Nasal-Associated Lymphoid Tissue: Phenotypic and Functional Evidence for the Primary Role of Peripheral Node Addressin in Naive Lymphocyte Adhesion to High Endothelial Venules in a Mucosal Site

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Nasal-Associated Lymphoid Tissue: Phenotypic and Functional Evidence for the Primary Role of Peripheral Node Addressin in Naive Lymphocyte Adhesion to High Endothelial Venules in a Mucosal Site

Keri L. Csencsits, Mark A. Jutila, and David W. Pascual

Nasal-associated lymphoid tissue (NALT), a mucosal inductive site for the upper respiratory tract, is important for the development of mucosal immunity locally and distally to intranasally introduced Ag. To more fully understand the induction of nasal mucosal immunity, we investigated the addressins that allow for lymphocyte trafficking to this tissue. To investigate the addressins responsible for naive lymphocyte binding, immunofluorescent and immunoperoxidase staining of frozen NALT sections were performed using anti-mucosal addressin cell adhesion molecule-1 (MAdCAM-1), anti-peripheral node addressin (PNAd), and anti-VCAM-1 mAbs. All NALT high endothelial venules (HEV) expressed PNAd, either associated with MAdCAM-1 or alone, whereas NALT follicular dendritic cells expressed both MAdCAM-1 and VCAM-1. These expression profiles were distinct from those of the gut mucosal inductive site, Peyer’s patches (PP). The functionality of NALT HEV was determined using a Stamper-Woodruff ex vivo assay. The anti-L-selectin MEL-14 mAb blocked >90% of naive lymphocyte binding to NALT HEV, whereas the anti-MAdCAM-1 mAb, which blocks almost all naive lymphocyte binding to PP, minimally blocked binding to NALT HEV. NALT lymphocytes exhibited a unique L-selectin expression profile, differing from both PP and peripheral lymph nodes. Finally, NALT HEV were found in increased amounts in the B cell zones, unlike PP HEV. These results suggest that NALT is distinct from the intestinal PP, that initial naive lymphocyte binding to NALT HEV involves predominantly L-selectin and PNAd rather than α4β7-MAdCAM-1 interactions, and that MAdCAM-1 and VCAM-1 expressed by NALT follicular dendritic cells may play an important role in lymphocyte recruitment and retention. The Journal of Immunology, 1999, 163: 1382–1389.

A unique nasal-associated lymphoid tissue (NALT) has been identified in rodents (1, 2) that represents the oropharyngeal lymphoid tissues of the upper respiratory airways. This structure is believed to be analogous to human Waldeyer’s ring (tonsils and adenoids) (3) and consists of bilateral lymphoid structures dorsal to the cartilaginous soft palate. It is thought, much like the Peyer’s patches (PP), to behave as a mucosal inductive site. This resemblance is evident in both the organization and the structure of NALT as well as its cellular composition. First, there is an epithelial layer overlaying the NALT that contains specialized M cells (1, 2), which are thought to mediate Ag entry much like that observed in the PP. In addition, there is a unique organization of both B cell zones and T cell areas much like that expected for the PP. In fact, the relative percentages of B and T cells in the NALT approximate those observed in the PP (4–6). Although the NALT may not be the only site that contributes significantly to the stimulation of mucosal effector precursors for the upper airways, its importance may be due to its proximity to the nasal lamina propria. Collectively, this evidence suggests that this unique structure is an important tissue for studying immunity to nasally introduced Ags.

Intranasal (i.n.) immunization is an effective route for stimulating mucosal immunity to a variety of pathogens (7–11), soluble proteins including cholera toxin (12, 13), and microparticle-delivered Ags (14). This route of immunization induces strong Ag-specific mucosal and systemic IgA and IgG Ab responses (6, 7, 9–13) and stimulates elevated CTL responses to viral and OVA peptides (15–17). Thus, the elevations in humoral and cell-mediated immunity coupled with the ease of administering Ags make this a favorable route of immunization not only for local respiratory immunity, but also for providing immunity at distal mucosal sites (8, 9, 12, 13, 18, 19). In particular, i.n. immunization provides immunity in the genito-urinary tract (11, 13, 20–22) where direct immunization is often hampered by epithelial cell turnover and hormonal influences. For instance, mice immunized i.n. with the HIV gp160 protein produced HIV-1-neutralizing IgA and IgG Abs in serum, lung, and vagina (11). Elevations in Ag-specific IgG and IgA were reported in vaginal secretions of mice following i.n. immunization with plasmid DNA as well as viral and bacterial vectors (8, 22, 23). Although i.n. immunization may prove to be a highly effective method of inducing immunity in the genito-urinary tract, it is unknown how immunization of the NALT leads to immunity at this distal mucosal site.
The ability of NALT to induce an immune response at a distal mucosal site may lie in part within the specialized ligands expressed by its high-walled endothelial venules (HEV). These ligands, or adhesion molecules, interact with specific homing receptors expressed by B and T lymphocytes and allow for trafficking of the lymphocytes from blood into lymph tissue. However, the addressin profiles displayed by these HEV have yet to be identified, although some functional attributes of preferential homing by NALT lymphocytes have been observed. Lymphocytes isolated from rat NALT preferentially homed back to NALT, to cervical lymph nodes, and to mesenteric lymph nodes (MLN) rather than to the PP (24). This evidence suggests that the NALT addressin profile may differ from the PP profile.

Naive lymphocyte homing to the PP has been well characterized. It has been shown that the mucosal vascular addressin, MadCAM-1, plays an important role in the trafficking of naive B and T cells into PP (25–27). MadCAM-1 is expressed in the PP, MLN, and gut lamina propria (28, 29). Both B and T cells interact with MadCAM-1 through the cellular ligand, α4β7 (30). In some tissues, MadCAM-1 can also bind cells expressing L-selectin through the expression of the peripheral node addressin (PNAd) carbohydrate on the MadCAM-1 glycoprotein backbone (26, 31). In contrast, naive lymphocyte trafficking into peripheral lymph nodes (PNL) is mediated by L-selectin binding to PNAd expressed on glycoproteins other than MadCAM-1 (32, 33). Thus, there appears to be a definite separation between mucosal and peripheral type lymphocyte recirculation pathways, and it has yet to be determined whether NALT HEV express an exclusive mucosal or peripheral phenotype, or a combination of both.

In this study we show that NALT HEV express a unique addressin profile that resembles neither a strict mucosal nor a peripheral phenotype. All NALT HEV express PNAd, either alone or in conjunction with MadCAM-1. This profile differs greatly from the PP, the inductive site for the gut-associated lymphoreticular tissues, MadCAM-1 can also bind cells expressing L-selectin through the expression of the peripheral node addressin (PNAd) carbohydrate on the MadCAM-1 glycoprotein backbone (26, 31). In contrast, naive lymphocyte trafficking into peripheral lymph nodes (PNL) is mediated by L-selectin binding to PNAd expressed on glycoproteins other than MadCAM-1 (32, 33). Thus, there appears to be a definite separation between mucosal and peripheral type lymphocyte recirculation pathways, and it has yet to be determined whether NALT HEV express an exclusive mucosal or peripheral phenotype, or a combination of both.

Tissue isolation and collection

MLN, PLN, and PP were isolated from normal BALB/c mice. Each set of lymphoid tissue was pooled from five different mice, washed in RPMI 1640 medium (BioWhittaker, Walkersville, MD), and frozen using Tissue-Tek O.C.T. compound embedding medium (Miles, Elkhart, IN) in a 15- by 15-mm Tissue Tek Cryomold. Samples were stored at –80°C until use. NALT tissues were collected by removing the ventral edge of the tongue previously described (5, 14). Briefly, euthanized mice were decapitated, their heads were immobilized, and the lower jaws, including tongue, were removed. Palates were scored along the outer edge and removed gently. Palates were washed in RPMI 1640 medium, blotted dry, and arranged in the cryomolds with their ventral faces (containing the NALT) oriented at the bottom of the mold and frozen in O.C.T. as described above. For double immunofluorescence and immunoperoxidase staining, 5-μm cryosections were cut, air-dried, fixed in acetone at 4°C, and air-dried before rehydration.

Double immunofluorescence staining for tissue addressins

Lymphoid tissue sections were incubated with MECAM 367 supernatant (rat anti-mouse MadCAM-1 mAb) or normal rat serum for 30 min at room temperature. Specific detection of MadCAM-1 was obtained upon incubation of a 1/150 dilution of TRITC conjugate goat anti-rat IgG (H+L) Ab (Southern Biotechnology Associates, Birmingham, AL) in the dark for 30 min at room temperature. Sections were then stained for PNAd using a 1/50 dilution of MECAM 79 (FITC-conjugated rat IgM anti-mouse PNAd) mAb in the dark for 30 min at room temperature. To prevent nonspecific binding of FITC-conjugated MECAM 79 mAb to free arms of the TRITC-goat anti-rat IgG Ab, sections were blocked with 1% rat serum for 30 min before addition of the FITC-conjugated MECAM 79 mAb. After rinsing, slides were coverslipped using Vecta-Shield mounting medium (Vector Laboratories, Burlingame, CA).

Immunohistochemical staining of MadCAM-1 and VCAM-1 location within NALT

Serially cut frozen sections were rehydrated in Dulbecco’s PBS (DPBS) containing 0.2% normal goat serum (NGS), and endogenous peroxidase was blocked with Dako peroxidase blocking reagent (Dako, Carpinteria, CA). Nonspecific binding was blocked using 10% NGS in DPBS for anti-MadCAM-1 and VCAM-1 mAb staining or 10% NGS with 2.5% mouse serum in DPBS for anti-B220 mAb staining for 30 min at room temperature. Endogenous avidin and biotin were blocked with avidin/biotin blocking solution (Vector Laboratories). Sections were incubated with a 1/200 dilution of biotinylated rat anti-mouse VCAM-1 mAb, a 1/500 dilution of biotinylated rat anti-mouse mAb B220, a MECA 367 mAb supernatant, or an isotype-matched rat IgG control for 30 min at room temperature. Sections incubated with MECAM 367 mAb were then treated with a 1/250 dilution of biotinylated goat F(ab’)2 anti-rat Ig (γ and light chain, adsorbed against mouse Ig; BioSource International, Camarillo, CA) for 30 min at room temperature. All sections were then incubated with a 1/500 dilution of streptavidin-HRP (SA-HRP; SA-HRP, BioSource International), and HRP was visualized with a precipitate of 3-amin-9-ethylcarbazole (AEC; Sigma, St. Louis, MO). After AEC development for 3–5 min, the sections were counterstained with hematoxylin (Richard-Allan Scientific, Kalamazoo, MI) and coverslipped using Immuno-Mount mounting medium (Shandon-Lipshaw, Pittsburgh, PA).

Double immunohistochemical staining of NALT lymphocytes and addressins

Frozen sections were rehydrated and blocked as described above. A 10% NGS and a 2.5% mouse serum block was used for the anti-B220 mAb staining, whereas a 10% NGS block was used for the anti-CD3 mAb staining. Sections were incubated with 100 μg/ml of MECAM 79, MECAM 367 supernatant, or mAb isotype control for 30 min at room temperature. Sections were then stained with a 1/500 dilution of biotinylated goat anti-rat Ig (BioSource International) for 30 min at room temperature, then treated with a 1/500 dilution of SA-HRP (BioSource International) for 20 min. Location of the HRP was visualized with an unchained diaminobenzidine (Vector Laboratories).

After diaminobenzidine development, sections were reblocked for endogenous peroxidase, and 0.2% BSA was added to the PBS wash to prevent nonspecific Ab binding. Sections were blocked with 10% NGS and 1% rat serum. Sections were then stained with a 1/100 dilution of biotinylated rat anti-mouse CD3 mAb (PharMingen), or isotype-matched mAb control for 30 min, followed by a 1/500 dilution of SA-HRP (BioSource International). SA-HRP was visualized with VIP substrate kit (Vector Laboratories). Sections were counterstained with hematoxylin

Materials and Methods

Mice

Specific pathogen-free BALB/c mice were purchased from the National Cancer Institute at 5–6 wk of age, or BALB/cBy mice were bred and maintained in the Animal Resources Center at Montana State University (Bozeman, MT). All mice were kept under pathogen-free conditions in horizontal laminar flow cabinets and were fed sterile food and water ad libitum. The mice were free of bacterial and viral pathogens as determined by Ab screening and by histopathologic analysis of major organs and tissues. The mice were used at 6–8 wk of age in these experiments.

Monoclonal Abs

The rat anti-mouse Abs used were anti-PNAd mAb MECAM 79 (32), anti-MadCAM-1 mAb MECAM 367 (28), anti-L-selectin mAb MEL-14 (34), anti-VCAM-1 mAb 426 (MVCAM.A; PharMingen, San Diego, CA), PE-conjugated MEL-14 (PharMingen), anti-β7 mAb FIB 30 (35), anti-CD55/B220 mAb RA3-6B2 (PharMingen), anti-CD4 mAb RM4-5 (PharMingen), and CD8α mAb 53-6.7 (PharMingen). Also used was a hamster anti-mouse CD3 mAb 500-A2 (PharMingen).

The Journal of Immunology 1383

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Ab blocking of naive lymphocyte adhesion to HEV

A modification of the Stamper-Woodruff protocol (36) was performed. Murine MLN were isolated, suspended in RPMI 1640 medium, and subjected to Dounce homogenization. The resulting cell suspension was filtered through NITEX fabric (Fairmont Fabrics, Mount View, CA). Following centrifugation, the MLN lymphocytes were resuspended in DMEM (Sigma) and 2.5% BSA at a concentration of 1 × 10⁶ cells/ml.

Frozen tissue samples were cut to a thickness of 7 μm and allowed to air-dry for 30 min. A hydrophobic circle was drawn around each section, and the sections were then placed on an orbital shaker (GeneMate, Intermountain Scientific, Bountiful, UT) at 4°C at 80 rpm. MECA 79 or MECA 367 mAb supernatant was added, and the Ab was rotated on the sections for 45–60 min to allow binding to MAdCAM-1 or PNAd and then decanted. One hundred microliters of lymphocytes (1 × 10⁷ cells/ml) were added and rotated over the sections for 30 min to allow binding to HEV. Lymphocytes bound to HEV were fixed by placing sections in a cold 1.5% glutaraldehyde solution.

To block lymphocyte binding to L-selectin or αβ, cells were treated with 50 μg/ml MEL-14 (rat anti-mouse L-selectin) mAb or FIB 30 (rat anti-mouse αβ) mAb respectively, for 30 min. Pretreated cells were then rotated over the sections as described above.

Comparison of L-selectin expressed on NALT, PP, MLN, and PLN lymphocytes

Lymphocytes were isolated from NALT using procedures previously described (4, 6). Briefly, dissected NALT was disrupted by Dounce homogenization, and released lymphocytes were collected in serum-free HBSS (Sigma). PP and PLN lymphocytes were obtained by Dounce homogenization of the tissues as described above, and the resulting cell suspensions were filtered through NITEX. Lymphocytes were stained with FITC-anti-B220, FITC-anti-CD4, or FITC-anti-CD6 mAbs and PE-MEL-14 rat anti-mouse L-selectin mAb. FL1 and FL2 gains and compensation were set by the analysis of single-color FITC or PE. Two-color analyses were performed using a FACSCalibur (Becton Dickinson, Mountain View, CA).

Ten thousand events per sample were collected.

Statistical analysis

Results were analyzed using paired Student’s t test. Significant p values are indicated.

Results

Murine NALT HEV express a unique addressin profile

Lymphocyte trafficking into the PP requires MAdCAM-1 expression by its HEV (25, 26). If the NALT is indeed analogous to PP and behaves as a mucosal inductive site, it would be expected that its HEV would also mediate lymphocyte binding primarily through MAdCAM-1. To assess the NALT addressin phenotype, its HEV were examined for expression of both mucosal and peripheral addressins. NALT HEV were stained simultaneously with mAb for MAdCAM-1 (MECA 367 mAb and TRITC-labeled secondary) and PNAd (FITC-conjugated MECA 79 mAb). The number of HEV expressing MAdCAM-1, PNAd, or both addressins in each NALT, PP, or MLN section was determined. As expected in a mucosal tissue, the NALT HEV expressed MAdCAM-1; however, the total percentage of HEV expressing mucosal addressin was significantly less than that observed in the PP (Fig. 1, A–F, and Table I). In fact, all the MAdCAM-1-positive HEV in the NALT also coexpressed PNAd. This coexpression was observed in 62.8% of the NALT HEV. No NALT HEV expressed MAdCAM-1 only. In contrast, 52.4% of PP HEV expressed MAdCAM-1 only, whereas the remaining 47.6% of PP HEV expressed MAdCAM-1 and PNAd (p < 0.0001). In addition, there was a significant difference in the number of HEV expressing PNAd alone in the NALT (37.2%) compared with that in the MLN (2.5%; p < 0.01). NALT also differs from the PLN, where nearly 100% of HEV expressed PNAd alone. Thus, NALT HEV express a unique addressin profile, one that differs from the strictly peripheral pheno-type of the PLN as well as from the intestinal PP and MLN HEV addressin profiles.

Staining of NALT sections with MECA 367 mAb revealed diffuse MAdCAM-1 located within the B cell area of the NALT as well as on HEV (Fig. 1, I and M). Staining for VCAM-1 showed localization within the B cell areas, but not on HEV within the NALT (Fig. 1Q). However, staining for MAdCAM-1 in the NALT consistently appeared diffuse, whereas staining for VCAM-1 appeared intense. This staining profile differed greatly from that in the PP, which showed dark staining with the anti-MAdCAM-1 mAb in the B cell areas (Fig. 1, G and K). In addition, NALT FDC displayed addressin profiles different from those of MLN, which showed very little VCAM-1 staining and a higher incidence of MAdCAM-1 (Fig. 1, H, L, and P). In contrast, although PLN displays VCAM-1 on its FDC, very little MAdCAM-1 staining is observed in the follicles (Fig. 1, J, N, and R). Taken together, these Ab stains provide further evidence for the expression of a distinct addressin profile in the NALT.

Initial naive lymphocyte binding to NALT HEV is mediated primarily by PNAd–L-selectin interaction

To determine whether the PNAd expressed by NALT HEV was functional, we used a Stamper-Woodruff ex vivo binding assay. Frozen tissue sections were treated with mAbs specific for MAdCAM-1 or PNAd or with irrelevant isotype-matched Abs. For blocking of cellular homing receptors, naive MLN lymphocytes were pretreated with mAbs specific for αβ, or L-selectin or with a rat IgG2a isotype control Ab. The number of cells bound per HEV per node in the presence of addressin-specific Ab was compared with control binding, defined as lymphocytes bound to HEV in the presence of isotype-matched control Ab. Blocking of the HEV expressed on the HEV of NALT with the MECA 79 mAb resulted in a 60% reduction of binding compared with that in controls (Fig. 2). This reduction of lymphocyte binding was similar to that observed in PLN sections treated with MECA 79 mAb. In contrast, treatment of PP with MECA 79 mAb resulted in no significant reduction in naive lymphocyte binding (p < 0.01). Treatment of NALT HEV with MECA 367 mAb had little effect (<20%) on the number of lymphocytes bound per HEV, indicating that the expressed MAdCAM-1 has no apparent role in naive lymphocyte binding. These results were in direct contrast with those for PP, where treatment of sections with MECA 367 mAb resulted in >90% reduction in binding. In addition, NALT HEV displayed no similarities in binding to MLN HEV, as 50% of naive lymphocyte binding to MLN HEV was blocked by MECA 367 mAb. Thus, initial naive lymphocyte binding to NALT HEV was most similar to naive lymphocyte binding to peripheral lymph node HEV and dissimilar to the binding observed in the PP, again providing evidence that NALT acts in a manner unique from other characterized mucosal tissues.

Blocking of naive lymphocyte homing receptors provided further evidence that the NALT HEV displayed a more peripheral addressin phenotype (Fig. 3). Blocking of L-selectin using MEL-14 mAb resulted in >90% reduction in binding of naive lymphocytes to NALT HEV. Binding of lymphocytes to NALT HEV was reduced to a lesser extent by blocking of αβ, with FIB 30 mAb, which reduced binding by about 60% compared with the control value. MEL-14 mAb blocking of naive lymphocyte binding to NALT was similar to that observed in the PLN, where this mAb blocked nearly all naive lymphocyte binding. However, as expected, treatment with FIB 30 mAb had no effect on binding in the PLN. In the PP, MEL-14 mAb reduced binding of naive lymphocytes to HEV by ~30% compared with the control value (p < 0.01), whereas FIB 30 mAb showed a >90% reduction in naive
lymphocyte binding ($p < 0.0001$). In the MLN, naive lymphocytes exhibit characteristics of binding to both mucosal and peripheral addressin, evidenced by an 80% reduction in binding by both FIB 30 and MEL-14 mAbs ($p < 0.01$). These results suggest that the NALT HEV express an addressin phenotype distinct from those of other characterized mucosal sites, and that naive lymphocyte binding to NALT HEV is primarily mediated by L-selectin-PNAd interactions. However, $\alpha_\beta$-MAdCAM-1 interactions may also play a role, as evidenced by the reduction in binding observed upon treatment of naive lymphocytes with FIB 30 mAb. These results were consistent with the NALT phenotype, in which all HEV coexpress PNAd, unlike PP HEV. NALT HEV mediated initial naive lymphocyte binding through different addressin-receptor pairs than did PP HEV. Thus, NALT HEV expressed more of a peripheral phenotype in both form and function. This preferential lymphocyte binding to the NALT HEV via PNAd rather than MAdCAM-1 suggested that the L-selectin expression on NALT B and T cells might be different from that on lymphocytes in the PP.

### Table 1. Percentages of MAdCAM-1, PNAd, and double positive HEV in NALT, PP, MLN, and PLN

<table>
<thead>
<tr>
<th>Tissue</th>
<th>n</th>
<th>MAdCAM-1 Positive</th>
<th>PNAd Positive</th>
<th>Double Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP</td>
<td>147</td>
<td>52.4 ± 6.4</td>
<td>0</td>
<td>47.6 ± 6.4</td>
</tr>
<tr>
<td>MLN</td>
<td>343</td>
<td>40.9 ± 4.8</td>
<td>2.5 ± 0.9</td>
<td>56.8 ± 4.6</td>
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<tr>
<td>NALT</td>
<td>921</td>
<td>0</td>
<td>37.2 ± 5.0</td>
<td>62.8 ± 5.0</td>
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<tr>
<td>PLN</td>
<td>209</td>
<td>0</td>
<td>95.5 ± 2.0</td>
<td>4.5 ± 2.0</td>
</tr>
</tbody>
</table>

$^a$ Mean values ± SEM are expressed as the percentage of HEV expressing MAdCAM-1, PNAd, or double positive/total number of HEV per node.

NALT lymphocytes express a distinct L-selectin profile

Based upon the observation that naive lymphocyte binding to NALT HEV was primarily mediated by L-selectin-PNAd interactions, it would be expected that NALT lymphocytes express profiles of L-selectin expression more similar to those of PLN than to those of PP lymphocytes. Two-color flow cytometric analyses of lymphocytes isolated from NALT, PP, and PLN were performed to determine the expression of L-selectin on B cells, CD4$^+$ T cells, and CD8$^+$ T cells (Fig. 4). As expected for naive lymphocytes, nearly all the NALT B and T cells expressed L-selectin, similar to the L-selectin$^+$ populations observed in the PLN. This result differed from that in the PP, where a significant portion of the B220$^+$
and CD4+ lymphocytes were L-selectin negative (Fig. 4 and Table II). The NALT L-selectin-positive lymphocyte homing receptor profile appeared to differ greatly from that in the PP, and these data substantiated the results of the ex vivo binding assays, where initial binding was mediated primarily through L-selectin interactions with PNAd.

Segregation of double-positive and PNAd-positive NALT HEV in B and T cell areas

Although van der Ven and Sminia had observed HEV within the T cell areas of murine NALT (2), the expressed mucosal and peripheral addressins had not yet been fully characterized. It was therefore unknown whether the various HEV phenotypes unique to the NALT segregated to specific B cell zones or T cell areas within the tissue. To determine whether the location of HEV within the NALT affected the type of addressin expressed, a sequential double immunoperoxidase staining was performed. Staining of murine NALT with anti-B220 and anti-CD3 mAbs showed clearly defined B and T cell regions. HEV were localized to both the T and B cell regions (Table III); 39.7% of HEV that expressed L-selectin were localized to both the T and B cell areas (Table III). 39.7% of HEV that expressed PNAd were located in the B cell areas, whereas the remaining HEV that expressed L-selectin were localized to the T cell areas (Table III); 58.6% of HEV that expressed L-selectin were located in the T cell areas. No significant difference was observed for the distribution of NALT HEV expressing PNAd and MadCAM-1 in the B and T cell areas. In addition, our evidence suggests that these double-positive HEV were more likely to be located within the B cell areas of the NALT, than were the HEV expressing PNAd alone (p = 0.05). However, compared with the PP, in which only 16.2% of double-positive HEV were located in the B cell areas, a significantly greater number of NALT double-positive HEV were located within the B cell area (p < 0.001; Table III).

Discussion

We have shown that murine NALT HEV express a distinct addressin phenotype. All NALT HEV expressed either PNAd alone or coexpressed PNAd with MadCAM-1. The addressin profile expressed by NALT HEV is considerably different from that expressed by the HEV of the gut mucosal inductive tissue, the PP. In addition, intense staining was obtained in the FDC of NALT with the mAb against VCAM-1. Together, these results suggest that although NALT behaves as a mucosal inductive site like the PP, it displays a distinct addressin profile, much different from the PP expression of MadCAM-1.

Our results show that naive lymphocytes from nonimmunized mice bind to the NALT HEV using a peripheral addressin homing receptor pair. The mucosal ligand-addressin \( \alpha_4\beta_7 \)-MadCAM-1 interactions have been shown to mediate both naive and memory lymphocyte homing to the PP and extralymphoid mucosal sites such as the gut lamina propria (27, 37). L-selectin can also play a role in naive lymphocyte trafficking into the PP (26), and this can be observed in the results of the ex vivo binding assay, where treatment of lymphocytes with MEL-14 mAb reduces binding to PP HEV (Fig. 3). Almost all naive lymphocytes display L-selectin on their cell surface and thus are able to traffic throughout many different lymphoid sites in the body through interactions with PNAd. NALT HEV may express PNAd to facilitate naive lymphocyte homing into the PP (26), and this can be observed in the results of the ex vivo binding assay, where treatment of lymphocytes with MEL-14 mAb reduces binding to PP HEV (Fig. 3). Almost all naive lymphocytes display L-selectin on their cell surface and thus are able to traffic throughout many different lymphoid sites in the body through interactions with PNAd. NALT HEV may express PNAd to facilitate naive lymphocyte trafficking through the nasal passages. Thus, naive lymphocytes can readily circulate through the lymphoid tissue that first encounters nasally introduced Ag.

However, this does not readily explain why all NALT HEV express PNAd. In this respect, NALT more resembles a PLN than a PP. Several experiments have shown that lymphocyte homing to the upper airways of humans and sheep does not appear to be mediated primarily by \( \alpha_4\beta_7 \)-MadCAM-1 interaction, (38–40).
Also, MECA 79 mAb has been shown to block naive lymphocyte binding to HEV of human tonsils (41). In.n. immunization induces B cells that have both L-selectin and $\alpha_4\beta_7$ on their cell surface, in contrast to enteric immunization, which induces mainly $\alpha_4\beta_7$-expressing B cells (42). In addition, i.n. immunization has been more effective than intragastric immunization at producing protective immune responses in the lower respiratory tract (18, 19). This may reflect a specific homing pathway of effector cells induced in the NALT rather than the PP. It appears as though L-selectin-PNAd interactions may be most important for lymphocyte trafficking into the respiratory tract, and this conclusion is reflective of the addressin profile of the NALT HEV. Because the respiratory tract contains both mucosal (upper airways and bronchi) and systemic (alveolar spaces) compartments, the coexpression of a peripheral phenotype with the mucosal addressin may enable trafficking of lymphocytes that facilitate both systemic and mucosal immune responses.

The presence of MAdCAM-1 as well as VCAM-1 and PNAd within the NALT presents interesting implications for the theory of the common mucosal immune system. Recent studies have shown that $\alpha_4\beta_7$-MAdCAM-1 interactions do not play a major role in lymphocyte trafficking into the lung or pulmonary tissues (39–42), but do provide a mechanism for protective immunity against gut pathogens (43). Therefore, it has been suggested that this addressin/homing receptor pair should be regarded as intestinal rather than mucosal (44). However, our studies in the mouse have shown that $\alpha_4\beta_7$-MAdCAM-1 interactions may appear to play some role in the recruitment of lymphocytes to the nasal tissues and may thus provide a method of dissemination of Ab-producing cells to distal mucosal effector sites, including the intestinal and reproductive tracts.

$\alpha_4\beta_7$-MAdCAM-1 interactions may also play a role in the initial binding of naive lymphocytes to NALT HEV. This is evidenced by a 60% reduction in binding in lymphocytes treated with the anti-$\beta_7$ FIB 30 mAb (Fig. 3). These data may suggest a mechanism for the

![FIGURE 4. Expression profiles of L-selectin on NALT lymphocytes more closely resemble PLN. NALT, PP, MLN, or PLN lymphocytes were treated simultaneously with FITC-conjugated anti-B220, anti-CD4, or anti-CD8 mAbs and PE-conjugated anti-L-selectin (MEL-14) mAb. Representative plots are shown for each tissue. As depicted, the majority of the NALT and PLN lymphocytes are L-selectin $^+$, in contrast to the PP, which contained both L-selectin $^+$ and L-selectin $^-$ lymphocyte populations.](http://www.jimmunol.org/)

### Table II. Comparison of levels of expression of L-selectin on NALT, PP, PLN, and MLN B and T lymphocytes

<table>
<thead>
<tr>
<th></th>
<th>PP$^a$</th>
<th>MLN$^b$</th>
<th>NALT$^b$</th>
<th>PLN$^b$</th>
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<tr>
<td>B220$^+$</td>
<td>71.2 ± 3.7</td>
<td>87.0 ± 2.3</td>
<td>86.7 ± 2.6</td>
<td>96.9 ± 0.4</td>
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<tr>
<td>CD4$^+$</td>
<td>60.2 ± 2.9</td>
<td>88.7 ± 0.6</td>
<td>89.3 ± 1.5</td>
<td>94.8 ± 0.8</td>
</tr>
<tr>
<td>CD8$^+$</td>
<td>62.7 ± 8.5</td>
<td>92.9 ± 0.2</td>
<td>87.2 ± 4.5</td>
<td>94.3 ± 1.6</td>
</tr>
</tbody>
</table>

$^a$ Percentage of NALT, PP, PLN, and MLN B220$^+$, CD4$^+$, and CD8$^+$ lymphocytes expressing L-selectin.

$^b$ Mean values ± SEM from nine experiments.

$^c$ Mean values ± SEM from three experiments.

### Table III. Locations of Double and Single Positive HEV within NALT and PP

<table>
<thead>
<tr>
<th></th>
<th>B Cell Area$^a$</th>
<th>T Cell Area$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NALT HEV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Double positive</td>
<td>57.4 ± 8.3</td>
<td>40.9 ± 7.9</td>
</tr>
<tr>
<td>PNAd positive</td>
<td>39.7 ± 3.8</td>
<td>58.6 ± 3.7</td>
</tr>
<tr>
<td>PP HEV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Double positive</td>
<td>16.2 ± 4.4</td>
<td>74.5 ± 8.0</td>
</tr>
<tr>
<td>MAdCAM-1 positive</td>
<td>19.1 ± 4.5</td>
<td>81.0 ± 4.5</td>
</tr>
</tbody>
</table>

$^a$ The number of HEV expressing both MAdCAM-1 and PNAd (double positive) or PNAd or MAdCAM-1 alone in the B and T cell areas was determined. The number of HEV expressing either PNAd or MAdCAM-1 (indicated with DAB substrate) and their relative locations in the B cell zones or T cell areas (indicated with VIP substrate) in the NALT were determined. The number of HEV/phenotype/area was divided by the total number of HEV/phenotype.

$^b$ The values represent the mean of 12 sections ± SEM.
entrance of intestinal lymphocytes, which have been shown to preferentially express αβββ and enter lymphoid tissue primarily through MadCAM-1-αβββ interactions (26, 28). Alternatively, the αβββ-MadCAM-1 interaction may play a significant role in tight adhesion of lymphocytes to NALT HEV. In the three-step model of lymphocyte homing, the initial binding of lymphocytes to NALT HEV may be primarily mediated by L-selectin-PNAd interactions, whereas subsequent tight binding might be mediated primarily by MadCAM-1-αβββ interactions (26, 27).

The αβββ-MadCAM-1 interaction also may play a significant role in the trafficking of memory lymphocytes to NALT. Some memory lymphocytes preferentially express αβββ, and home to MadCAM-1 in the mucosal PP and lamina propria (45, 46). In addition, MadCAM-1 has been shown to be up-regulated in inflamed mucosal tissue (29, 47, 48). It has yet to be determined, however, whether MadCAM-1 is up-regulated on the HEV of inflamed NALT and if such an up-regulation would result in a higher percentage of lymphocytes binding through αβββ interaction.

Another method for naïve lymphocyte recruitment and/or retention in NALT is suggested by the expression of MadCAM-1 and VCAM-1 by the FDC in the NALT. MadCAM-1 expression on PP FDC has been recently characterized (49). MadCAM-1 expression within the PP follicles could mediate binding by αβββ memory lymphocytes, whereas binding to the follicles of PLN appeared to be primarily mediated by VCAM-1-αβββ interaction. It has also been shown (50) that VCAM-1 interactions with the α4 subunit can mediate binding to the germinal centers of human tonsil. However, VCAM-1 also binds both αβββ- as well as αβββ- expressing lymphocytes (51), suggesting that the αβββ lymphocytes might bind in the germinal centers through this route as well. In addition, adhesion to VCAM-1 may prevent apoptosis and allow positive selection of B cells within the follicle (52). Although binding studies of lymphocytes to the NALT follicles have yet to be determined, intense staining with anti-VCAM-1 mAb in the NALT suggests that lymphocytes expressing both αβββ and αβββ integrins are able to traffic into these germinal centers, where they then mature and differentiate.

Finally, our results indicated that compared with PP, a greater percentage of the NALT HEV were located within B cell areas. It appeared as though the double-positive HEV would more likely be located within the B cell areas of the NALT. The reason for this apparent localization of the MadCAM-1+ HEV is unclear, but this localization of HEV that can support binding of αβββ in the B cell areas might provide a new mechanism for B cell localization. Rather than entering the lymphoid tissue in the paracortex and migrating to the B cell areas in response to chemotactic factors, as has been recently reported (53), these lymphocytes might simply enter the B cell areas directly, where MadCAM-1 and VCAM-1 expression on dendritic cells might also enable B cell survival and differentiation. However, if T lymphocytes enter these B cell areas as well, some type of migration would still occur.

In summary, characterization of addressin expression revealed that NALT HEV were phenotypically and functionally distinct. This addressin profile was demonstrated to be important for initial naïve lymphocyte binding mediated by PNAd. Lymphocytes isolated from the NALT also displayed a homing receptor profile that more resembled lymphocytes derived from peripheral tissues. Finally, the location of the HEV within the NALT differed dramatically from that observed in the PP. Collectively, these results suggest that NALT is a truly unique inductive mucosal tissue that cannot be equated with the intestinal PP. In addition, these data may provide evidence for a unique lymphocyte homing pathway, where T and B cells activated in the NALT are able to preferentially circulate to alternate mucosal tissues.

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References


