII polyclonal antibody was affinity purified using a fusion protein containing the anthranilatesynthase sequence from *E. coli,* fused to the Fuc-TVII sequence. This Fuc-TVII fusion protein was electroblotted onto a membrane and incubated with rabbit anti-mouse Fuc-TVII antiserum. The membrane was then washed and the bound antibody was eluted and collected for immunohistochemical procedures.

In our experiments Fuc-TVII staining was performed according to a protocol developed by Smith et al. [10]. Briefly, 5-μm cryostat sections of popliteal LN were air-dried for maximal 15 min after cutting, and fixed for 20 min on ice in paraformaldehyde (4 % paraformaldehyde dissolved in PBS at 60 °C and immediately used after preparation). After washing with PBS, sections were fixed in 100 % methanol for 20 min on ice. The sections were permeabilized and washed in blocking solution (PBS containing 2 % normal goat serum, 0.1 % Triton X-100 and 0.1 % Tween-20) for 20 min at room temperature. Incubation of the sections with anti-Fuc-TVII was carried out overnight at 4 °C in the blocking solution at a concentration of 5 μg/ml. Sections were thoroughly washed followed by incubation with horseradish peroxidase-conjugated swine anti-rabbit Ig (Dako) at a 1:200 dilution in blocking solution containing 2 % NMS for 1 h at room temperature. Staining was visualized using DAB and $CuSO_4$ as described above.

### 4.4 *In situ* hybridization procedures

*In situ* hybridization was performed as described previously [10], on paraformaldehyde-fixed frozen sections of LN. The sections were digested and acetylated and incubated with $^{35}$S-labeled RNA in sense or antisense orientation. After hybridization the slides were treated with RNase A, and after dehydration coated with liquid emulsion (NTB2, Kodak). Sections were developed according to the manufacturer's instructions and examined with bright field and dark field optics after staining with hematoxylin and eosin.

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