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The mature phenotype of peripheral lymph node (LN) high endothelial venules (HEVs), defined as MAdCAM-1highPNAdhighLTβRhigh HEC-6SThigh, is dependent on signaling through the lymphotxin-β receptor (LTβR). Plasticity of PLN HEVs during immunization with oxazolone was apparent as a reversion to an immature phenotype (MAdCAM-1highPNAdlowLTβRlow HEC-6STlow) followed by recovery to the mature phenotype. The recovery was dependent on B cells and was inhibited by LTβR-Ig treatment. Concurrent with HEV reversion, at day 4 following oxazolone or OVA immunization, reduced accumulation of Evans blue dye and newly activated DCs in the draining LNs revealed a temporary afferent lymphatic vessel (LV) functional insufficiency. T cell priming to a second Ag was temporarily inhibited. At day 7, lymphangiogenesis peaked in both the skin and draining LN, and afferent LV function was restored at the same time as HEV phenotype recovery. This process was delayed in the absence of B cells. LV and HEV both express the LTβR. During lymphangiogenesis in the draining LN, HEV, and LV were directly apposed; some vessels appeared to express both PNAd and LYVE-1. Pretreatment with LTβR-Ig drastically reduced the number of PNAdLYVE-1+ vessels, suggesting a reduction in LV and HEV cross-talk. The concordance in time and function and the close physical contact between LVs and HEVs in the remodeling process after immunization indicate that the two vascular systems are in synchrony and engage in cross-talk through B cells and LTβR. The Journal of Immunology, 2006, 177: 3369–3379.

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3 Abbreviations used in this paper: LN, lymph node; LT, lymphotxin; PLN, peripheral LN; ILN, inguinal LN; MLN, mesenteric LN; LV, lymphatic vessel; HEV, high endothelial venule; PNAd, peripheral node addressin; OX, oxazolone; SRBC, sheep RBC; WT, wild type.

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of GAPDH. Primer sequences for real-time PCR are as follows: GAPDH, (forward) 5'-CTGACACCAACACTCTGTTAG; (reverse) 5'-GATGGCA TGGACTGGTGTAC; G10; CAM-1, (forward) 5'-CTCGCTGTGGTCT CAAAGATGTA; (reverse) 5'-CTCGTTGATAGTCGGGAGTGGAC; MadCAM-1, (forward) 5'-GTCCGCGGAGCGACGACGAC; (reverse) 5'-CACGACGGAGGGCAAGAAGGAG; CD34, (forward) 5'-AAACG GCCAATTCGAAGAGCAACCA; (reverse) 5'-TCGCCAACCAACAA CTAAAG; FucTIV, (forward) 5'-AGCCCGCGCCCACCCCTAAA; (reverse) 5'-AGCCCGCGCCCACCCCTAAA; FucTVII, (forward) 5'-GCC CATTCGGGCCACCTCAG; (reverse) 5'-CGCCAAGAACAGAAGCCACAGATA; HEC-6ST, (forward) 5'-TGGCCCAAGCCTTCAAG; (reverse) 5'-TGGCCCAAGCCTTCAAG.

Injection of LTβR-Ig and control-Ig (LF3-IAg)

Five-week-old C57BL/6 mice were injected i.p. once with 100 μg of either LTβR-Ig or LF3-IAg (a gift from J. Browning, Biogen Idec). PLNs were analyzed 7 days later. These experiments were performed twice with comparable results. To identify the role of LTβR in HEV gene recovery, 5-week-old mice were immunized with OX, and 4 days later injected i.p. with 100 μg of either LTβR-Ig or LF3-IAg. PLNs were analyzed 3 days after the injection (i.e., OXd3). For the role of LTβR in LV and HEV cross-talk after immunization, LTβR-Ig or control-Ig were injected i.p. 24 h before OX immunization. PLNs were analyzed at OXd4.

Evans blue dye accumulation in ILNs

Thirty microliters of 1% Evans blue dye (Eastman Kodak) was injected s.c. into each of the rear footpads. Dye accumulation in skin and in inguinal LNs was evaluated 6 h later.

Cell proliferation experiments

Seven days after immunization with OVA in CFA as above, draining PLN cells were isolated, and 2 × 10^5 cells per well were cultured with 1 mg/ml OVA for 2 days. [3H]Thymidine (1 μCi) (Amersham Biosciences) was added to each well and cultured for an additional 20 h. All proliferation assays were performed in triplicate in 96-well flat-bottom plates (BD Biosciences), and the incorporation of ^3H was evaluated by standard liquid scintillation (Wallac). Results are expressed as the stimulation index of cpm obtained with cells plus Ag divided by cpm of cells in medium alone.

Results

LTβR and HEC-6ST (mature HEV markers) are down-regulated and then recover after OX; converse regulation of MadCAM-1 (immature HEV marker)

HEV plasticity is apparent after skin painting with OX or immunization with SRBC in adjuvant (18, 19). The pattern of HEV gene regulation after Ag exposure, apparent as a decrease in the expression of some genes, but not of others, is puzzling. We adopted the OX skin painting model to analyze the plasticity that had been revealed in previous studies because OX is a standard reagent for investigating LN remodeling (25, 26). We analyzed protein and mRNA expression, concentrating on three important HEV genes: HEC-6ST, MadCAM-1, and LTβR. These genes respectively represent a mature HEV marker, an immature HEV marker, and the critical receptor in their development. PNA expression also was evaluated as the MECA-79 epitope, but the data for this particular series of experiments are not reported in this study because the changes in the cellular pattern and intensity of staining are the product of several genes, including HEC-6ST. Furthermore, because PNA is a product of several genes, it cannot be evaluated at the mRNA level. Mice were immunized with OX (in acetone), and axillary, brachial, and inguinal LNs (PLNs) from a minimum of five mice per time point were harvested at several different times after the single immunization and analyzed by immunohistochemistry and quantitative RT-PCR. Several changes were seen in HEVs after OX, but not after vehicle-alone (acetone) treatment. LTβR and HEC-6ST were regulated in a similar pattern. An initial decrease was apparent by 2 days (OXd2, data not shown). The
lowest level was seen at 4 days (OXd4; Fig. 1A, b and f). A rebound was apparent by 7 days (OXd7; Fig. 1A, c and g), with full recovery by 14 days (OXd14) (Fig. 1A, d and h). Surprisingly and importantly, in contrast with these two genes, MAdCAM-1 expression, which is normally very low in PLNs (Fig. 1A), increased after OX immunization. There was an increase not only in the number of MAdCAM-1 expressing vessels (from 4 ± 2 to 21 ± 7 per 7-μm LN section, apparent from counting 12 random LN sections from five mice), but also in the level of MAdCAM-1 expression on those vessels (Fig. 1A, j and k). By OXd14, MAdCAM-1 returned to the normal very low level seen in PLN HEVs (Fig. 1A). Interestingly, at OXd7, MAdCAM-1 expression was localized to those individual high endothelial cells that expressed low HEC-6ST (green) expression; a typical HEV (green) was decreased at OXd4 and then rebounded. MAdCAM-1 mRNA expression level was designated as 100% (n = 5). Nontreated ( ), OXd4 ( ■ ), OXd7 ( □ ), OXd14 ( ■ ).

To identify the mechanism of the changes in HEVs, we next investigated which cells and cytokines were responsible for these effects. First, we asked whether the remodeling of HEVs was the consequence of lymphocyte activity by evaluating HEV gene regulation in LNs of RAG-/- mice. Although these mice have no mature T or B cells, they exhibit HEV networks, although the level of constitutive HEC-6ST expression was considerably lower than in wild-type (WT) PLNs, and many of the vessels exhibited abluminal PNAd staining (Fig. 2A). The initial down-regulation of HEC-6ST still occurred at OXd4 (Fig. 2B, a–c). Therefore, the down-regulation of HEC-6ST after OX was not due to LN enlargement subsequent to infiltrating Ag-specific cells. However, the rebound of HEV gene expression at OXd7 was not as vigorous as in WT mice (Fig. 2B, a–c; compare with Fig. 1A, e–g), indicating a requirement for T and/or B cells for this later stage. The results indicate that the mature HEV phenotype and the optimal rebound of HEV gene expression after OX require T and/or B cells.

Next, we investigated the individual role of T and B cells in HEV remodeling. Either T or B cells were sufficient to maintain the mature HEV phenotype, because HEC-6ST expression was present in both μMT mice (B cell deficient) and nu/nu mice (T cell deficient) PLNs (Fig. 2A). The initial down-regulation of HEC-6ST occurred in both μMT and nu/nu LNs (Fig. 2B, e and h), again suggesting that an Ag-specific immune response is not necessary for the initiation of HEV remodeling after OX. However, the rebound of HEC-6ST expression after OX was delayed in μMT (Fig. 2Bf), although not in nu/nu LNs (Fig. 2Bf). These data suggest that B cells are required for optimal HEV gene rebound after OX.

Given the importance of TNFR1 in inflammation and its somewhat less prominent role in secondary lymphoid organ development, we asked whether HEV gene expression after OX also was
regulated through this pathway. We investigated HEV gene expression in LNs of three TNFRI (p55) knockout mice after OX. The pattern of HEV gene regulation after OX was similar to that seen in WT mice (Fig. 2B,j–l). Therefore, the TNFR1 signal is not important in HEV gene regulation after OX.

Mature HEV phenotype recovery from OX is dependent on LTβR and B cells

The critical importance of LTβR signaling for HEV gene expression in development and chronic inflammation was shown in our previous experiments conducted in gene knockout and transgenic mice (3). Furthermore, HEVs express LTβR (27), suggesting that the effect of LTβR ligands was directly on the vessels themselves. In a recent publication, Browning et al. (29) found that long-term, repeated treatment with LTβR-Ig, an inhibitory fusion protein of the LTβR with human Ig Fc region (28), reduced the expression of HEV genes. In agreement with their observations, we found that by 7 days after even a single i.p. injection of 100 μg of LTβR-Ig, expression of several HEV genes was dramatically down-regulated. Quantitative real-time RT-PCR revealed down-regulation of mRNAs of several HEV genes, including the posttranslational modifying enzymes HEC-6ST, FucT-IV, FucT-VII, HEV core glycoprotein, GlyCAM-1, and MAdCAM-1 (Fig. 3A). Interestingly and importantly, this treatment also resulted in ~65% reduction of the LTβR itself (Fig. 3A) (n = 6) suggesting, for the first time, feedback regulation of the LTβR.

We then asked whether the rebound of HEV gene expression that occurred after HEV remodeling also was dependent on LTβR activity. Mice were preimmunized with OX 4 days before (OXd4) and then injected i.p. with 100 μg of LTβR-Ig or control LFA3-Ig. PLNs were harvested 3 days later (i.e., OXd7) and analyzed by immunofluorescent double staining for PNAd and HEC-6ST expression. The rebound of HEC-6ST expression occurred in HEVs of mice injected with LFA3-Ig (Fig. 3Bb), but not in those of mice injected with LTβR-Ig (Fig. 3Br). In fact, some HEVs of LTβR-Ig treated mice appeared to express only abluminal PNAd (Fig. 3B,d and e) (n = 3 mice per group). Because we had determined that B cells were crucial for the recovery of mature HEVs, we then asked whether this B cell activity was through LTβR. In OXd7 LN, HEC-6SThigh HEVs were located in the interfollicular region and in isolated B cell clusters (Fig. 3Bc). After LTβR-Ig treatment, some HEVs and B cells still colocalized, but HEC-6ST did not recover (Fig. 3B, e and f), suggesting that B cell-regulated HEV gene recovery is mediated through the LTβR. Taken together, these data strongly support the concept that the role of the LTβR ligand on HEVs is direct and crucial, not only for initiation and maintenance of HEVs, but also for the recovery of the mature phenotype.

Because B cells play such an important role in HEV gene regulation, we next investigated the cell number and composition in the draining LN after immunization. There was an increase in total cell number, and interestingly, a disproportionate increase in B cells (Fig. 3C). The proportion of B cells in the draining LN drastically increased from 17.8 ± 1.1% at steady state to 49.4 ± 0.1% at day 4 and remained at 40.4 ± 3.8% at day 7.
FIGURE 3. Maintenance of mature HEV phenotype and recovery from OX depend on LTβR signaling mediated by B cells. A, Real-time RT-PCR of mRNAs isolated from PLNs after LTβR-Ig treatment. Control LFA3-Ig (□) or LTβR-Ig (■) (n = 6 LNs per group). B, LTβR-Ig suppresses the recovery of HEC-6ST after OX. Mice were immunized with OX, and 4 days later injected i.p. with LTβR-Ig. Three days after LTβR-Ig injection (i.e., OXd7), PLNs were analyzed. a–c, Control-Ig treatment; d–f, LTβR-Ig treatment. PNAd (red); a and d, HEC6ST (green); b and e, Insets show representative HEVs with merged HEC-6ST and PNAd expression; original magnification ×20; c and f, B220 (red) and HEC6ST (green) double staining; original magnification ×10; n = 3 mice per group. C, Total cell number per PLN and the proportion of B cells per LN after OX immunization (data are the average of ILNs from five mice per time point).

Afferent LV function is impaired at day 4 and recovers at day 7 after OX skin painting

The above data indicate two stages of HEV plasticity after immunization: reversion to an immature phenotype and then recovery (Fig. 1). They also suggest that HEV recovery after immunization requires B cells and LTβR signaling (Figs. 2 and 3). However, neither B cells nor cytokine signals explain the initiation of HEV remodeling after immunization. The early effects on HEV gene regulation after OX appeared to mimic those in previous reports of severed afferent lymphatic vessels (19, 23, 24). This caused us to wonder whether HEV phenotypic reversion after OX was due to the exclusion of factor(s) arriving through the afferent LVs.

In this study, we investigated the effect of OX treatment on the accumulation of lymph and cells arriving from afferent lymph in the draining LNs. Afferent lymph access was investigated by monitoring Evans blue dye accumulation in draining inguinal LNs (ILNs). Mice were preimmunized with OX for either 4 or 7 days (OXd4 or OXd7). Evans blue was injected s.c. into the rear footpads of these and control nontreated mice. Although Evans blue can be visualized in ILNs within 2 h and is apparent in efferent lymph within 4 h, we found that 6 h after dye injection was the optimal time point to distinguish the accumulation pattern in ILNs and the surrounding skin. Six hours after Evans blue injection, dye accumulated in the ILNs of control mice (Fig. 4A, a and d), and the surrounding skin was only slightly blue, indicating normal LV function. In contrast, much less dye accumulated in OXd4 ILNs (Fig. 4A, b and e) and, in fact, it collected in the skin around the nodes, which appeared edematous (Fig. 4Ab). This indicates an insufficiency in afferent LV function at this time point. Blood vessel angiogenesis and efferent LV dilation was apparent, consistent with the previous observations regarding blood and efferent lymph flow after immunization (Fig. 4Ab). In OXd7 mice, the amount of dye that accumulated in ILN was nearly normal (Fig. 4A, c and f) and the edema subsided, indicating that LV function had recovered (Fig. 4Ac). ILNs were sectioned and evaluated microscopically to take advantage of Evans blue autofluorescence (red). In nontreated ILNs, a dense fluorescence was apparent encircling the medullary area, the site of the highest lymphatic vessel density; the fluorescence was much fainter in the OXd4 ILNs and recovered in OXd7 ILNs (Fig. 4A, d and f) (n = 5).

Previous studies had shown similar effects on HEVs of immunization with either OX or SRBC, and we extended these observations to OVA. Mice were preimmunized with OVA+CFA by s.c. injection in flanks and tail 4 or 7 days previously, and Evans blue was injected i.c. into the rear footpads and evaluated as above. The pattern of dye accumulation was identical with that seen after OX immunization: it was reduced at day 4 (Fig. 4B, a and c) and the edema subsided, indicating that LV function had recovered at day 7 after immunization with OX (Fig. 4B, c and f). Although edema was less apparent in skin surrounding the LN, compared with OX (Fig. 4, Ab and Bb), the dye collected at the Ag injection site (Fig. 4B, b and c, circled with white dashed line). Thus, the effects on afferent LV function after immunization are not unique to OX.

Afferent LV function, evaluated as DC access and T cell priming, is impaired at day 4 and recovers at day 7 after immunization with OX

Having shown that LV function impairment is a consequence of immunization, we next asked whether there was an immunologic consequence to the reduction in lymph access. We investigated whether the accumulation of cells arriving from afferent lymph was reduced. By FITC skin painting, the number of newly activated DCs in draining LNs can be evaluated by FACS. Individual mice were preimmunized with OX at different time points (day 0, 1, 2, etc., accordingly OXd0, OXd1, OXd2, etc.) on the abdomen, and then painted on the flanks with FITC. Twenty-four hours after FITC painting, the accumulation of FITC-labeled DCs was evaluated in ILNs. In non-OX-treated mice, 1.3% of the cells in ILNs were FITC+CD11c+ DCs. A comparable proportion was obtained
in ILNs of vehicle-only (acetone)-treated mice (Fig. 5A). A lower proportion of FITC⁺CD11c⁺ cells was obtained from ILNs of OXd0 mice, with an even greater decrease in OXd1 ILNs, reaching a nadir in OXd2 and OXd3 ILNs (0.2%). Recovery to the levels found in nodes of nonimmunized mice was apparent in OXd5, and the restored/enhanced accumulation of newly activated DCs continued to OXd14 (Fig. 5A). Thus, the accumulation of newly activated APCs in sensitized LNs was transiently reduced between days 2 and 5 after OX, even though the total number of CD11c⁺ cells was actually increased after immunization (Fig. 5A and inset). Fig. 5A shows representative results (n = 3). The decrease in access of afferent lymph to the immunized LNs and/or the increase of efferent lymph output could explain the reduction of Evans blue accumulation in OXd4 ILNs. However, because APCs are rare in efferent lymph (9), the reduction of FITC⁺CD11c⁺ cell accumulation in OXd4 ILNs is likely due to impaired afferent LV access to the LN.

The decreased accumulation of newly activated DCs in the draining ILN also could be due to a reduced number of DCs in the skin after immunization because of their rapid egress from that site. However, no obvious difference in the number of MHC class II-expressing cells was apparent in nonimmunized, OXd4 (nonimmunized side), OXd7 (nonimmunized side) skin (Fig. 5B).

Because the accumulation of newly activated APCs was transiently reduced in draining LNs, we asked whether T cells could be primed to another Ag during this period. Nontreated, OXd2, OXd4, and OXd7 mice were immunized with OVA in CFA in both flanks and in the tail. Seven days later, PLNs were removed and evaluated for T cell recall proliferation to OVA. OVA-specific proliferation was reduced when cells were obtained from OVA-OXd2 mice, compared with those from OVA-only immunized control mice. The proliferative response of cells from OVA-OXd4 mice approached that of non-OX-treated mice and was actually over compensated in OVA-OXd7 cells (Fig. 5B). This overcompensation which is apparent in LNs from mice treated with OX, immunized with OVA 7 days later and then analyzed after an additional 7 days is likely due in part to the recovery of DC arrival and is probably also influenced by the remodeled HEVs and lymphatic vessels (see below). These results indicate that inhibition of T cell priming to a second Ag occurs after immunization, is transient, and correlates in time with the effects on LV vessel function.

**Lymphangiogenesis in the skin after immunization is associated with recovery of afferent lymphatic vessel function**

Because afferent LV function was impaired after immunization, we next investigated whether the vessels were disrupted at the skin-painting site. We quantified LV number by counting the number of CD31⁺ or LYVE-1⁺ vessels under fluorescent microscopy per entire ×40 field. Twenty-five random fields were counted in skin sections in the epidermal and dermal area, excluding s.c. tissue (20 random skin sections per mouse, two mice per group). The number of CD31⁺ vessels increased, reaching a peak at OXd4 (p < 0.001), and was maintained at OXd7, indicating angiogenesis. The number of LYVE-1⁺ vessels was comparable in OXd4 skin and nontreated skin but almost doubled in OXd7 skin (p < 0.001), indicating that lymphangiogenesis in the skin occurred and peaked at day 7 after immunization (Fig. 6A).

**LTβR and B cells mediate HEV and LV cross-talk after immunization**

Because afferent LV biological function is transiently impaired at day 4 after immunization and then recovers and correlates with the recovery of the mature HEV phenotype, we next investigated whether there was an effect on LVs in the draining LNs. First, we evaluated LV and HEV morphology in LNs by LYVE-1 and PNAd double staining at various times after immunization. Lymphangiogenesis in the skin after immunization is associated with recovery of afferent lymphatic vessel function. LTβR and B cells mediate HEV and LV cross-talk after immunization. Because afferent LV biological function is transiently impaired at day 4 after immunization and then recovers and correlates with the recovery of the mature HEV phenotype, we next investigated whether there was an effect on LVs in the draining LNs. First, we evaluated LV and HEV morphology in LNs by LYVE-1 and PNAd double staining at various times after immunization. Lymphangiogenesis in the skin after immunization is associated with recovery of afferent lymphatic vessel function. LTβR and B cells mediate HEV and LV cross-talk after immunization. Because afferent LV biological function is transiently impaired at day 4 after immunization and then recovers and correlates with the recovery of the mature HEV phenotype, we next investigated whether there was an effect on LVs in the draining LNs. First, we evaluated LV and HEV morphology in LNs by LYVE-1 and PNAd double staining at various times after immunization. Lymphangiogenesis in the skin after immunization is associated with recovery of afferent lymphatic vessel function. LTβR and B cells mediate HEV and LV cross-talk after immunization. Because afferent LV biological function is transiently impaired at day 4 after immunization and then recovers and correlates with the recovery of the mature HEV phenotype, we next investigated whether there was an effect on LVs in the draining LNs. First, we evaluated LV and HEV morphology in LNs by LYVE-1 and PNAd double staining at various times after immunization. Lymphangiogenesis in the skin after immunization is associated with recovery of afferent lymphatic vessel function. LTβR and B cells mediate HEV and LV cross-talk after immunization. Because afferent LV biological function is transiently impaired at day 4 after immunization and then recovers and correlates with the recovery of the mature HEV phenotype, we next investigated whether there was an effect on LVs in the draining LNs. First, we evaluated LV and HEV morphology in LNs by LYVE-1 and PNAd double staining at various times after immunization. Lymphangiogenesis in the skin after immunization is associated with recovery of afferent lymphatic vessel function. LTβR and B cells mediate HEV and LV cross-talk after immunization.
functional consequences. These double-positive vessels were rare in control and OXd7 LN (n = 3 mice per group).

Because HEV gene recovery was contingent on B cells, we then asked whether B cells also contribute to the lymphangiogenesis in the draining LNs. No LN lymphangiogenesis occurred in OX day 4 or 7 μMT LN (Fig. 6c, a–c), indicating that B cells are important in triggering lymphangiogenesis in the early time after immunization, confirming a previous observation (31). However, at day 14 after immunization, lymphangiogenesis occurred even in the absence of B cells, indicating that factors in addition to those derived from B cells contribute to this process (Fig. 6d). Furthermore, during the extended period before lymphangiogenesis occurred in the LNs of μMT mice, the effects on HEV gene expression regulation were exaggerated; PNAd expression was extremely low and MadCAM-1 expression was dramatically increased, compared with WT mice (Fig. 6c, e–h, compare with Fig. 1a, i–l). By day 14, lymphangiogenesis occurred, PNAd recovered, and MadCAM-1 was reduced to its normal low level (Fig. 6c, e–h). The correlation between lymphangiogenesis and HEV phenotype regulation after immunization in μMT mice emphasizes the synchrony between LV and HEV.

We previously found that LTβR is expressed on PNAd-positive HEVs (27). In this study, we extended these findings and also found coexpression of LYVE-1 and LTβR on HEVs from LNs of unimmunized mice (Fig. 6d, a and b). Thus, both HEVs and LVs express the LTβR.

We next asked whether LV and HEV cross-talk is mediated by LTβR. Mice were injected i.p. with 100 μg/mouse of either LTβR-Ig or control-Ig 24 h before OX immunization. PLNs were analyzed 4 days after OX (OXd4). PNAd “LYVE-1+” vessels are yellow in the merged image (arrowhead) (Fig. 6ea). Pretreatment with LTβR-Ig did not significantly affect the number of vessels that stained individually with MECA-79 or anti-LYVE-1 but did drastically reduce the number of vessels positive for both markers (p < 0.001) (Fig. 6eb), indicating that signaling through the LTβR mediates LV and HEV cross-talk.

Discussion

Data in this communication describe the regulation and plasticity of HEVs and the regulation and plasticity of LVs and thus reveal the intimate functional relationship between these vascular systems. They confirm the previous observations of our group (3, 27) and others (29) that the LTβR is crucial for regulation of HEV gene expression. We extend these observations to situations of immunization, demonstrate the significant role of B cells in recovery of HEV maturity after immunization, and, importantly, reveal that control of the LTβR itself, is a crucial aspect of HEV regulation. Next, we show the plasticity of LVs with loss of function and subsequent lymphangiogenesis in the course of immunization, with remodeling in both the skin and the LN. The LTβR is again implicated in its expression on LVs, and B cells are seen as important, but not the sole, players in the remodeling of LVs. There is a temporal concordance in the function of LVs and the maturity of HEVs. The concordance in time, function, LTβR expression, the importance of B cells, and the close physical contact between LVs and HEVs in the remodeling process provide insight into the nature of the cross-talk and synchrony of HEVs and LVs.

Remodeling after immunization reveals HEV plasticity and involves the LTβR

The plasticity of HEVs during an immune response is evidenced by the HEV remodeling process, which involves down-regulation
of mature HEV genes on existing HEVs, and, most likely, generation of new HEVs. This remodeling process is a common phenomenon after immunization with a variety of Ags: contact allergens, OX as reported in this study, and 2,4-dinitrofluorobenzene (32), SRBC (18, 19), and OVA (Ref. 29 and data not shown). The initiation of HEV remodeling does not require an adaptive immune response, because it occurs in the absence of T and/or B cells (RAG−/−, μMT, and nu/nu mice). Our data provide evidence that the LTβR is not only crucial for induction of HEV gene expression and maintenance, but also plays a critical role during the HEV remodeling period. Continuous LTβR signaling is required to maintain HEVs; LTβR signaling is required for HEV recovery after OX; the LTβR itself requires LTβR activity to maintain its expression; the LTβR is down-regulated at OXd4, which impairs LTβR signaling activity on HEVs. Finally, B cells, a likely source of LTβR, were found to be essential for HEV recovery. Treatment with LTβR-Ig results in down-regulation of MAdCAM-1, whereas immunization initially results in an increase in expression of that adhesion molecule, and then a reversion. This difference suggests that HEV remodeling after OX is more complicated than

**FIGURE 6.** LTβR and B cells mediate LV and HEV cross-talk after immunization. A, The number of CD31+ and LYVE-1+ vessels after OX were counted in skin. Results are the average number of CD31+ or LYVE-1+ vessels under fluorescent microscopy per entire ×40 field. Twenty-five random fields from 40 random skin sections, two mice per group. B, PNAd (red) and LYVE-1 (green) staining demonstrate the kinetics of lymphangiogenesis in LNs. HEVs were surrounded by LVs after immunization. Direct interaction between LV and HEV is indicated by arrowhead. d–f; Hematoxylin counter staining is shown. C, No lymphangiogenesis in μMT mice in the first 7 days after immunization (a–d), original magnification ×5. e–h, MAdCAM-1 expression was exaggerated and protracted in μMT mice. n = 3 mice per group, original magnification ×20. D, LTβR is expressed on HEVs and lymphatic vessels (a). Coexpression of LTβR (red) and LYVE-1 (green) on lymphatic vessels in medullary area of LN from an unimmunized mouse (b), the circled areas show LYVE-1 negative vessels (HEVs) that express LTβR, original magnification ×40, n = 3 mice. E. The occurrence of vessels positive for both LYVE-1 and PNAd is apparent after immunization and depends on the LTβR. Mice were pretreated with control-Ig or LTβR-Ig 24 h before OX immunization. PLNs were analyzed at OX d4. a. representative merged pictures of PNAd and LYVE-1 immunofluorescence double staining, the double-positive vessel show in yellow (arrowhead); pictures were taken at areas of HEV-rich but away from the major LV network, original amplification ×20. b, The number of vessels per ×20 image as described above, 10 random images were counted (*, p = 0.23; **, p = 0.21; ***, p < 0.001, n = 2 mice per group).
simply blocking LTβR signaling. MAdCAM-1 might respond differently to the transient down-regulation of LTβR after OX treatment than to the sustained blockage of LTβR signaling following LTβR-Ig treatment.

Remodeling of LVs after immunization
Between 2 and 5 days after immunization, the function of afferent LVs, but not LNs or efferent LVs, is severely compromised, as demonstrated by restricted access of Evans blue and FITC+ DCs; a later rebound occurs. The fact that Angeli et al. (31) did not observe the transient insufficiency but did see the later rebound is most likely due to difference in immunization regimens. Several nonexclusive mechanisms most likely contribute to the early functional insufficiency. First, the steady-state function of LVs is probably inadequate to offset the increased interstitial fluid induced by vasodilation at OXd4. The fact that insufficiency also was induced by OVA immunization, a condition that results in less edema, suggests that fluid accumulation is not the sole explanation for these effects. Second, OX immunization might impair the LVs themselves. The strong acute inflammation might disrupt LVs and/or the increased afferent lymph cell content might obstruct LVs. The enlarged LNs also might increase LN internal pressure, causing the collapse of the afferent LVs. A third possibility is that in the course of lymphangiogenesis in the skin, immature LVs might not facilitate APC travel to the LNs. The increased numbers of LYVE-1+ vessels observed in OXd7 skin suggests that lymphangiogenesis occurs at the immunization site. The increased numbers of LVs thereby offset the increased interstitial fluid, and the skin edema subsides by OXd7.

As noted above, LV insufficiency at day 4 after a single immunization is not restricted to OX immunization, because the reduced accumulation of Evans blue also was seen in the draining ILNs of mice immunized with OVA+CFA. Even though there was no obvious edema observed at day 4 after OVA immunization, the dye actually accumulated in the OVA injection site and not in the LN. Therefore, the edema observed in OXd4 skin is the result of LV insufficiency rather than the cause of impaired LV function. Nevertheless, OX might be an extreme situation with regards to LV insufficiency at day 4 after immunization, because immunization with OX (in acetone), presumably toxic, affects a much wider area of skin than does OVA s.c. injection. Lymphangiogenesis also was observed in OVA+CFA injection site at day 7 (data not shown), indicating that immunization-induced lymphangiogenesis in skin is not limited to OX immunization.

Lymphangiogenesis also occurs in LNs after immunization and, as in HEV regulation, is influenced by B cells (Ref. 31 and Fig. 6B). The expression of LTβR on LVs, as on HEVs, suggests that signaling through that receptor could contribute to these events. The important role of B cells in the early time point after immunization also was indicated by the dramatic influx of B cells in the draining LN. However, lymphangiogenesis occurred in μMT mice in the absence of B cells, albeit at a slower pace, indicating that factors in addition to B cells contribute to lymphangiogenesis. Lymphangiogenesis has been noted in chronic inflammation (33, 34) and even in acute inflammation in which macrophages have been implicated.

LVs and HEVs are tightly coordinated
Mature HEVs are maintained by LTβR and factors arriving through afferent LV. Severing afferent LVs prevents the accumulation of afferent lymph factor(s) in LNs; immunization temporarily impairs afferent LV function, resulting in the transient reduction of lymph

FIGURE 7. The mechanism of LV and HEV remodeling after immunization. HEV gene regulation after immunization is in synchrony with the alterations in lymphatic vessel morphology and function through B cells and LTβR.

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factor(s) accumulation in the draining LNs. Both procedures result in HEV reversion to an immature phenotype (MadCAM-1(HOE)HEC-6ST(W)), suggesting that HEV remodeling is actually the consequence of LV functional inefficiency after immunization.

We propose a model for LV and HEV remodeling after immunization (Fig. 7). After an immunization-priming period (day 0–2), HEVs undergo remodeling. During the remodeling period (day 2–5), angiogenesis in the skin peaks at day 4 and the number of HEVs increases. The efferent LV function remains elevated. B cells trigger early lymphangiogenesis in the LN. In the contrast, aff erent LVS are functionally insufficient, resulting in accumulation of fluid at the immunization site. Consequently, the accumulation of aff erent lymph factors (lymph and cells) is reduced in draining LNs. T cell priming to a second Ag also is inhibited at this time point. HEVs are thus exposed to a reduced amount of newly arrived lymph factors. Meanwhile, LTβR expression on HEVs is diminished. HEVs therefore lack continuous stimulator(s) arriving from LVS and reduce their LTβR activity and display an immature HEV phenotype (MadCAM-1(HOE)HEC-6ST(W)), the same gene expression pattern observed after severing aff erent LVS. After the remodeling period (days 7–10), lymphangiogenesis peaks at day 7 in both LNs and the immunization site (skin) and LV function recovers. HEVs are surrounded by LV network. LTβR also is restored. Sufficient lymph factors arrive in the LN to stimulate HEVs and LTβR activity, and the mature phenotype is recovered (Fig. 7).

The expression of LTβR on LVS and HEVs is consistent with the notion that LTβR signaling coordinates these two vascular systems, and we suggest that, in addition to B cells, LTβR signal activator(s) are delivered by aff erent lymph. It is likely that cells in lymph, expressing or capable of inducing the LTβR ligands, LTα1β2 or LIGHT, are these LTβR signal activator(s). During the steady state, immature DCs continually migrate into LNs through aff erent LV and die locally within several days (35–37). Because immature DCs express LIGHT (38), they could migrate to HEVs to directly activate the LTβR signal, although it has been reported that activated DCs do not contact HEVs (39). DCs also express LTβR, and signaling through that receptor is important in their homeostasis (40). The activated DCs line up around HEVs to interact with newly homed T cells (41), and thus they might interact with T cells and activate them to increase expression of LTβR or LIGHT and thus indirectly signal LTβR on HEVs. The tight interaction between LVS and HEVs in the remodeling period suggests the intriguing possibility that factors may directly diffuse from LVS to HEVs and influence gene expression. Support for this hypothesis is provided by the observations of Gertz et al. (42) and Sixt and colleagues (43), who noted the presence of tracer in the lumen of HEVs after s.c. injection of labeled dextran.

What could be the purpose of the extensive remodeling of HEVs and LVS after immunization? The reduction in Ag access and HEV maturity may serve to prevent an immune response from escalating out of control and may reduce the possibility of an inappropriate response to self Ags in a highly activated environment. We have found that the transient reduction in LN access does have functional consequences when mice are immunized in that period (S. Liao and N. H. Ruddle, manuscript in preparation). In contrast, in the later time after immunization, the generation of new LVS, the maturation of HEVs, and the overcompensated response to a second immunization may set the scene for the generation of memory.

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