



Importance of L-selectin in the induction of immune responses in the upper respiratory tract
by Keri Lin Csencsits

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Abstract:

Recent evidence suggests that the “common mucosal immune system” (CMIS) is, in fact, compartmentalized. Intranasal (i.n.) immunization preferentially induces effector immune responses in the nasal passages (NP) and reproductive tract (RT), while oral immunization induces robust intestinal response, and little to no response in NP or RT. Furthermore, intestinal mucosal addressin cell adhesion molecule-1 (MAdCAM-1) - $\alpha 4\beta 7$ homing interactions do not appear to be important for the induction of immunity after i.n. immunization. Therefore, we hypothesized that L-selectin-peripheral node addressin (PNAd) interactions are important for induction of immune responses in the NP and RT. Identification of addressins on naive nasal associated tissue (NALT) and cranial, oral and nasal associated tissue (CONALT) revealed that these sites express primarily PNAd, rather than MAdCAM-1, and that naive lymphocyte binding is mediated through L-selectin-PNAd interactions. In fact, NALT and CONALT more closely resemble peripheral lymph node (PLN) than the intestinal mucosal inductive site, the Peyer’s patch (PP). Studies of addressin expression in NALT after i.n. cholera toxin (CT) immunization revealed increase in expression and functionality of MAdCAM-1 on HEV as well as MAdCAM-1 expression by dendritic cells (DC). Studies of effector immune responses in L-selectin-deficient (L-Sel^{-/-}) mice revealed that Ab responses in NP and RT, but not intestinal lamina propria (iLP) were abated 16 days post-i.n. CT immunization. Investigation of lymphocyte homing receptors revealed that an L-selectin^{low}/ $\beta 7$ ^{low}($\alpha 4\beta 7$ ⁺) B lymphocyte population provides effector immunity in NP and RT, while iLP effector immune response is contained within the L-selectin^{low}/ $\beta 7$ ^{low}($\alpha 4\beta 7$ ⁺) B lymphocyte subpopulation as well as a unique L-selectin^{low}/ $\beta 7$ ^{high}(αE ⁺) B lymphocyte population that is unaffected by the loss of L-selectin. Moreover, oral immunization of L-Sel^{-/-} mice induced response in all effector sites, and increased response in CONALT suggesting a definite difference in the effect of loss of L-selectin on inductive immune responses in PP. Finally, long-term immunization studies revealed that effector immune responses in L-Sel^{-/-} NP and RT are delayed, not eliminated, and Ab response was significantly increased in L-Sel^{-/-} CONALT. Together, these results highlight the important of L-selectin-PNAd homing interactions in induction of immunity after i.n. immunization, and support the concept of the compartmentalization of the CMIS.

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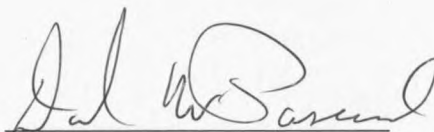
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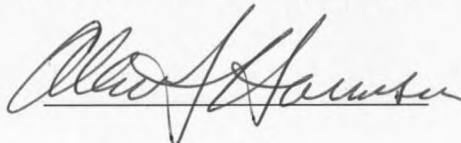
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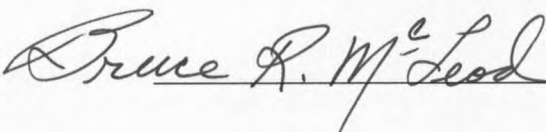
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ABSTRACT

Recent evidence suggests that the "common mucosal immune system" (CMIS) is, in fact, compartmentalized. Intranasal (i.n.) immunization preferentially induces effector immune responses in the nasal passages (NP) and reproductive tract (RT), while oral immunization induces robust intestinal response, and little to no response in NP or RT. Furthermore, intestinal mucosal addressin cell adhesion molecule-1 (MAdCAM-1) – $\alpha_4\beta_7$ homing interactions do not appear to be important for the induction of immunity after i.n. immunization. Therefore, we hypothesized that L-selectin-peripheral node addressin (PNAd) interactions are important for induction of immune responses in the NP and RT. Identification of addressins on naive nasal associated tissue (NALT) and cranial, oral and nasal associated tissue (CONALT) revealed that these sites express primarily PNAd, rather than MAdCAM-1, and that naive lymphocyte binding is mediated through L-selectin-PNAd interactions. In fact, NALT and CONALT more closely resemble peripheral lymph node (PLN) than the intestinal mucosal inductive site, the Peyer's patch (PP). Studies of addressin expression in NALT after i.n. cholera toxin (CT) immunization revealed increase in expression and functionality of MAdCAM-1 on HEV as well as MAdCAM-1 expression by dendritic cells (DC). Studies of effector immune responses in L-selectin-deficient (L-Sel^{-/-}) mice revealed that Ab responses in NP and RT, but not intestinal lamina propria (iLP) were abated 16 days post-i.n. CT immunization. Investigation of lymphocyte homing receptors revealed that an L-selectin^{low}/ $\beta 7^{\text{low}}$ ($\alpha_4\beta_7^+$) B lymphocyte population provides effector immunity in NP and RT, while iLP effector immune response is contained within the L-selectin^{low}/ $\beta 7^{\text{low}}$ ($\alpha_4\beta_7^+$) B lymphocyte subpopulation as well as a unique L-selectin^{low}/ $\beta 7^{\text{high}}$ (α_E^+) B lymphocyte population that is unaffected by the loss of L-selectin. Moreover, oral immunization of L-Sel^{-/-} mice induced response in all effector sites, and increased response in CONALT suggesting a definite difference in the effect of loss of L-selectin on inductive immune responses in PP. Finally, long-term immunization studies revealed that effector immune responses in L-Sel^{-/-} NP and RT are delayed, not eliminated, and Ab response was significantly increased in L-Sel^{-/-} CONALT. Together, these results highlight the important of L-selectin-PNAd homing interactions in induction of immunity after i.n. immunization, and support the concept of the compartmentalization of the CMIS.

CHAPTER 1

LYMPHOCYTE HOMING AND THE COMMON MUCOSAL IMMUNE SYSTEM

Mucosal ImmunologyThe common mucosal immune system

The ability of immunization at one mucosal site to effect protective immune responses at all mucosal surfaces was first suggested in the 1890's. Paul Erlich demonstrated that feeding increasing doses of ricin to rodents not only prevented killing after subcutaneous administration of the toxin (oral tolerance), but also prevented toxicity in the eye (reviewed in 1). In much later studies it was learned that secretory IgA (S-IgA) (reviewed in 2) antibodies specific to the mucosal surfaces, such as the intestine, nasal passages, salivary glands, and mammary glands, are protective against pathogens (3-8). Anatomical studies of lymphoid tissue in the gut revealed that the unique lymphatic nodule, the Peyer's patch (PP), first identified by Johannes Conrad Peyer in 1673, is where lymphocytes first encounter antigen (9). Subsequent intestinal immunity depends upon lymphocyte activation in these structures, collectively referred to as the gut-associated lymphoid tissue (GALT), and eventual homing to intestinal effector sites (10). Likewise, anatomical studies revealed similar lymphoid structures in the bronchi (bronchus-associated lymphoid tissue, or BALT) (11, 12) and a similarity to the architecture of the PP in the structures of the tonsils and adenoids. Such similarities in mucosal structure and antibody secretion led to characterization of the mucosal surfaces

as part of a greater "common mucosal immune system" (CMIS) (13). Through the 1970's, many experiments revealed that oral immunization induced antigen-specific IgA responses at mucosal surfaces throughout the body, from salivary and ocular secretions (14, 15), to mammary secretions (16), to reproductive tract (RT) immunity (17, 18). Such observations have led to the speculation that "...the mucosal immune system may be more generalized even than originally thought" (1).

Compartmentalization of the common mucosal immune system

More recent studies have revealed that the CMIS may, in fact, be compartmentalized. Oral vaccination in humans induces a robust intestinal response and a response in the RT, but little to no response in the upper respiratory tract (19). In contrast, intranasal (i.n) vaccination induces the expected strong IgA antibody (Ab) response in the nasal passages (NP) and upper respiratory tract and systemic IgG responses (20-30). I.n. immunization induces elevated CTL responses to viral and ovalbumin peptides as well (31-33). I.n. immunization also induces IgA response in the intestine, and most importantly, Ab production in the RT (19, 27, 30, 34-42). Throughout the past decade, many investigators have focused on i.n. immunization as a potent method for inducing RT immunity for several reasons. First, i.n. immunization provides a noninvasive route of administration of antigen, particularly advantageous when booster doses are required. Moreover direct vaginal immunization can be affected by hormonal influences and epithelial cell turnover during the estrous cycle (43). Finally, direct vaginal immunization does not stimulate immunity at other mucosal surfaces and

produces variable systemic Ab responses (39, 41). Successful protection in murine RT has been achieved using i.n. immunization with bacterial proteins (39, 40, 44), viral proteins and peptides (27, 34, 45), attenuated virus (41), recombinant viral vectors (32, 36, 37), and plasmid DNA (42, 46). In addition, studies in horses, non-human primates, and humans also show that i.n. immunization is a viable route for inducing upper and lower respiratory tract as well as reproductive tract immunity in various species (19, 30, 47-49). I.n. immunization shows promise as an effective means to stimulate local respiratory immunity and induce protective immunity in the RT. Thus, it is important to determine the mechanisms by which lymphocytes traffic from inductive sites in the NP to distal mucosal sites.

The mucosal immune system consists of inductive and effector sites

In order to effectively induce immunity throughout mucosal surfaces, antigen must first be processed in the organized lymphoid structures of the mucosal inductive sites. Lymphocytes are activated and assume memory phenotypes in these sites, and then disseminate to the lamina propria of the mucosal effector sites. The hallmark of effector site immunity is the production of S-IgA, though CD4⁺ and CD8⁺ lymphocytes also contribute to protection at these sites (50).

Antigen is first encountered and processed, and initial activation and expansion of B and T cells takes place in mucosal inductive sites. The intestinal Peyer's patch (PP) is the most widely characterized inductive site. While the PP shares morphology with other lymphoid sites, such as the PLN, it is highly specialized to induce mucosal immunity. B

cell follicles, and T cell areas are contained within the PP. The PP also contain a specialized lymph epithelium known as the sub epithelial "dome" area (SED; Figure 1). At the top of this dome lie specialized epithelial cells, known as "M" cells (51, 52). These cells take up antigens from the intestinal lamina propria, in effect "sampling" the contents of the intestine for antigens and microorganisms (52-56). "M" cells transport antigen to the underlying region of the SED which contains a high concentration of dendritic cells (DC) with a smaller population of macrophages. It is thought that immature DC ($CD11c^{+}$, NLDC-145 $^{-}$) take up and process antigen in the SED and then migrate to the T cell regions of the PP, where they assume a more mature phenotype ($CD11c^{+}$, NLDC-145 $^{+}$) and present antigen to the T cells (57). Subsequent activation of $CD4^{+}$ T cells provides the necessary T helper (Th) functions for the activation of and differentiation of B lymphocytes. The majority (60-70%) of B lymphocytes in the germinal centers of the PP are surface IgA (sIgA) $^{+}$ and are considered to be precursors of IgA plasma cells (58, 59). These lymphocytes then drain from the Peyer's patch, into the mesenteric lymph node, and to the thoracic duct (60). Activated lymphocytes then enter the bloodstream, where they eventually migrate to the lamina propria of mucosal effector sites, and differentiate into IgA plasma cells. The migration of activated lymphocytes to specific effector sites has yet to be completely understood; however, it is likely that specific addressin-homing receptor interactions facilitate the trafficking of lymphocytes to the effector sites.

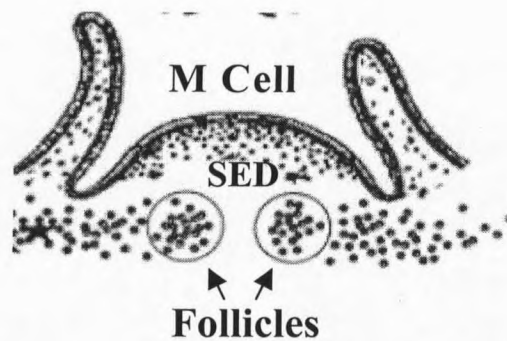


Figure 1. Structure of the Peyer's patch, the mucosal inductive site for the intestine. The epithelial cell layer containing M cells, the underlying subepithelial dome (SED) region, and B cell follicles are depicted

Effector sites of the mucosal immune system are defined as sites protected by mucus secretions. These sites include the nasal lamina propria (LP), intestinal LP, oral cavity, and the reproductive tract. Activated B cells migrate from inductive sites to the LP of the effector sites, where they mature into IgA plasma cells. Polymeric IgA produced by the plasma cells in the LP compartment binds to the polymeric immunoglobulin receptor (pIgR) expressed on the basolateral surface of the overlying epithelial cells. This binding interaction triggers transcytosis of the IgA-pIgR receptor complex to the apical surface of the epithelial cells, and the pIgR receptor is cleaved enzymatically. The released polymeric IgA retains a fragment of the pIgR referred to as secretory component (SC), which is thought to protect the newly synthesized secretory IgA (S-IgA) from proteolytic cleavage. (61-63). While small amounts of IgG and IgM secreting plasma cells (10-30% combined) are also found in the mucosal effector sites, particularly in the human reproductive tract (64-66), the majority of immunoglobulin produced is S-IgA (50).

Th and cytotoxic T cells (CTLs) also play key roles in the LP. Depending on the type of antigen administered during immunization, CD4⁺ Th cells secrete cytokines to drive a Th1- or a Th2-type response. Intracellular pathogens, such as *Salmonella* induce a Th1 type response, characterized by the secretion of IFN- γ , IL-2, and the induction of cell-mediated immunity. Extracellular pathogens, such as enterotoxigenic *E. coli* (ETEC), or mucosal adjuvants such as cholera toxin (CT), induce a Th2-type response, characterized by IL-4, IL-5, IL-6, and IL-10 production. Th2-type cytokines are more efficient in promoting B cell responses and preferentially stimulate IgG1, IgE, and IgA in the mouse (Reviewed in 67). Although Th2-type responses seem to predominate in mucosal sites, the type of pathogen that induces an immune response can also impact the type of Th cell response produced.

NALT is an inductive site for the nasal passages

A unique nasal-associated lymphoid tissue (NALT) has been identified in rodents (68-70) and birds (71) 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) (72), and consists of bilateral lymphoid structures dorsal to the cartilaginous soft palate. It is thought, much like the PP, to behave as a mucosal inductive site. This resemblance is evident in both the organization and structure of NALT as well as its cellular composition. First, there is an epithelial layer overlaying the NALT which contains "M" cells and an SED region thought to mediate antigen entry, uptake, and presentation much like that observed in the PP (68, 69, 73, 74). In addition,

there is a unique organization of both B cell zones and T cell areas similar to the structure of the PP, though the percentage of B220⁺ cells (55%), CD4⁺ cells (32%), and CD8⁺ cells (9%) in NALT more closely resembles that of the spleen than the PP, which contains nearly 70-80% B cells. (26, 75, 76). Given its proximity to the nasal LP, and its similarity in structure to the gut-associated lymphoid inductive tissue, the PP, NALT is assumed to significantly contribute to the stimulation of mucosal effector precursors for the upper airways.

Studies of NALT response after i.n. immunization have provided insight into the unique role of this putative nasal inductive site. After i.n. immunization with cholera toxin (CT) antibody-forming cells (AFC) were present in NALT, whereas oral immunization induced only CT-specific IgA cells in the PP (26). In addition, both IgA and IgG responses were observed in NALT 7 days post influenza immunization in mice (77), and AFC of all isotypes have been observed as rapidly as three days following i.n. immunization with bacterial proteins (78, 79). I.n. immunization with influenza virus stimulates Ag-specific IgA, IgG, and IgM AFC response in NALT by day 8 post-infection, and these Ab responses are maintained as long as five months post-infection (80). In addition, both Th and CTL responses, as well as long term memory proliferation are induced in NALT subsequent to i.n. immunization with various antigens (79, 81-83). While these studies show that NALT likely plays an important role in the induction of mucosal immunity following nasal immunization, responses to antigen also occur in the lymph nodes (LN) that drain the NALT.

Role of CONALT in the induction of nasal immunity

Several LN have been identified that drain the NP, brain, and the skin of the head and neck. We refer to these tissues collectively as the cranial, oral, and nasal-associated lymphoid tissues, or CONALT (84). The CONALT encompasses the facial or parotid gland LN (PRLN) located posterior to the parotid gland, the submaxillary gland LN (SMLN), and the deep cervical LN (CLN) located dorsal to the brachial plexus deep within the musculature of the neck (60). Studies of drainage patterns revealed that the SMLN drain NALT, nasal passages and tongue area, while the PRLN drain the skin of the head and neck region as well as the conjunctiva (78, 85). The deep CLN drain the NALT, SMLN, PRLN, and, in the case of antigen overload, the NP directly (60, 86, 87). CLN have also been shown to contain antigens draining from the brain (88-90), suggesting that the deep CLN play a role as a central node of the head and neck. Lymphocytes that collect in CLN drain via the cervical duct and then re-enter circulation via the subclavian vein (60). The relationship between antigen drainage in the NALT and CONALT has been further elucidated by microsphere immunization experiments, where it was determined that i.n. administered fluorescent microspheres first appeared in NALT 15 minutes post immunization, in SMLN at 24 hours post immunization, and in the CLN as late as 10 days post-immunization (91). IL-4 and IFN- γ secretion in response to i.n. microparticle injection appears first in NALT, then in the CLN and SMLN a week later (83). In addition, activated lymphocytes draining from the NALT pass through the SMLN and CLN as they are disseminated throughout the immune system.

It has been suggested that CONALT and NALT response to antigen must be

stimulated in order to effect intranasal immunity. I.n. administration of bacterial protein induces IgG and IgA AFC in SMLN and CLN, and also stimulates antigen-specific proliferation mainly in the CLN that begins two days after immunization and remains as late as six months post-immunization (78). More importantly, similar responses were observed after oral immunization with the same antigen but only in the presence of cholera toxin B subunit (CT-B) adjuvant. These data indicate that antigen-specific responses formed directly as a result of i.n., but not intragastric route of immunization in these LN. Also, IgA response following nasal immunizations with T cell dependant antigens appears restricted to the NALT and CLN (86). In addition, CLN have been shown to produce strong CTL responses after i.n. HIV peptide immunization (33). Finally, after nasal infection with influenza virus, there is greater accumulation and recovery of virus-specific CD8⁺ cells in the SMLN than in NALT, leading to speculation that cell-mediated immunity to influenza virus infection might be induced in the SMLN, rather than in the NALT (81).

CONALT also play a role in the induction of immune responses at sites other than the NP. While oral immunization induces specific salivary Abs, i.n. immunization appears to be more efficient at stimulating sustained, elevated salivary immune responses (92). This effect is also evident in the CONALT where i.n. and to a lesser extent, oral immunization, with bacterial protein antigens coupled to CT-B produces strong IgA and IgG responses in the SMLN and CLN, resulting in immune S-IgA Abs in saliva (78). However, IgG responses in the PRLN are more effectively stimulated by subcutaneous (s.c.) rather than i.n. immunization. CLN have been proven to be important in the

triggering of allergic responses to particulate antigens (93) and have been shown to play an important role in the induction of immunity in the central nervous system, as removal of the CLN ablates experimental autoimmune encephalomyelitis (94).

Though it is clear that immune responses to i.n. introduced antigen first begin in NALT and CONALT, it is unknown by what mechanisms mucosal immunity is induced at distal mucosal effector sites, and if lymphocyte trafficking from the nasal inductive sites follows unique and specific patterns.

Lymphocyte Homing

Lymphocytes display specific trafficking patterns

Since 1964, it has been known that lymphocytes are capable of continuously trafficking from the blood to the lymph and back again (95). More importantly, these studies showed that naive lymphocytes migrate according to their site of origin, that is, mucosally derived lymphocytes return to the intestine and PP, while peripherally derived lymphocytes return to peripheral LN (PLN) (96). Memory lymphocytes also possess the ability to return to the site of initial encounter of antigen (97). More importantly, memory lymphocytes display mucosal vs. peripheral routes of recirculation as cutaneous immunization induced subsets of T and B lymphocytes that trafficked to the skin while lymphocytes activated in the GALT preferentially returned to iLP, PP, and mesenteric LN (MLN) (97-100). Lymphocytes that preferentially trafficked to gut expressed primarily IgA, while lymphocytes that returned to peripheral sites were more likely to express IgG. Interestingly, such studies also provided early evidence for the

compartmentalization of CMIS, as lymphocytes derived from BALT preferentially migrated to the lung rather than intestine, and expressed IgG rather than IgA (17). Clearly, specialized cell-to-cell interactions exist that control tissue-specific homing and provide for mucosal vs. peripheral lymphocyte trafficking.

The multistep model of lymphocyte homing

Lymphocyte homing to LN is controlled via a complex series of interactions between specialized vascular endothelium and adhesion receptors on microvilli of circulating lymphocytes (101, 102). The adhesion receptors, known as lymphocyte homing receptors, consist of selectins and integrins, which interact with specialized addressin ligands expressed by high endothelial venules (HEV) (103, 104), or adhesion molecules up-regulated as a result of inflammatory signals in the flat-walled endothelium of the extralymphoid sites, such as gut (105, 106). Selectins initiate the initial rolling and reversible tethering interaction and allow for lymphocyte "sampling" of the local microenvironment. If the required chemoattractants are present (107, 108), integrins present on the lymphocyte surface are activated through G-protein linked receptor signaling, leading to the firm arrest of the lymphocyte to the endothelium (109). Additional chemokine signals are then required to complete the final step of lymphocyte homing: transendothelial migration (110). All of the steps of lymphocyte homing up to transendothelial migration are reversible if the right chemical signals are not present, leading to an exquisitely controlled system that can regulate the recruitment of specific lymphocyte subsets into different tissues.

Addressin expression is tissue specific

A member of the immunoglobulin superfamily has been identified as the addressin that mediates initial lymphocyte binding to PP HEV. This mucosal cell adhesion molecule-1 (MAdCAM-1) binds to the integrin $\alpha_4\beta_7$, and L-selectin on naive lymphocytes, and can mediate both the initial tethering and tight binding interactions in the three-step model of lymphocyte homing (101, 111). While MAdCAM-1 is expressed at high levels on the HEV of the PP and the MLN, as well as flat-walled endothelium in the intestinal LP (106, 112), and vessels in the marginal sinus of spleen (113, 114), it is present at very low levels or not at all on the HEV of PLN (115).

In contrast, the predominant addressin expressed in PLN is peripheral node addressin (PNAd). PNAd is defined as several carbohydrate epitopes recognized by the MECA 79 antibody. These carbohydrate moieties decorate different glycoprotein backbones on the HEV (116), and all must be sulfated for recognition by the PNAd ligand, L-selectin (117, 118). MECA 79 binds to a specific carbohydrate expressed by the MAdCAM-1 glycoprotein backbone (119) as well as to carbohydrate expressed by soluble glycosylation-dependent cell adhesion molecule (GlyCAM)-1 (120), cell associated CD34 (121), and soluble and cell associated sulfated glycoprotein (Sgp) 200 (118). L-selectin-PNAd interactions mediate almost all naive lymphocyte binding to PLN HEV (122, 123), as well as some binding in the MLN (106). L-selectin-PNAd interactions also contribute to binding of naive lymphocytes to the PP through the expression of PNAd carbohydrate on the MAdCAM-1 glycoprotein backbone (101, 119). High levels of PNAd are also expressed on the HEV of human tonsil (124). Therefore,

while L-selectin-PNAd interactions are thought to mediate the majority of naive lymphocyte homing to PLN, there appears to be a role for these interactions in mucosal tissues as well. It is currently unknown if the NALT behaves like the human tonsil and expresses PNAd, though preliminary homing results suggest that this tissue may indeed express a more "peripheral" addressin phenotype (125).

Lymphocyte homing receptors

Addressin ligands that mediate binding to endothelium consist of selectins and integrins. The extracellular protein domains of the selectins contain an NH₂ terminal lectin domain, which binds sialyl Lewis-X carbohydrates, followed a single epidermal growth factor (EGF) - like repeat, and a varying number of repeating short consensus sequences followed by a transmembrane domain anchored by a short cytoplasmic tail. (126, 127). The ligand for PNAd, L-selectin, is expressed on all naive lymphocytes and mediates the initial rolling interaction in the multistep model of lymphocyte homing (102, 128). In fact, binding via L-selectin interaction requires a threshold hydrodynamic shear (129), which contributes to the regulation of the function of L-selectin on lymphocytes as they traffic through the bloodstream.

Integrins can also mediate the initial, as well as the secondary, steps of lymphocyte homing. Integrins are heterodimers that consist of noncovalently associated α and β subunits. To date, fourteen different α subunits and eight different β subunits, which can combine to form twenty different heterodimers have been identified. The larger α subunits, which determine the specificity of the integrin binding, have large

extracellular binding domains consisting of three to four cation binding domains, a transmembrane region, and short cytoplasmic tails. The smaller β subunits contain extracellular domains consisting of cysteine residues clustered in four repeated motifs followed by a transmembrane domain and also have short cytoplasmic tails (reviewed in (130)). The $\alpha_4\beta_7$ heterodimer, expressed at low levels on nearly all naive lymphocytes, can participate in both rolling and adhesion to the HEV of PP (101), and may act to slow selectin-initiated rolling (mediated via PNAd expression on MAdCAM-1) in order to allow for tight adhesion of lymphocytes. However, in the case of the $\alpha_4\beta_7$ heterodimer interaction with MAdCAM-1 expressed in the iLP, the integrin provides both rolling and tight adhesion interactions (102).

$\alpha_E\beta_7$ is an intestine-specific integrin

Unlike other integrins, the $\alpha_E\beta_7$ integrin is present mainly on some $CD4^+$ and most $CD8^+$ T lymphocytes located within the iLP and intestinal epithelial lymphocyte (IEL) compartments of mouse and human (131-133). This integrin has also been observed on $CD8^+$ thymocytes and is expressed at higher levels on $CD4^+$ and $CD8^+$ PP and MLN lymphocytes than those in spleen or PLN (134). No expression of $\alpha_E\beta_7$ integrin has been observed on B lymphocytes. Unlike $\alpha_4\beta_7$, $\alpha_E\beta_7$ is not capable of binding to MAdCAM-1 nor does it appear to play a role in lymphocyte homing to the intestine (135). Instead, $\alpha_E\beta_7$ appears to be induced and maintained primarily by the secretion of TGF- β_1 by epithelial cells, and binds to epithelial cadherin (E-cadherin) expressed on the basolateral surface of epithelial cells (136-139). It is believed that $\alpha_E\beta_7$ contributes to the retention

of lymphocytes, particularly in the IEL compartment, since α_4 deficient mice (lacking $\alpha_4\beta_7$) have normal IEL development (140), while β_7 deficient mice (lacking both $\alpha_4\beta_7$ and $\alpha_E\beta_7$) show reduced numbers of IEL (141). However, recent studies of antiviral CD8⁺ cell homing have shown that $\alpha_E\beta_7$ is not required for trafficking or long term retention in the IEL (142). Still more studies have shown that loss of α_E does indeed reduce the number of T cells found in iLP, and that $\alpha_E\beta_7$ can bind to a non-E-cadherin ligand expressed on human endothelial cells (143, 144). Together, these results suggest that while $\alpha_E\beta_7$ may not play a role in selective CD8⁺ lymphocyte trafficking to iLP and IEL, this integrin may contribute to formation of the IEL and possibly to extravasation into the iLP. More importantly, $\alpha_E\beta_7$ may also represent an intestinal-specific integrin that is not present in the non-intestinal mucosal effector sites.

Expression of addressins by follicular dendritic cells may contribute to lymphocyte recruitment and retention

Recent evidence suggests that stromal elements, most likely follicular dendritic cells (FDC), may express addressins (145). While the function of this addressin expressed on the FDC is unknown, it has been suggested that stromal MAdCAM-1 and vascular cell adhesion molecule-1 (VCAM-1) play a role in lymphocyte retention within the follicle. Szabo *et al* (145) showed that both MAdCAM-1 and VCAM-1 in the follicles of PP and PLN bound lymphocytes expressing their respective ligands, $\alpha_4\beta_7$ and $\alpha_4\beta_1$. In addition, it has been reported that binding to VCAM-1 plays a role in B cell selection, as lymphocytes bound to VCAM-1 showed resistance to apoptosis (146). These results

suggest that addressins expressed in the follicles of lymphoid sites play an important role in the retention of antigen-specific T and B cells.

This evidence also suggests that the division of addressins into "mucosal" and "peripheral" types holds true for FDC-expressed MAdCAM-1 and VCAM-1. Szabo *et al* (145) found that FDC in the PP expressed MAdCAM-1, while FDC in the PLN expressed VCAM-1. More importantly, the tonsil, which is considered analogous to NALT, expresses VCAM-1 but not MAdCAM-1 within its follicles (147). This suggests that NALT might express a more "peripheral" phenotype of follicular addressins and supports our contention that addressin expression varies between mucosal tissues.

Different homing receptor-addressin pairs mediate memory lymphocyte homing to peripheral and mucosal sites

Immune responses that induce memory result in upregulation of specific homing receptors on lymphocytes and addressins that mediate binding in the HEV of LN. In particular, MAdCAM-1 is expressed in normal and inflamed intestine of humans and mice, in both the PP and LP, as well as in inflamed mucosal sites (148). Lymphocytes that home preferentially to these sites express high levels of $\alpha_4\beta_7$ (149, 150). Only $\alpha_4\beta_7^{\text{high}}$ B lymphocytes produced protective antibody against rotavirus infection (151). In contrast, lymphocyte homing to non-gut mucosal sites, such as the upper airways in humans and sheep, does not appear to be mediated by MAdCAM-1 - $\alpha_4\beta_7$ interactions (152-154). Since the NALT acts as an inductive site for the respiratory tract, which contains both mucosal (upper airways and bronchi) and systemic (alveolar spaces), it seems likely that memory lymphocytes induced in the NALT may not be dependent upon

$\alpha_4\beta_7$ -MAdCAM-1 interactions for homing.

The roles of L-selectin and $\alpha_4\beta_7$ have been further defined by the creation of L-selectin deficient (L-Sel^{-/-}) (155, 156) and β_7 deficient (141) mice. L-Sel^{-/-} mice show a marked reduction in cellularity in PLN, while the size of PP, where homing of lymphocytes is compensated by $\alpha_4\beta_7$ - MAdCAM-1 interactions, are not affected (155). Loss of L-selectin illustrates the role this homing receptor plays in the migration of memory lymphocytes to peripheral sites of inflammation, as L-Sel^{-/-} mice show reduced peripheral delayed-type hypersensitivity responses and delayed skin graft rejection (157-159). In contrast, β_7 - deficient mice, which lack both $\alpha_4\beta_7$ and $\alpha_E\beta_7$, show a drastically impaired formation of GALT but not PLN, highlighting the importance of β_7 integrin in lymphocyte homing to the intestinal, but not the peripheral tissues (141). Interestingly, studies of β_7 /L-selectin double deficient mice revealed that lymphocyte homing to MLN, which expresses both MAdCAM-1 and PNA^d is mediated by both L-selectin and $\alpha_4\beta_7$ (160). These studies underscore the important differences between L-selectin and $\alpha_4\beta_7$ in the formation of peripheral and mucosal sites and the role of L-selectin in peripheral immune responses. However, no studies have yet examined the effect of loss of L-selectin on mucosal effector sites.

Roles of L-selectin and $\alpha_4\beta_7$ in memory B lymphocyte homing

Though studies in mice and humans indicate that $\alpha_4\beta_7$ expression is necessary for the induction of mucosal immunity to rotavirus (149, 151, 161), new evidence suggests that the mediation of lymphocyte homing to various effector sites may be controlled or

compensated for by other lymphocyte homing receptors. β_7 -deficient mice still mount effective immune responses to rotavirus, and Ag-specific $CD8^+$ lymphocytes remain capable of trafficking to the intestine in these mice (142, 162). Much less is known about the trafficking of B cells in β_7 -deficient mice, though IgA response in rotavirus infected β_7 -deficient intestine was decreased.

Immediately following activation, B lymphocytes shed L-selectin, whereas memory B lymphocytes up-regulate L-selectin after secondary immunization, allowing for the re-entry of memory B lymphocytes into the circulation (163). Decrease or loss of L-selectin expression on B lymphocytes limits their ability to traffic to LN and suggesting that humoral responses in $L\text{-Sel}^{-/-}$ mice must be abated as well (164, 165). However, IgM and IgG1 responses following intraperitoneal (i.p.) immunization were actually increased in $L\text{-Sel}^{-/-}$ mice. Responses immediately following s.c. immunization were delayed but secondary immune responses in $L\text{-Sel}^{-/-}$ mice were greater than those in their $L\text{-Sel}^{+/+}$ counterparts. These results indicate that B cell responses might not be dependent upon L-selectin mediated trafficking; however, the role of L-selectin in generation of memory humoral responses in humans has yet to be defined. Immunohistochemical staining of PP B lymphocytes revealed that the majority of naive ($sIgD^+ CD19^+ CD20^+ CD45RA^+$) B lymphocytes were weakly positive or negative for $\alpha_4\beta_7$ and expressed some L-selectin, though samples were variable. Memory B lymphocytes showed more intense $\alpha_4\beta_7$ staining and very few of these cells expressed L-selectin (166). Other studies of normal human lymphoid tissue revealed the presence of L-selectin on mantle zone B lymphocytes, but not on memory lymphocytes at the site of

inflammation (167). However, identification of AFC after oral, rectal, and parenteral administration of *Salmonella typhi* revealed that Ag-specific AFC in the blood showed both $\alpha_4\beta_7$ and L-selectin expression, though expression levels of these homing receptors varied depending on the route of immunization (168, 169). These results suggest that L-selectin, and $\alpha_4\beta_7$ play a role in the induction of memory B cell responses in both rodents and humans.

Statement of the Hypothesis

NALT has been identified as a unique mucosal inductive site that stimulates responses in the NP and upper airways, and in the reproductive tract. Given the remarkable ability of i.n. immunization, but not oral immunization to preferentially induce immunity in the RT it is likely that there exists a compartmentalization of the CMIS. The ability of NALT to induce immunity at local sites such as the NP and the RT may be related to the homing receptor-addressin interactions that allow for lymphocyte trafficking to and from this site. Whereas lymphocyte homing receptor-addressin interactions that mediate lymphocyte trafficking to the GALT have been well defined, it is unknown what homing receptors mediate lymphocyte trafficking to NALT. Evidence does indicate that trafficking to and from the NP and RT is not mediated by intestinal $\alpha_4\beta_7$ - MAdCAM-1 interactions. Therefore, I hypothesize that lymphocyte trafficking interactions to and from the NALT are mediated via PNAd-L-selectin interactions. Furthermore, L-selectin plays an important role in the induction of immunity in the non-intestinal mucosal effector sites.

In Chapter 3 of the text I describe the identification of the addressins expressed by

the HEV of naive NALT, the homing receptors expressed by the lymphocytes of this tissue, and determine the functionality of the expressed addressins and homing receptors in lymphocyte binding to NALT. The same studies were performed on the related draining CONALT and these results are discussed in Chapter 4, further elucidating the role of peripheral addressin-homing receptor interactions in the inductive tissues of the head and neck. In Chapter 5, I describe the changes that occur in lymphocyte homing to NALT following immunization with a potent mucosal adjuvant, cholera toxin (CT). The role of L-selectin in the induction of immunity in the distal effector sites after both i.n. and oral immunization was determined in and is described in Chapters 6 and 7. An unexpected effect of loss of L-selectin in inductive tissue and long term effector immune response after i.n. immunization is detailed in Chapter 8.

Together, these results support my hypothesis that lymphocyte homing to and from the inductive sites of the head and neck is mediated via peripheral addressin – homing receptor interactions and suggests that the CMIS may be compartmentalized into “intestinal” and “non-intestinal” mucosal effector sites.

CHAPTER 2

MATERIALS AND METHODS

Mice

Specific pathogen-free BALB/c or C57BL/6N mice were purchased from the National Cancer Institute at 5-6 wk of age and maintained in the Animal Resources Center at Montana State University (Bozeman, MT). Breeding pairs of C57BL/6-TgN(ACTbEGFP)1Osb mice (C57BL/6-GFP; (170)) and L-Sel^{-/-} mice on a B6 background (155, 156) were purchased from The Jackson Laboratories (Bar Harbor, ME), and colonies were established and maintained in the Animal Resources Center at Montana State University. 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 used in these experiments were between 5-8 wk of age.

Immunizations

Mice were immunized via intranasal drip on day 0 with 5 µg CT in 10 µl sterile PBS (List Biological Laboratories, Campbell, CA), and boosted on days 7 and 14 post-primary immunization with 2.5 µg CT. For oral administration of CT, mice were orally

gavaged with 200 μ l of 50%-saturated sodium bicarbonate solution, followed by 10 μ g CT/200 μ l sterile PBS using a #22 oral gavage needle. Mice were orally boosted with 10 μ g CT at days 7 and 14 post-primary immunization.

Collection of serum, fecal, and vaginal samples

Blood was collected from mice through saphenous vein bleeding. Fresh fecal pellets were collected from individual mice. Pellets were treated with 10X volume/weight of 50 μ g/ml soybean trypsin inhibitor (Sigma, St. Louis, MO) and vortexed for 30 minutes at 4°C. Vaginal secretions were collected by gently pipetting 75 μ l of sterile PBS in and out of the vaginal vault. All samples were placed in microcentrifuge tubes and held on ice until centrifugation. Samples were centrifuged in an Eppendorf 5415 C microcentrifuge (Brinkman Instruments Inc. Westbury, NY) for 30 minutes at 13,000 rpm at 4°C for 30 minutes, and supernatants containing secreted Ab were collected and stored frozen until use.

Anti-CT-B ELISA

Falcon® Micro-test III™ Flexible assay microtiter plates (Becton-Dickinson, Oxnard, CA) were coated with 50 μ l/well of 5 μ g/ml CT-B (List Biological Labs) in sterile PBS and incubated overnight at room temperature. The plates were blocked with 200 μ l/well PBS + 1.0% BSA for 1 hour at 37°C. Plates were washed three times with PBS and twice with PBS-Tween-20®, and serum dilutions in ELISA buffer (PBS + 0.5% BSA + 0.05% Tween-20®) were added at 50 μ l/well, and plates incubated at 4°C overnight.

Plates were washed, and 50 μ l/well of detecting goat anti- mouse IgG or IgA-horseradish peroxidase (HRP) conjugate (1 μ g/ml; Southern Biotechnology Associates) was added and the plates were incubated at 37°C for 1.5 hours. HRP was visualized by the addition of 50 μ l/well of ABTS substrate (Moss Inc.). Optical density was determined by reading the plates at 415 nm, and endpoint titers were expressed as the reciprocal of the last sample dilution giving an absorbance > 0.1 over the value of negative control wells after a 1 hour incubation.

Tissue isolation and collection

MLN, PLN, PP, PRLN, SMLN, and CLN were isolated from normal BALB/c and C57BL/6N mice. Each set of lymphoid tissue was pooled from five mice, washed in RPMI-1640 medium (BioWhittaker, Walkersville, MD), and frozen using Tissue Tek[®] O.C.T. compound embedding medium (Miles Inc., Elkhart, IN) in a 15 mm by 15 mm Tissue Tek[®] Cryomold. Samples were stored at -80°C until use. For double immunofluorescent and immunoperoxidase staining, frozen sections were cut at a thickness of 5 μ m, air-dried, fixed in acetone at 4°C, and air-dried again before rehydration.

NALT tissues were collected by removing the soft palates as previously described (76, 83). Briefly, euthanized mice were decapitated, their heads immobilized, and the lower jaws, including tongue, were removed. Palates were scored along the outer edge and gently removed. Palates were then washed in RPMI 1640 medium, blotted dry, and arranged in cryomolds with their ventral faces (containing NALT) oriented at the bottom of the mold and frozen in O.C.T. as described above.

For flow cytometry and ELISPOT analysis, LN were removed and subjected to Dounce homogenization. The resulting cell suspensions were filtered through Nitex fabric (Fairview Fabrics, Hercules, CA), washed with RPMI 1640 medium and centrifuged at in a Beckman GPR tabletop centrifuge (Beckman Instruments Inc. Palo Alto, CA) for 1500 rpm for 5 minutes. The resulting cell pellet was resuspended in FACS buffer (Dulbecco's phosphate buffered saline [dPBS]; + 2% Fetal Bovine Serum; Hyclone), or complete medium (CM; RPMI 1640 + 10% FBS + 10 mM HEPES Buffer + 10 mM non-essential amino acids + 10 mM sodium pyruvate + 10 units/mL penicillin/streptomycin).

NALT lymphocytes were isolated for flow cytometry by Dounce homogenization as described for lymph node cells. For ELISPOT cell isolation, the soft palate was removed from the head of the mouse and placed in a 200U/ml 200 U/ml collagenase type IV solution (Sigma; (171)) in RPMI-1640 media containing .08U/ml DNase (Promega, Madison, WI) in a scintillation vial with a 2 cm magnetic stir bar. The palate was vigorously agitated on a magnetic stir plate for 45 minutes at 37°C, the resulting cell supernatant removed, filtered through Nitex, and cells were washed and resuspended in CM.

NP Lymphocyte Isolation

Mice were euthanized and NP removed from the head by scraping the turbinates from the nasal cavity, in a modification of a previous protocol (28) The nasal tissue was digested with 200 U/mL collagenase type IV solution containing 0.08 U/ml DNase in a glass 50 ml flask containing a magnetic stir bar. Following rapid agitation during

digestion at 37°C for 30 minutes, released NP lymphocytes were removed and fresh collagenase solution was added back to the flask. This procedure was repeated until digestion of the tissue was complete. Isolated cells were washed in complete medium and resuspended in a 40% Percoll® solution (Pharmacia, Uppsala, Sweden), and then layered over a 60% Percoll solution and subjected to gradient centrifugation. Lymphocytes were removed from the interface layer, washed, and resuspended in CM.

Intestinal LP lymphocyte isolation

A modification of a previous protocol was performed (172). Intestines were extracted from the mouse and the PP were carefully removed. Fecal material and mucous were flushed from the intestine using RPMI-1640 medium pushed through a #22 gavage needle. Intestines were then slid onto the needle and flayed open, minced into ~1mm pieces, shaken vigorously in CM to remove remaining mucous and fecal material, and the waste was filtered through a mesh screen. Intestinal tissues were then placed in RPMI 1640 medium containing 5% FBS (Hy-Clone) and 2 mM DTT (Sigma) in a 50 ml Teflon® flask containing a magnetic stir bar, and gently agitated on a stir plate at room temperature for 5 -10 minutes. This process results in the removal of the intestinal epithelial cell fraction. Supernatant was discarded, and DTT was rinsed from the intestinal pieces with RPMI-1640 medium. Intestinal tissues were returned to the Teflon flask, 50 U/ml collagenase type IV solution containing .08 U/ml DNase as previously described was added and the suspension was agitated at 37°C. After 10 minutes, the supernatant containing intestinal lamina propria cells was removed and washed, and fresh collagenase was added to the remaining intestine. The process was repeated 2 more

times, and lymphocytes isolated by Percoll gradient centrifugation as described above.

RT lymphocyte isolation

In a modification of a previous protocol (173), reproductive tracts, which include vagina, cervix, and uteri, were removed from mice, and the mucous and epithelium were flushed using RPMI 1640 medium. RT were then flayed open on a blunted 23 gauge needle, and minced into 1-2 mm pieces. These pieces were added to a 200 U/ml collagenase Type IV solution in a glass 50 ml flask containing a magnetic stir bar, and cells were released from the RT tissue by vigorous agitation on a magnetic stir plate at 37°C for 1 hour. Supernatant resulting from this process was removed and cells were washed in CM, and lymphocytes were isolated via Percoll gradient centrifugation as described above.

Double immunofluorescent staining for tissue addressins

Lymphoid tissue sections were incubated with MECA 367 mAb supernatant fluid (Table 1) or 10% normal rat serum for 30 minutes at room temperature (RT). Specific detection of MAdCAM-1 was obtained upon incubation of a 1:200 dilution of tetramethylrhodamine-5-(and-6-) isothiocyanate (TRITC) conjugated goat anti-rat IgG (H + L) Ab (Southern Biotechnology Associates, Birmingham, AL) in the dark for 30 min at RT. Sections were blocked with 1% rat serum for 15 minutes to bind any free arms of the goat anti-rat secondary Ab. Sections were then stained for PNAd using a 1:50 dilution of MECA 79 mAb (FITC; Table 1) in the dark for 30 minutes at RT. After rinsing, the slides were cover slipped using Immuno-Mount mounting media (Shandon-

Lipshaw, Pittsburgh, PA).

Immunohistochemical staining of MAdCAM-1 and VCAM-1

Serially cut frozen sections were rehydrated in 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 by incubating the sections with 10% NGS in DPBS for 30 minutes at RT, followed by blocking for endogenous avidin and biotin with avidin/biotin blocking solution (Vector Laboratories, Burlingame, CA). Sections were incubated with 1:200 dilution of biotinylated rat anti-mouse vascular cell adhesion molecule-1 (VCAM-1) mAb (Table 1) or rat anti-mouse MECA 367 mAb supernatant fluid or with an isotype matched IgG control for 30 minutes at RT. Sections incubated with MECA 367 mAb were then treated with 1:250 dilution of biotinylated goat F(ab')₂ anti-rat IgG (γ and light chain, absorbed against mouse Ig; Biosource International, Camarillo, CA) for 30 minutes, and all sections were then incubated with a 1:500 dilution of streptavidin-HRP (SA-HRP; BioSource International). HRP was visualized by use of a 3-amino-9-ethyl-carbazole (AEC) staining kit (Vector Laboratories). After AEC development for 10 - 20 minutes, the sections were counterstained with hematoxylin (Richard-Allan Scientific, Kalamazoo, MI) and cover slipped using Immuno-Mount mounting medium.

Blocking of lymphocyte adhesion to HEV

A modification of the Stamper-Woodruff protocol was performed (174). Murine MLN were isolated and suspended in RPMI-1640 media and then subjected to Dounce

homogenization to release lymphocytes. The resulting cell suspension was filtered through NITEX fabric (Fairmont Fabrics, Hercules, CA). Following centrifugation, the MLN lymphocytes were resuspended in DMEM (Sigma, St. Louis, MO) containing 2.5% BSA at a concentration of 1×10^7 cells/ml.

Frozen tissue samples were cut to a thickness of 7 μm and allowed to air-dry for 30 minutes. A circle was drawn around each section with a hydrophobic pen. Sections were then placed on an orbital shaker (GeneMate, Intermountain Scientific Co., Bountiful, UT) at 4°C and 80 rpm. MECA 79 or MECA 367 mAb supernatant, or 5 $\mu\text{g/ml}$ solution of MVCAM.A mAb was added, and the Ab was rotated on the sections for 45-60 minutes to allow binding to MAdCAM-1, PNA_d, or VCAM-1, and then decanted. Lymphocytes (1×10^6) were added at a volume of 100 μl and rotated over the sections for 30 minutes 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 via L-selectin, $\alpha_4\beta_7$, or $\alpha_4\beta_1$, cells were treated with 50 $\mu\text{g/ml}$ MEL-14 (rat anti-mouse L-selectin) mAb, FIB 30 (rat anti-mouse β_7) mAb, or 9EG7 (rat anti-mouse β_1) mAb respectively, for 30 minutes. Pretreated cells were then rotated over the sections as described above. Binding assays were analyzed by counting the number of cells bound/HEV in each LN and then comparing the average number of cells bound/HEV in the presence of Ab to the average number of cells bound/HEV in control sections.

Comparison of $\alpha_4\beta_7$ and L-selectin expression on lymphocytes

Lymphocytes were stained with either FITC-anti-B220, FITC-anti-CD4, or FITC-

anti-CD8 mAbs together with PE-MEL-14 rat anti-mouse L-selectin mAb, and biotinylated DATK 32 rat anti-mouse $\alpha_4\beta_7$ mAb for 30 minutes, washed, and then incubated with SA-Cychrome[®] (BD PharMingen) for 30 minutes. FL1, FL2, and FL3 parameters and compensation were set by the analysis of single-color FITC, PE, or CyChrome[®], respectively, while SA-CyChrome[®] conjugate was added to cells alone in order to determine a baseline fluorescence on the FL3 channel. Three-color analysis was performed using a FACSCalibur (Becton Dickinson, Mountain View, CA). Ten thousand events/sample were collected.

In vivo lymphocyte homing

Donor C57BL/6-GFP mice, whose nucleated cells constitutively express green fluorescent protein (GFP) (170) were euthanized, and SMLN, PRLN, and CLN were removed from 15-20 animals/experiment. Nodes were subjected to dounce homogenization, and lymphocytes were filtered through NITEX and combined. Lymphocytes were treated with 10 μ g of purified MEL-14, FIB-30, or with IgG2a isotype control in sterile PBS for 30 minutes at RT, and $12-15 \times 10^6$ lymphocytes were then injected into recipient C57BL/6 mice via the tail vein with 100-250 μ g excess Ab. Recipient mice were sacrificed 4 hours or 24 hours post injection, and lymphocytes from NALT, CONALT, PLN, spleen, MLN, and PP were isolated. Percentage of GFP⁺ cells in lymphocyte suspensions was determined by FL-1 analysis on a FACSCaliber. Fifty thousand events/sample were collected.

Table 1. Monoclonal antibodies used in experiments

mAb	Isotype	Recognizes	Source
MECA 79	rat IgM	PNAd	(116)
MECA 367	rat IgG2a	MAdCAM-1	(106)
MEL 14	rat IgG2a	CD62L (L- selectin)	(123) BD PharMingen
FIB 30	rat IgG2a	β_7 integrin	(175)
FIB 504	rat IgG2a	β_7 integrin	(175), ATCC
MVCAM.A	rat IgG2a	CD106 (VCAM-1)	BD PharMingen
M290	hamster IgG	CD103 (α_E integrin)	BD PharMingen
9EG7	rat IgG2a	CD29 (β_1 integrin)	BD PharMingen
Ha2/5	hamster IgG	CD29 (β_1 integrin)	BD PharMingen
DATK 32	rat IgG2a	$\alpha_4\beta_7$ integrin (LPAM-1)	BD PharMingen
RA3-6B2	rat IgG2a	CD45R/B220	BD PharMingen
RM4-5	rat IgG2a	CD4	BD PharMingen
53-6.7	rat IgG2a	CD8 α	BD PharMingen
N418	hamster IgG	CD11c	ATCC
NLDC-145	rat IgG2a	DEC 205	ATCC

Flow cytometry and sorting of effector lymphocytes

NP, iLP, and RT lymphocytes combined from ten mice were stained for three or four color flow cytometry analysis as follows: cells were stained with anti- $\beta 7$ FIB 504 mAb supernatant fluid, followed by a biotinylated goat anti-rat IgG (BioSource, Camarillo, CA), then by addition of 1% rat serum, followed by streptavidin-APC. For three color analysis, anti-L-selectin mAb MEL-14-PE, and anti-B220-Cychrome mAb were used, while anti- $\alpha 4\beta 7$ heterodimer DATK-32-PE mAb, anti- αE M290-FITC mAb, and anti-B220-Cychrome mAb (see Table 1) were used for four color analysis. B220⁺ lymphocytes were sorted on a FACSVantage (BD-Biosciences) according to $\beta 7$ and L-selectin staining. $\beta 7^{\text{high}}$ and $\beta 7^{\text{low}}$ populations were sorted from L-selectin deficient (L-Sel.^{-/-}) and L-selectin positive C57BL/6 (L-Sel^{+/+}) iLP, while $\beta 7^{\text{low}}$ / L-selectin^{high} and $\beta 7^{\text{low}}$ L-selectin^{low} populations were sorted from L-Sel^{+/+} NP and RT. All cells were sorted at $\geq 97\%$ purity, counted, and the respective populations used in a B cell ELISPOT.

CT-B-specific ELISPOT

Mixed cellulose ester membrane-bottomed microtiter plates (Multi-Screen®-HA, Millipore Corp., Bedford, MA) were coated with 5 $\mu\text{g/ml}$ CT-B (List Biological Labs) in sterile PBS overnight at room temperature. The plates were blocked at 37°C for 2 hours with CM. A total of 100 μl of cells from each tissue at varying concentrations (2×10^6 - 1.25×10^5 lymphocytes/ml) were added to the wells, and the plates were incubated at 37°C overnight. Cells were removed, and the plates were washed three times with PBS +

0.1% Tween 20 and twice with PBS. For detection of mouse antibody, 100 μ l of 1 μ g/ml goat anti-mouse IgG and IgA-HRP conjugates (South. Biotech. Assoc.) were added to the wells, and the plates incubated overnight at 4°C. After washing as described above, the wells were developed with 100 μ l of AEC (Moss Inc., Pasadena, MD), and the reaction allowed to continue until spots developed (~ 30 minutes). The reaction was stopped with H₂O, the plates were allowed to dry overnight, and spot-forming cells were enumerated by counting under a low-power dissecting microscope (Leica Microscopy Systems LTD & Co. Heerbrugg, Switzerland).

Statistical analysis

Results were analyzed using a paired Student's *t* test. Significant *p* values are indicated.

CHAPTER 3

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

Introduction

NALT represents the oropharyngeal lymphoid tissues of the upper respiratory airways (68, 69). This structure is believed to be analogous to human Waldeyer's ring (tonsils and adenoids) (72), and consists of bilateral lymphoid structures dorsal to the cartilaginous soft palate. It is thought, much like the PP, to behave as a mucosal inductive site. This resemblance is evident in both the organization and structure of NALT as well as its cellular composition. First, there is an epithelial layer overlaying the NALT which contains specialized "M" cells (68, 69) thought to mediate Ag entry much like that observed in the PP. 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 for the PP (75, 76, 83). While 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 LP. Collectively, this evidence suggests that this unique structure is an important tissue for studying immunity to nasally-introduced antigens.

I.n. immunization is an effective route for stimulating mucosal immunity to a variety of pathogens (20, 21, 27, 28, 35), soluble proteins including CT (29, 30), and

microparticle-delivered Ags (83). This route of immunization induces strong antigen-specific mucosal and systemic IgA and IgG antibody responses (20, 27-30, 35, 83) and stimulates elevated CTL responses to viral and ovalbumin peptides (31, 33, 35). Thus, the elevations in humoral and cell-mediated immunity coupled with the ease of administering Ags make this a favorable route of immunization for local respiratory immunity, and immunity at distal mucosal sites (29, 30, 176, 177). In particular, i.n. immunization provides immunity in the genitourinary tract (27, 30, 32, 34, 65) where direct immunization is often hampered by epithelial cell turnover and hormonal influences. For instance, mice i.n.-immunized with the HIV gp160 protein produced HIV-1 neutralizing IgA and IgG antibodies in serum, lung, and vagina (27). Elevations in antigen-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 (21, 46, 65). While i.n. immunization may prove to be a highly effective method of inducing immunity in the genitourinary 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 HEV. These ligands, or "addressins", interact with specific homing receptors expressed by B and T lymphocytes and allow 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 CLN, and

to MLN rather than to the PP (125). This evidence suggests that the NALT addressin profile may differ from the PP.

Naive lymphocyte homing to the PP has been well characterized. It has been shown that MAdCAM-1 plays an important role in trafficking of naive B and T cells into PP (101, 178, 179). MAdCAM-1 is expressed in the PP, MLN, and in the gut LP (106, 112). Both B and T cells interact with MAdCAM-1 through the cellular ligand, $\alpha_4\beta_7$ (180). In some tissues, MAdCAM-1 also binds cells expressing L-selectin through the expression of the PNAd carbohydrate on the MAdCAM-1 glycoprotein backbone (101, 119). In contrast, naive lymphocyte trafficking into PLN is mediated by L-selectin binding to PNAd expressed on glycoproteins other than MAdCAM-1 (116, 122). 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 the NALT HEV express a unique addressin profile that resembles neither a strictly mucosal nor a peripheral phenotype. All NALT HEV express PNAd, either alone or in conjunction with MAdCAM-1. This unique profile differs greatly from the PP, the inductive site for the GALT. The functionality of this PNAd expression was explored through an *ex-vivo* binding assay, where lymphocytes bound to NALT HEV primarily through PNAd - L-selectin interactions. NALT lymphocytes also displayed unique expression of L-selectin profiles. In addition, NALT displayed both MAdCAM-1 and VCAM-1 on the follicular dendritic cells within its B cell areas. Finally, the relative location of HEV within the NALT differed greatly from

the PP. This study suggests that NALT is a unique mucosal tissue, where lymphocyte binding is primarily mediated by PNAd, and where the location of MAdCAM-1 and VCAM-1 expressed by FDC may play an important role in lymphocyte recruitment and retention.

Results and Discussion

Murine NALT HEV express a unique addressin profile

Lymphocyte trafficking into the PP requires MAdCAM-1 expression by its HEV (101, 179). 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 + TRITC labeled secondary) and PNAd (FITC conjugated MECA 79). 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 (Figure 1: A-F, Table 2). In fact, all of the MAdCAM-1 positive HEV in the NALT also co-expressed PNAd. This co-expression was observed in 64.2% of the NALT HEV. No NALT HEV expressed MAdCAM-1 only. In contrast, 52.4% of PP HEV expressed MAdCAM-1 only, while the remaining 47.6% of PP HEV expressed MAdCAM-1 and PNAd ($p < .0001$). In addition, there was a significant difference in the number of HEV expressing PNAd alone in the NALT (37.2%) when

compared to MLN (2.5%) ($p < .01$). NALT also differs from the PLN, where nearly 100% of HEV express PNAd alone. Thus, NALT HEV express a unique addressin profile, one that differs from the strictly peripheral phenotype of the PLN, as well as from the intestinal PP and MLN HEV addressin profiles.

Table 2. Percentages of MAdCAM-1, PNAd, and double positive HEV^a in NALT, PP, MLN and PLN

Tissue	<i>n</i>	MAdCAM-1 only positive	PNAd only positive	Double positive
PP	147	52.4 ± 6.4	0	47.6 ± 6.4
MLN	343	40.9 ± 4.8	2.5 ± 0.9	56.8 ± 4.6
NALT	921	0	37.2 ± 5.0	62.8 ± 5.0
PLN	209	0	95.5 ± 2.0	4.5 ± 2.0

^aValues are expressed as the percentage of HEV expressing MAdCAM-1 alone, PNAd alone, or double positive/total number of HEV per node. SEM reported.

Staining of NALT sections with MECA 367 revealed diffuse MAdCAM-1 located within the B cell area of the NALT as well as on HEV (Figure 2: I, M.). Staining for VCAM-1 showed localization within the B cell areas, but not on HEV within the NALT (Figure 2 Q). However, staining for MAdCAM-1 in the NALT consistently appeared very diffuse, while staining for VCAM-1 appeared very intense. This staining profile differed greatly from the PP, which showed dark staining with the anti-MAdCAM-1 antibody (Figure 2: G, K). In addition, NALT FDC displayed different addressin

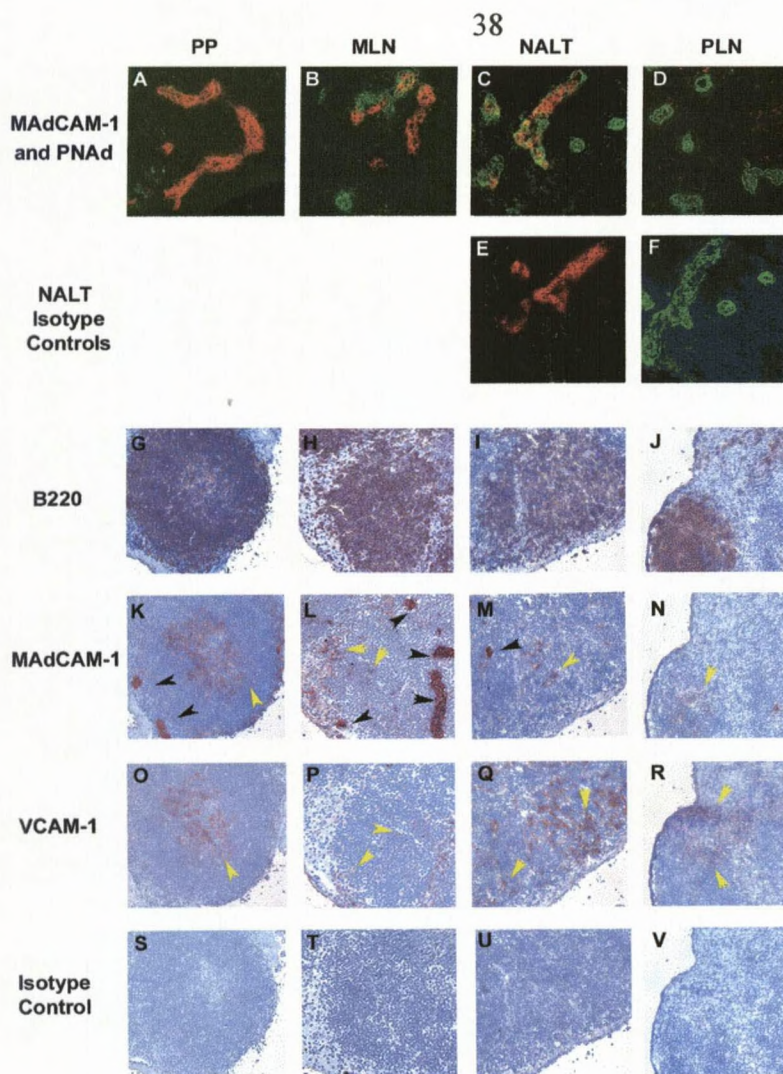


Figure 2. (A-F) Murine NALT HEV display an addressin phenotype different from PP. Fixed tissue sections were treated with MECA 367 + TRITC conjugated goat anti-rat IgG (red color) and FITC conjugated MECA 79 (green color). Results are visualized at 180X magnification, and images were captured using a Spot digital imaging system (Diagnostic Instruments, Inc. Sterling Heights, MI). Red and green images were overlaid to produce the double positive images shown in A-D. Negative isotype-matched controls are shown for the NALT tissue, where the MECA 367 + anti-rat TRITC was followed by treatment with a FITC labeled irrelevant IgM isotype control (E), or an IgG2a isotype control + anti-rat TRITC was followed by treatment with MECA 79 FITC (F). (G-V) NALT FDC display both MAdCAM-1 and VCAM-1. NALT, PP, and PLN serially cut frozen sections were treated with anti-B220 in order to visualize the B cell areas (G-J) with an AEC substrate (red color). Results are visualized at 200X magnification. The same areas were then observed for diffuse staining with MECA 367 (K-N) or anti-VCAM-1 (O-R) antibodies. Black arrows denote HEV staining, while yellow arrows indicate diffuse addressin staining. Specificity of staining is shown by treatment of the tissues with an irrelevant IgG2a isotype control (S-V).

profiles from MLN, which showed very little VCAM-1 staining and a higher incidence of MAdCAM-1. In contrast, although PLN displays VCAM-1 on its FDC, very little MAdCAM-1 staining is observed in the follicles (Figure 2: J, N, R). Taken together, these Ab stains provide further evidence for the expression of a unique addressin profile in the NALT.

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

In order to determine if the PNAd expressed by NALT HEV is functional, we utilized a Stamper-Woodruff *ex vivo* binding assay. Frozen tissue sections were treated with mAbs specific for MAdCAM-1, PNAd, or with irrelevant isotype matched antibodies. For blocking of cellular homing receptors, naive MLN lymphocytes were pretreated with mAbs specific for the $\alpha_4\beta_7$ or L-selectin, or with a rat IgG2a isotype control antibody. The number of cells bound/HEV/node in the presence of addressin-specific antibody was compared to control binding, defined as lymphocytes bound to HEV in the presence of isotype-matched control antibody. Blocking of the PNAd expressed on the HEV of NALT with the MECA 79 antibody resulted in a 60% reduction of binding when compared with control (Figure 3). This reduction of lymphocyte binding was similar to that observed in peripheral lymph node sections treated with MECA 79. In contrast, treatment of PP with MECA 79 resulted in no significant reduction in naive lymphocyte binding ($p < 0.01$). Treatment of NALT HEV with MECA 367 antibody had little effect ($< 20\%$) upon the number of lymphocytes bound/HEV, indicating that the expressed MAdCAM-1 has no apparent role in naive

lymphocyte binding. These results were in direct contrast with PP, where treatment of sections with MECA 367 resulted in greater than 90% reduction in binding. In addition, NALT HEV displayed no similarities in binding to MLN HEV as 50% of naive lymphocyte binding to MLN was blocked by MECA 367. Thus, initial naive lymphocyte binding to NALT HEV was most similar to naive lymphocyte binding to peripheral lymph node HEV, and was 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 (Figure 4). Blocking of L-selectin using MEL-14 resulted in a $> 90\%$ reduction in binding of naive lymphocytes to NALT HEV. Binding of lymphocytes to NALT HEV was reduced to a lesser extent by blocking of $\alpha_4\beta_7$ with FIB 30, which reduced binding by about 60% when compared with controls. MEL-14 blocking of naive lymphocyte binding to NALT was similar to that observed in the PLN, where this antibody blocked nearly all naive lymphocyte binding. However, as expected, treatment with FIB 30 antibody had no effect on binding in the PLN. In the PP, MEL-14 reduced binding of naive lymphocytes to HEV by approximately 30% when compared with controls ($p < 0.01$), while the FIB 30 antibody 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 approximately 80% reduction in binding by both FIB 30 and MEL-14 ($p < 0.01$). These results suggest that the NALT HEV express a unique addressin phenotype, and that naive lymphocyte binding to NALT HEV is

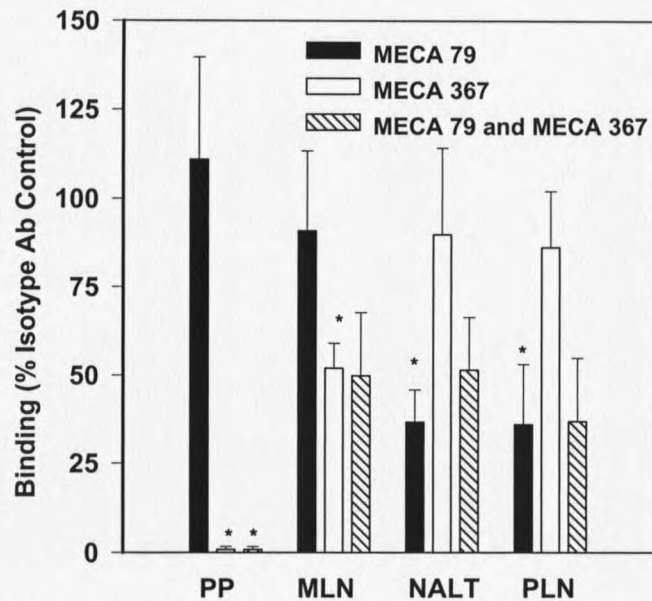


Figure 3. An anti-PNAd Ab blocks initial naive lymphocyte binding to NALT HEV. Fresh tissue sections were treated with 50 μ g/ml of MECA 79 (anti-PNAd), MECA 367 (anti-MAdCAM-1) mAbs or purified rat IgG2a, or IgM isotype matched Ab control for one hour before the addition of MLN lymphocytes. The average number of lymphocytes binding/HEV in each node was determined. Lymphocyte binding is expressed as the percentage of the numbers of cells bound/HEV in the presence of a non-specific isotype matched control Ab. 10-20 HEV/node were counted in each tissue type, and an average of 7.5 lymphocytes were bound/HEV on isotype control treated sections. MECA 79 mAb blocked > 60% of naive lymphocyte binding to both NALT and PLN HEV, but not to PP HEV. The indicated values are the mean of three experiments $\pm$ SEM. * $p < 0.01$

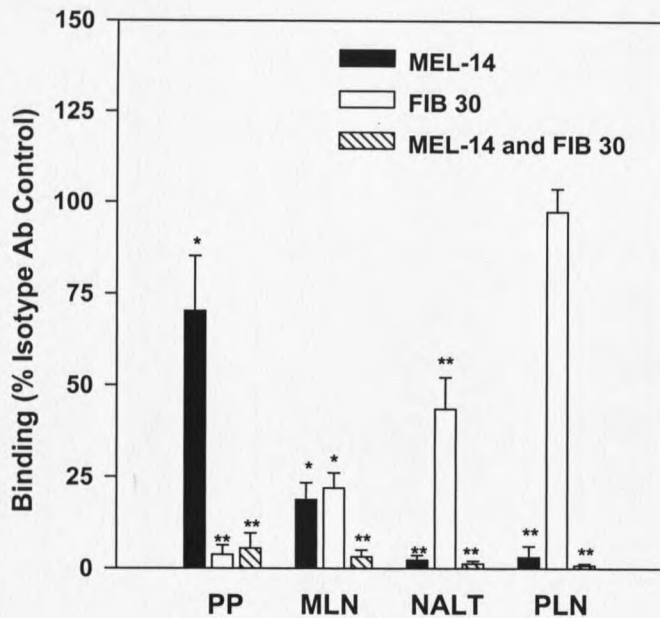


Figure 4. An anti-L-selectin Ab blocks initial naive lymphocyte binding to NALT HEV. MLN lymphocytes ($10^7/\text{ml}$) were treated with $50\text{ }\mu\text{g/ml}$ of purified MEL 14 (anti-L-selectin) mAb, FIB 30 (anti- β_7) mAb, or purified rat IgG2a, isotype matched Ab control for 30 minutes before their addition to tissue sections. The average number of lymphocytes binding/HEV in each node was determined. Lymphocyte binding is expressed as the percentage of the numbers of cells bound/HEV in the presence of a non-specific isotype matched control Ab. 10-20 HEV/node were counted in each tissue type, and an average of 6.5 lymphocytes were bound/HEV on isotype control treated sections. MEL 14 mAb blocked > 90% of naive lymphocyte binding to both NALT HEV, but had a negligible effect on naive lymphocyte binding to PP HEV. The indicated values are the mean of three experiments $\pm$ SEM. * $p < 0.01$ * $p < 0.0001$

primarily mediated by L-selectin - PNAd interactions. However, $\alpha_4\beta_7$ - MAdCAM-1 interactions may also play a role, as evidenced by the reduction in binding observed upon treatment of naive lymphocytes with FIB 30 antibody. These data are in contrast to the pattern observed in PLN HEV, where initial binding is blocked almost completely by MEL-14, but not by FIB 30. These results were consistent with the NALT phenotype, in which all HEV are decorated with PNAd, unlike PP HEV. NALT HEV mediated initial naive lymphocyte binding through different addressin-receptor pairs than did PP HEV. Thus, NALT HEV expressed a more "peripheral" phenotype, both in form and in function. Lymphocyte binding to the NALT primarily through PNAd rather than MAdCAM-1 suggested that the profiles of $\alpha_4\beta_7$ and L-selectin expression on the B and T cells of the NALT might be different from those observed in the PP as well.

NALT lymphocytes express a unique 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 PLN than to PP lymphocytes. Two color flow cytometry 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 (Figure 5). As expected for naive lymphocytes, nearly all of the NALT B and T cells expressed L-selectin, similar to the L-selectin⁺ populations observed in the PLN. This result differed from the PP where a significant portion of the B220⁺ and CD4⁺ lymphocytes were L-selectin⁻ (Figure 5, Table 3).

observed in the PLN. This result differed from the PP where a significant portion of the B220⁺ and CD4⁺ lymphocytes were L-selectin⁻ (Figure 5, Table 3).

The NALT L-selectin⁺ lymphocyte homing receptor profile appeared to more closely resemble that of a PLN rather than a 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.

Table 3. Comparison of the percentages^a of NALT, PP, PLN^b, and MLN^c B and T lymphocytes that express L-selectin.

	<i>PP</i>	<i>MLN</i>	<i>NALT</i>	<i>PLN</i>
B220 ⁺	71.2 ± 3.7	87.0 ± 2.3	86.7 ± 2.6	96.9 ± 0.4
CD4 ⁺	60.2 ± 2.9	88.7 ± 0.6	89.3 ± 1.5	94.8 ± 0.8
CD8 ⁺	62.7 ± 8.5	92.9 ± 0.2	87.2 ± 4.5	94.3 ± 1.6

^aPercentage of NALT, Peyer's patch (PP), peripheral lymph node (PLN), and mesenteric lymph node (MLN) B220⁺, CD4⁺ and CD8⁺ lymphocytes expressing L-selectin.

^b average values for NALT, PP, and PLN from 9 experiments.

^c average values for MLN from 3 experiments ± SEM reported.

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 (69), the expressed mucosal and peripheral addressins had not yet been fully characterized. It was therefore unknown if the various HEV phenotypes unique to the NALT segregated to specific B cell zones or T cell areas within the tissue.

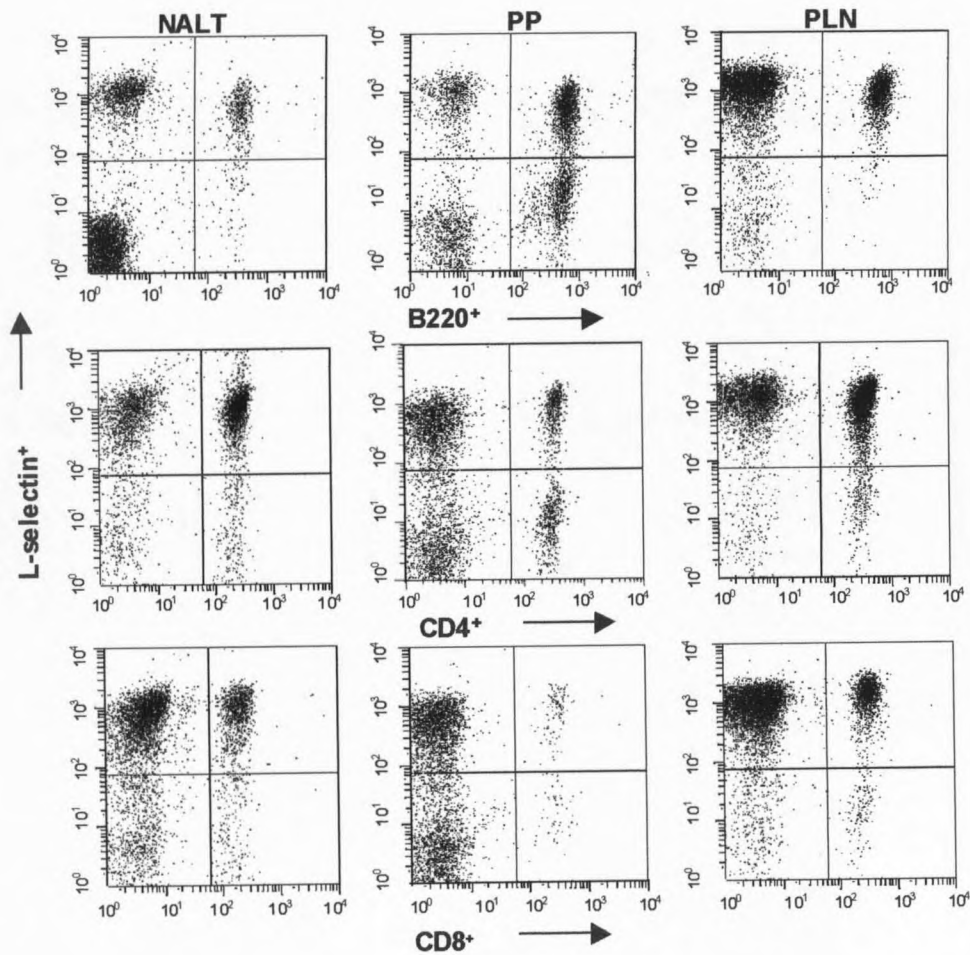


Figure 5. Expression profiles of L-selectin on NALT resemble PLN. NALT, PP, MLN, or PLN lymphocytes were treated simultaneously with FITC conjugated anti-B220, anti-CD4, or anti-CD8 mAbs and PE-conjugated anti-L-selectin (MEL-14) mAb. Representative plots are shown for each tissue. As depicted, the majority of NALT and PLN lymphocytes are L-selectin⁺, in contrast to the PP, which contained both L-selectin⁺ and L-selectin⁻ lymphocyte populations.

In order to determine if 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 monoclonal antibodies showed clearly defined B and T cell regions. HEV were localized to both the T and B cell regions (Table 4): 39.7% of HEV that expressed PNAd were located in the B cell areas, while the remaining PNAd⁺ HEV (58.6%) were located within the T cell areas. No significant difference was observed for the distribution of NALT HEV co-expressing PNAd and MAdCAM-1 in the B and T cell areas. In addition, our evidence also 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, in comparison to 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 ($p < .001$) (Table 4).

Murine NALT is a unique mucosal inductive site

We have shown that murine NALT HEV express a unique addressin phenotype. All NALT HEV expressed either PNAd alone, or co-expressed 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, strong staining with a monoclonal antibody against VCAM-1 was observed in the FDC of NALT. Together, these results suggest that although NALT behaves as a mucosal inductive site like the PP, it displays a unique addressin profile, much different from the

Table 4. Locations^a of Double and Single Positive HEV within NALT and PP^b

	B cell area	T cell area
NALT HEV		
Double positive	57.4 $\pm$ 8.3	40.9 $\pm$ 7.9
PNAd positive	39.7 $\pm$ 3.8	58.6 $\pm$ 3.7
PP HEV		
Double positive	16.2 $\pm$ 4.4	74.5 $\pm$ 8.0
MAdCAM-1 positive	19.1 $\pm$ 4.5	81.0 $\pm$ 4.5

^a The number of HEV expressing either PNAd or MAdCAM₇-1 (indicated with DAB substrate) and their relative locations in the B cell zones or T cell area (indicated with VIP substrate) in the NALT were determined. The number of HEV/phenotype/area was divided by the total number of HEV/phenotype.

^bSEM reported for the average of the results from 12 sections.

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 mediate both naive and memory lymphocyte homing to the PP and extralymphoid mucosal sites such as the gut lamina propria (178, 181). L-selectin also plays a role in naive lymphocyte trafficking into the PP (101), and this can be observed in the results of the *ex vivo* binding assay, where treatment of lymphocytes with MEL-14 reduces binding to PP HEV (Figure 4). 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 in order 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 model 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 (152-154). Also, MECA 79 blocks naive lymphocyte binding to HEV of human tonsils (124). Intranasal immunization induces B cells that have both L-selectin and $\alpha_4\beta_7$ on their cell surface, in contrast to oral immunization, which induces mainly $\alpha_4\beta_7$ expressing B cells (182). In addition, i.n. immunization has been shown to be far more effective than oral immunization at producing protective immune responses in the lower respiratory tract

(176, 177). 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. Since the respiratory tract contains both mucosal (upper airways and bronchi) and systemic (alveolar spaces) compartments, the co-expression 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, 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 appear to play a major role in lymphocyte trafficking into the lung or pulmonary tissues (124, 153, 154, 182), but do provide a mechanism for protective immunity against gut pathogens (151). Therefore, it has been suggested that this addressin/homing receptor pair should be regarded as "intestinal" rather than "mucosal" (183). However, our studies in the mouse suggest $\alpha_4\beta_7$ - MAdCAM-1 interactions may 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- β_7 antibody FIB 30 (Figure 4). These data suggest a mechanism for the entrance of intestinal lymphocytes, which have been shown to

preferentially express $\alpha_4\beta_7$ and enter lymphoid tissue primarily through MAdCAM-1 - $\alpha_4\beta_7$ interactions (106, 178). Alternatively, the $\alpha_4\beta_7$ - 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 - PNA_d interactions, while subsequent tight binding might be mediated primarily by MAdCAM-1 - $\alpha_4\beta_7$ interactions (101, 178).

The $\alpha_4\beta_7$ - MAdCAM-1 interaction also may play a significant role in the trafficking of memory lymphocytes to NALT. Memory lymphocytes preferentially express $\alpha_4\beta_7$ and home to MAdCAM-1 in the mucosal PP and LP (149, 150). In addition, MAdCAM-1 is upregulated in inflamed mucosal tissue (148, 178, 184). It has yet to be determined, however, if MAdCAM-1 is upregulated on the HEV of inflamed NALT, and if such an upregulation would result in a higher percentage of lymphocytes binding through $\alpha_4\beta_7$ interaction.

Another method for naive 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 (145). MAdCAM-1 expression within the PP follicles could mediate binding by $\alpha_4\beta_7^+$ memory lymphocytes, while binding to the follicles of PLN appeared to be primarily mediated by VCAM-1 - $\alpha_4\beta_1$ interaction. It has also been shown (147) that VCAM-1 interactions with the α_4 subunit can mediate binding to the germinal centers of human tonsil. However, VCAM-1 also binds both $\alpha_4\beta_7$, as well as $\alpha_4\beta_1$ expressing lymphocytes (185), suggesting that the $\alpha_4\beta_7^+$ lymphocytes might bind in the germinal centers through this route as well. In

addition, adhesion to VCAM-1 may prevent apoptosis and allow for positive selection of B cells within the follicle (146). Although binding studies of lymphocytes to the NALT follicles have yet to be undertaken, the presence of relatively high levels of VCAM-1 within the NALT suggests that lymphocytes expressing both $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins are able to traffic into these germinal centers where they then mature and differentiate.

Finally, our results indicated that in comparison to the PP, a greater percentage of the NALT HEV were located within the B cell areas. It appeared as though expression of MAdCAM-1 correlates with location of NALT HEV as well, as more double positive than single positive HEV were found within the B cell areas. The reason for this apparent localization of the HEV is unclear, but the localization of the HEV that can support binding of $\alpha_4\beta_7^+$ in the B cell area might provide a new mechanism for the localization of B cells to the B cell areas. 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 (186), these lymphocytes might simply enter the B cell areas directly, where the 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 naive lymphocyte binding mediated by PNAd expressed by NALT. Lymphocytes isolated from the NALT also displayed a homing receptor profile that more closely 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. These data 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.

CHAPTER 4

MAdCAM-1 EXPRESSION AND NAIVE LYMPHOCYTE BINDING
INTERACTIONS VARY AMONG THE CONALTIntroduction

Intranasal (i.n.) immunization offers a productive means for inducing mucosal immunity at local and distal mucosal tissues. Eliciting effective local immunity in the nasal cavity is possible because of the proximity of the draining LN where initial encounters between Ag and lymphocytes occur. These tissues support the induction of immunity in the oral cavity and associated salivary glands and are collectively referred to as the cranial, oral, and nasal-associated lymphoid tissues (CONALT). The CONALT encompasses the facial or parotid gland LN (PRLN) located posterior to the parotid gland, the submaxillary gland LN (SMLN), commonly referred to as the superficial cervical LN located anterior to the submaxillary gland (SMG), and the deep cervical LN (CLN) located dorsal to the brachial plexus deep within the musculature of the neck (60). The role of CONALT has been determined through i.n. immunization studies with soluble proteins, microparticles, and bacterial Ags. From these studies, it appears that the SMLN drain the nasal submucosa while the CLN drain the NALT (60, 86, 87), the SMLN, and the brain (88, 89). In contrast, the PRLN appear to be important for draining the skin of the head and neck as well as the conjunctiva (78, 85).

Salivary IgA responses can be stimulated via various modes of immunization (75, 187, 188). While oral immunization can induce specific salivary Abs, i.n. immunization

appears to be more efficient at stimulating sustained, elevated salivary immune responses (92). This effect is also evident in the CONALT where i.n. and, to a lesser extent, oral immunization with bacterial protein Ags coupled to CT-B produces strong IgA and IgG responses in the SMLN and CLN, resulting in immune S-IgA Abs in saliva (78). IgG responses in the PRLN are more effectively stimulated by s.c. rather than i.n. immunization. Furthermore, i.n. immunization with polymer microparticles induces IgG production in both the SMLN and the CLN (83), the latter having proven to be important in the triggering of allergic responses to particulate Ags (93) and to produce strong CTL responses after i.n. HIV peptide immunization (33). Though CONALT consists of lymph nodes from a similar area in the neck, immunization studies show that the responses of these LN can differ vastly depending on the route of immunization.

Varied immune responses in CONALT may be due to different addressin-homing receptor interactions in SMLN, PRLN, and CLN. Lymphocyte trafficking to intestinal sites is mediated by mucosal addressin cell MAdCAM-1 - $\alpha_4\beta_7$ interactions and PNAd-L-selectin interactions (101, 178, 179). The MAdCAM-1 glycoprotein is primarily expressed on HEV of the PP, the MLN, the intestinal LP, and in the minor vessels in the marginal sinus of the spleen (106, 112-114). MAdCAM-1 - $\alpha_4\beta_7$ interactions mediate both the initial tethering and tight binding interactions in the three-step model of lymphocyte homing (101). MAdCAM-1 also binds to L-selectin through expression of the PNAd carbohydrate on the MAdCAM-1 glycoprotein backbone (101, 119). In contrast, lymphocyte trafficking to the lungs appears to be rely on peripheral, rather than mucosal addressin - homing receptor interactions (122, 153). Initial naive lymphocyte

binding in the peripheral sites takes place through the interaction of PNAd with its cellular ligand, L-selectin (122, 123). PNAd, defined as several different carbohydrate epitopes recognized by the MECA 79 mAb, is predominantly expressed on glycoprotein backbones other than MAdCAM-1 on the HEV of peripheral LN (116). Interestingly, HEV in the mucosal inductive site for the upper airways, NALT, also primarily expresses PNAd (189). It is unknown what addressin-homing receptor interactions mediate naive lymphocyte trafficking to the salivary gland and associated LN; however, studies of lymphocyte homing receptors in the rat SMG revealed that $\alpha_4\beta_7$ and as a high percentage of L-selectin was expressed on SMG lymphocytes (190). To date, the addressin-homing receptor interactions in the CONALT have not been defined in detail.

In this study, we show that the addressin profiles expressed on the HEV of the SMLN, CLN, and PRLN more closely resemble those of peripheral LN rather than intestinal tissues. Significantly, the majority of lymphocyte binding to these tissues is mediated primarily by PNAd-L-selectin interactions. However, important differences in addressin expression and lymphocyte binding exist among the CONALT, as the PRLN appear more peripheral due to lymphocyte binding mediated by PNAd-L-selectin interactions. In contrast, the CLN appear to be relatively more mucosal with some lymphocyte binding mediated by MAdCAM-1- $\alpha_4\beta_7$ interactions. These experiments suggest the importance of peripheral addressin-homing receptor interactions in the CONALT, while indicating the role that varied expression of addressins plays in the functions of LN.

Results and Discussion

The CONALT display different addressin phenotypes

It has been postulated that Ag-specific lymphocytes disseminate from the NALT to the CONALT, which eventually provides effector IgA B cell immunity in the salivary glands and NP (60, 81, 83, 78, 91). If this is the case, it would be expected that the CONALT might express the same addressin phenotype as the NALT. To assess the addressins expressed on the HEV of the SMLN, PRLN, and CLN, double immunofluorescent staining with anti-MAdCAM-1 (MECA 367) and anti-PNAd (MECA 79) mAbs was performed (Fig. 6). Results from the immunostaining revealed that the CONALT expressed a variety of mucosal and peripheral addressin phenotypes. Most importantly, the CLN expressed a more mucosal phenotype than did the SMLN or PRLN. The majority (63%) of HEV in the CLN expressed PNAd, and 24% also co-expressed MAdCAM-1 (Figure 6 C, Table 5). HEV in the CLN expressing MAdCAM-1 alone were limited to only 8%. These results contrasted with the addressin phenotypes observed on the HEV of the SMLN and PRLN, which appeared more peripheral-like. In the SMLN, 86% of HEV expressed PNAd alone; 10% expressed both MAdCAM-1 and PNAd, and less than 1% of HEV expressed MAdCAM-1 alone (Fig. 6 B, Table 5). Of the three LN, the PRLN appeared to be the most peripheral, since 93% of its HEV expressed PNAd alone, 6% co-expressed MAdCAM-1, and approximately 1% of the HEV expressed MAdCAM-1 alone (Fig. 6 A, Table 5). Although these LN may drain related tissues, their varying addressin profiles might indicate selective trafficking of naive lymphocyte subsets.

Table 5. Percentages of MAdCAM-1, PNAd, and double positive HEV in PRLN, SMLN, and CLN^a.

Tissue	<i>n</i>	MAdCAM-1 positive	PNAd positive	Double Positive
PRLN	25	1.3 ± 1.3	92.9 ± 2.8	5.7 ± 2.6
SMLN	34	0.9 ± 0.5	85.8 ± 4.0	10.4 ± 3.1
CLN	18	7.6 ± 5.6	62.7 ± 10.0	24.2 ± 8.6

^a Mean values ± SEM are expressed as the percentage of HEV expressing MAdCAM-1, PNAd, or double positive/total number of HEV per node. PRLN from 13 mice, SMLN from 17 mice, and CLN from 9 mice were analyzed. *n* values represent the number of nodes analyzed.

Extravascular MAdCAM-1 and VCAM-1 are expressed within CONALT

Studies have shown that addressin expression by interfollicular dendritic cells and macrophages is important in lymphocyte recruitment and retention in LN (145, 147, 191). In order to determine additional addressins that might mediate lymphocyte binding in the CONALT, immunoperoxidase staining for non-vascular MAdCAM-1 and VCAM-1 was performed. Staining of CONALT tissue sections with MECA 367 mAb revealed intense staining for MAdCAM-1 in the follicular stromal elements in the CLN and SMLN (Fig. 6, G and H). In the CLN, this intense staining for diffuse MAdCAM-1 correlated with the expression of MAdCAM-1 on the HEV. In contrast, staining for diffuse MAdCAM-1 in PRLN, where less than 10% of HEV expressed MAdCAM-1, appeared considerably less intense (Fig 6 F). However, staining with the anti-VCAM-1 mAb in the PRLN revealed intense diffuse staining for VCAM-1 in the follicular and parafollicular regions

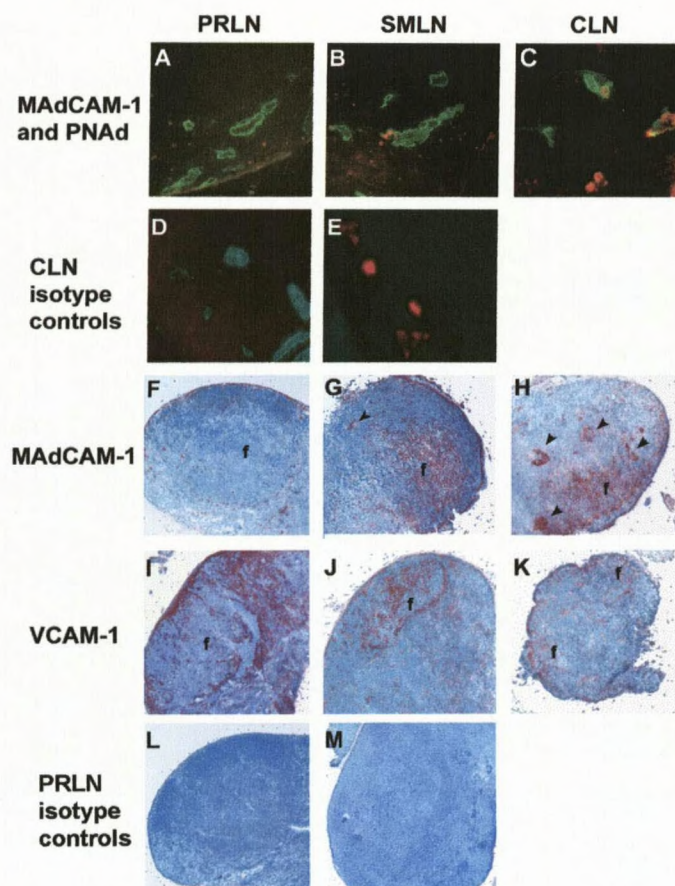


Figure 6. A-E, The CLN have a more mucosal phenotype than the SMLN or PRLN. Fixed tissue sections were treated with MECA 367 mAb TRITC conjugated goat anti-rat IgG (red color), and FITC conjugated MECA 79 mAb (green color). Results are visualized at 180X magnification, and images were captured using a Spot digital imaging system (Diagnostic Instruments, Inc). Red and green images were overlaid to produce the double positive images shown in A-C. Isotype matched negative controls are shown for the CLN, where a rat IgG2a isotype control and TRITC anti-rat IgG were followed by treatment with FITC MECA 79 mAb (D) or MECA 367 mAb, and TRITC-anti-rat IgG was followed by treatment with FITC-labeled irrelevant rat IgM isotype control (E). F-M, Extravascular MAdCAM-1 and VCAM-1 are expressed in CONALT. Immunohistochemical staining with MECA 367 mAb revealed MAdCAM-1 expression in the parafollicular region of PRLN (F) and in the follicles (denoted by "f") and on HEV (arrows) of the SMLN and the CLN (G-H). Intense VCAM-1 staining was observed in both follicular and medullary regions of PRLN (I), while lighter staining was observed in the same regions of the SMLN and CLN (J-K). Isotype matched negative control staining is shown for the PRLN, where nonspecific unlabeled (L) and biotinylated rat IgG2a (M) was added to sections, followed by secondary Ab and AEC development. PRLN are visualized at 26X magnification, while SMLN and CLN are magnified 50X.

of this node (Fig 6 I). The SMLN also displayed diffuse staining of VCAM-1 in the follicle, while CLN showed less intense VCAM-1 staining in its follicles (Fig 6 J and K). The varying expression of diffuse MAdCAM-1 and VCAM-1 among the CONALT supports the notion that the PRLN is the most peripheral of these nodes, while the CLN might be considered more mucosal. These results suggest differing patterns of lymphocyte retention in the CONALT tissues.

Naive lymphocyte binding to the HEV of the CONALT is mediated primarily by PNAd-L-selectin interactions

Since the HEV of the CLN displayed a more mucosal phenotype, we hypothesized that naive lymphocyte binding to this LN might be mediated by both MAdCAM-1- $\alpha_4\beta_7$ and PNAd-L-selectin interactions, while binding to the SMLN and the PRLN would be mediated almost exclusively by PNAd-L-selectin interactions. To investigate these possibilities, a modification of the Stamper-Woodruff *ex vivo* binding assay (174, 189) was performed using a sample population of cells isolated from MLN, which contain both L-selectin- and $\alpha_4\beta_7$ -bearing lymphocytes. These cells were rotated above tissue sections of PRLN, SMLN, or CLN in the presence of mAbs against $\alpha_4\beta_7$, L-selectin, MAdCAM-1, or PNAd, or in the presence of unrelated isotype -matched controls. Blocking of non-stimulated lymphocyte binding to the HEV of the nodes was measured as a percentage of cells/bound/HEV in the presence of blocking Abs versus cells/bound/HEV with isotype matched negative controls.

These experiments revealed that nearly all initial naive lymphocyte binding in CONALT is mediated primarily by PNAd-L-selectin interactions, though some

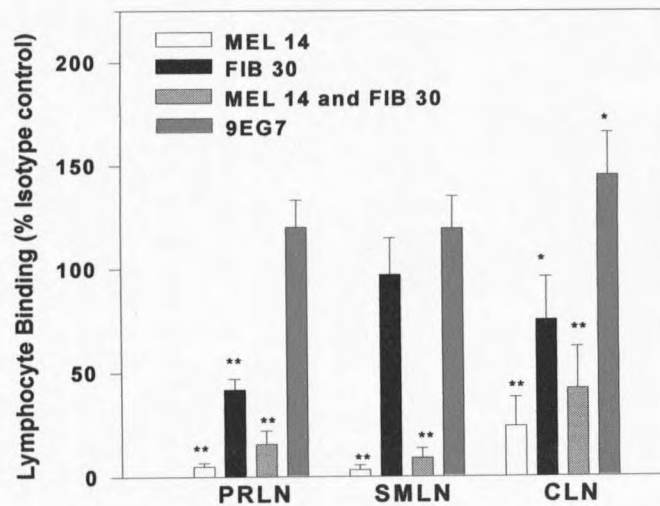


Figure 7. An anti-L-selectin mAb blocks initial naive lymphocyte binding to CONALT HEV. MLN lymphocytes ($10^7/\text{ml}$) were treated with 50 $\mu\text{g}/\text{ml}$ of purified rat anti-mouse MEL-14 (anti-L-selectin) mAb, FIB 30 (anti- β_7) mAb, 9EG7 (anti- β_1) mAb, or purified rat IgG2a isotype control for 30 min prior to their addition over the tissue sections. The average number of lymphocytes bound/HEV in each node was determined. Lymphocyte binding is expressed as a percentage of the number of cells bound/HEV with treatment of a non-specific isotype-matched Ab. Two to twenty-two HEV were counted per node in each tissue type with an average of 10.2 cells bound/HEV in PRLN, 6.7 cells bound/HEV in SMLN, and 1.9 cells bound/HEV in CLN. MEL-14 blocked > 95% of naive lymphocyte binding to PRLN and SMLN HEV and approximately 90% of naive lymphocyte binding to CLN HEV. The 9EG7 mAb failed to block lymphocyte binding to any of the CONALT. The indicated values are the results of three experiments $\pm$ SEM. * $p < 0.01$, ** $p < 0.0001$

interactions are mediated by MAdCAM-1- $\alpha_4\beta_7$. Treatment with the MEL 14 (anti-L-selectin) mAb blocked $> 95\%$ of lymphocyte binding to the PRLN and the SMLN ($p < 0.0001$) (Fig. 7) and approximately 75% of lymphocyte binding to the CLN ($p < 0.0001$). The FIB 30 (anti- β_7) mAb treatment of lymphocytes did not significantly reduce lymphocyte binding to SMLN, but it did reduce binding of lymphocytes to the HEV of the PRLN and the CLN by approximately 60% ($p < 0.0001$) and 40% ($p < 0.01$), respectively, indicating a possible role for MAdCAM-1- $\alpha_4\beta_7$ binding interactions in the HEV of these LN. In addition, since intense staining of VCAM-1 was noted in the PRLN and CLN, we performed blocking experiments with an anti- β_1 9EG7 mAb. However, no reduction in binding was observed, indicating that the $\alpha_4\beta_1$ integrin may have no role in initial naive lymphocyte binding to the CONALT. In fact, a significant increase in binding was observed in the CLN ($p < 0.01$). Identical results were observed with blocking with Ha2/5 anti- β_1 mAb (data not shown).

These results correlated with what was observed with the anti-addressin mAbs, MECA 367 (anti-MAdCAM-1), MECA 79 (anti-PNAd), and anti-VCAM-1 treatments of the CONALT (Fig. 8). In the CLN, MECA 79 and MECA 367 mAbs both blocked 40% of binding to HEV ($p \leq 0.02$). In the SMLN, MECA 79 mAb blocked 80% of binding to HEV ($p \leq 0.001$), while MECA 367 mAb had no effect on lymphocyte binding. Finally, in the PRLN, treatment with MECA 79 mAb reduced binding by nearly 40% ($p \leq 0.001$). While MECA 367 mAb had appeared to block some lymphocyte binding to this tissue, a paired Student's *t* test showed that this effect was not significant. However, treatment of PRLN with MVCAM.A mAb resulted in an approximately 45% reduction in lymphocyte

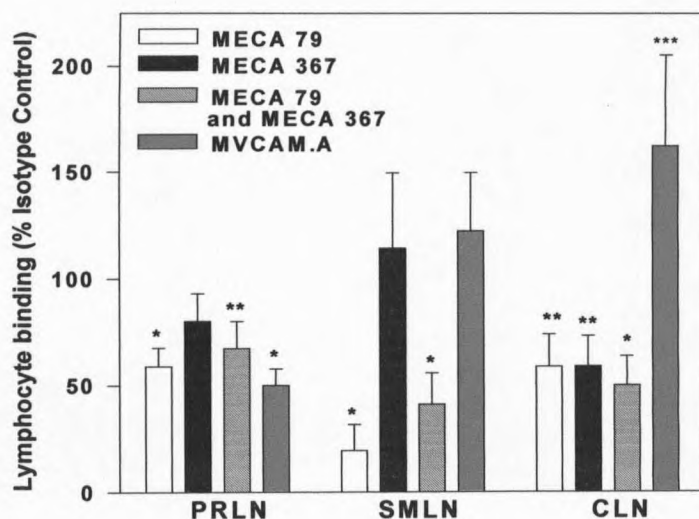


Figure 8. An anti-PNAd mAb blocks initial naive lymphocyte binding to the CONALT. Fresh tissue sections were treated with 50 μ g/ml MECA 79 (anti-PNAd), MECA 367 (anti-MAdCAM-1), or 50 μ g/ml purified MVCAM.A (anti-VCAM) mAb supernatant, or the same concentration of purified rat IgM or IgG2a isotype control for 1 hr prior to the addition of MLN lymphocytes. The average number of lymphocytes bound/HEV in each LN was determined. Lymphocyte binding is expressed as a percentage of the number of cells bound/HEV in the presence of a non-specific, isotype-matched control Ab. Two to twenty-two HEV were counted per node in each tissue type with an average of 11.7 cells bound/HEV in PRLN, 15.9 cells bound/HEV in SMLN, and 3.2 cells bound/HEV in CLN. MECA 79 (anti-PNAd) mAb blocked at least 40% of naive lymphocyte binding to the PRLN, SMLN, and the CLN, while MECA 367 (anti-MAdCAM-1) mAb blocked 40% of lymphocyte binding to the CLN only. Anti-VCAM-1 mAb blocked binding in PRLN only. The indicated values are the mean of three experiments $\pm$ SEM. * $p \leq 0.001$, ** $p \leq 0.02$, *** $p = 0.03$.

binding ($p < 0.001$), correlating with the blocking observed with treatment of FIB 30 mAb. Although there appeared to be a significant amount of staining for VCAM-1 in the PRLN, an anti- β_1 mAb did not block binding. Since the ability of $\alpha_4\beta_7$ to bind VCAM-1 and MAdCAM-1 has been well documented (111, 185, 192), it is likely that $\alpha_4\beta_7$ binds to VCAM-1 in the PRLN as well as the small number of MAdCAM-1 positive HEV. This inhibition by the anti-VCAM-1 mAb was limited to the PRLN, since similar treatment did not significantly block binding in the SMLN or the CLN. Similar to the results observed with the anti- β_1 antibody, blocking of VCAM led to a significant increase in binding to CLN HEV ($p = 0.03$). Together, with evidence from the cell ligand blocking experiments, these results indicate that PNAd-L-selectin interactions mediate the majority of naive lymphocyte binding in the CONALT, with lesser roles for MAdCAM-1- $\alpha_4\beta_7$ and VCAM-1- $\alpha_4\beta_7$ interactions.

Lymphocytes of the CONALT primarily express L-selectin

Results from the Stamper-Woodruff assay suggested that since naive lymphocyte binding in the CONALT was primarily mediated by PNAd-L-selectin interactions, the percentages of L-selectin⁺ and $\alpha_4\beta_7$ ⁺ lymphocytes in the three LN might be similar as well. Three-color flow cytometry analyses of CONALT lymphocytes revealed that there was not a significant difference in the populations of L-selectin⁺ lymphocytes in these LN (Fig 9; Table 6). B220⁺ lymphocytes in the SMLN and CLN expressed slightly lower percentages of L-selectin (91%) than did the B220⁺ lymphocyte population in the PRLN (95%), but this difference was not statistically significant (Table 6). The CD4⁺

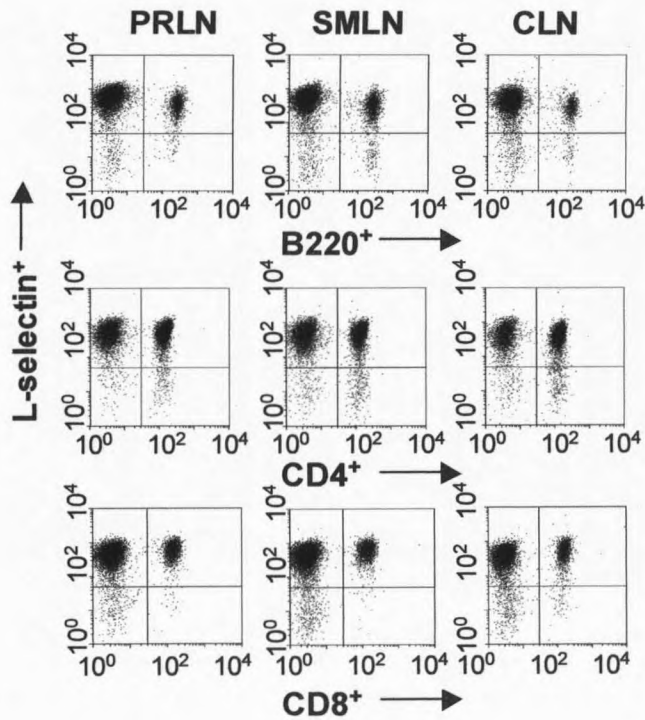


Figure 9. L-selectin is expressed on the majority of CONALT lymphocytes. Plots depict expression of L-selectin by B220⁺, CD4⁺, and CD8⁺ lymphocytes. Plots are representative of three experiments.

lymphocytes in the SMLN, PRLN, and CLN expressed slightly higher percentages of L-selectin (~ 95%) than did the B220⁺ lymphocytes. Finally, nearly all (97-98%) of the CD8⁺ lymphocytes in the CONALT expressed L-selectin. Here again, the percentage of L-selectin⁺, B220⁺, CD4⁺, and CD8⁺ lymphocytes did not vary significantly among the three LN.

Table 6. Comparison of percentage of B and T lymphocytes expressing L-selectin in the CONALT^a.

	PRLN	SMLN	CLN
B220 ⁺	95.2 ± 2.2	90.8 ± 1.6	90.6 ± 4.1
CD4 ⁺	95.9 ± 1.1	94.1 ± 1.6	95.0 ± 0.5
CD8 ⁺	97.1 ± 1.2	97.6 ± 0.8	97.2 ± 0.6

^aPercentage of PRLN, SMLN, and CLN B220⁺, CD4⁺, and CD8⁺ lymphocytes expressing L-selectin. Mean values ± SEM from three experiments, tissues were pooled from five mice in each experiment.

In contrast, lymphocytes in the CONALT expressed mostly low levels of $\alpha_4\beta_7$ (Table 7). B220⁺ lymphocytes expressed the highest percentage of $\alpha_4\beta_7$ in the CLN (86.6%), SMLN (81.8%), and PRLN (78%). The percentage of $\alpha_4\beta_7$ expression on CD4⁺ lymphocytes in the CONALT was much lower (46-50%), and 53-59% of CD8⁺ lymphocytes expressed $\alpha_4\beta_7$. However, $\alpha_4\beta_7^{\text{high}}$ lymphocytes were also found in CONALT (Fig. 10). Combined results from 5 experiments revealed that fifty percent of

$\alpha_4\beta_7^+$ B cells in the CLN were classified as high-expressing (Table 7), while only 33% and 37% of $\alpha_4\beta_7^+$ B cells in the SMLN and PRLN, respectively, were high-expressing. In addition, very few $\alpha_4\beta_7^{\text{high}}$ CD8⁺ cells (7-15%) were present in the CONALT. These results clearly show that the majority of lymphocytes that enter the CONALT are L-selectin⁺ and suggest that peripheral addressin-homing receptor interactions dominate in the CONALT, though some mucosal interactions may be important in CLN.

Table 7. Comparison of percentage of B and T lymphocytes expressing $\alpha_4\beta_7$ in the CONALT^a.

	PRLN	SMLN	CLN
B220 ⁺ total	78.0 ± 9.5	81.8 ± 4.3	86.6 ± 5.6
high	35.3 ± 11.0	32.7 ± 7.1	50.6 ± 5.8
CD4 ⁺ total	46.7 ± 11.8	46.6 ± 8.3	46.4 ± 9.1
high	7.3 ± 2.9	7.4 ± 3.7	8.4 ± 2.7
CD8 ⁺ total	53.8 ± 14.4	56.7 ± 6.8	59.2 ± 9.5
high	11.8 ± 5.1	10.0 ± 6.4	15.7 ± 3.3

^aPercentage of PRLN, SMLN, and CLN B220⁺, CD4⁺, and CD8⁺ lymphocytes expressing total $\alpha_4\beta_7$ and percentage of total $\alpha_4\beta_7^+$ lymphocytes that are $\alpha_4\beta_7^{\text{high}}$. Mean values ± SEM from three experiments, tissues were pooled from five mice in each experiment.

L-selectin primarily mediates in vivo homing of CONALT lymphocytes

Though the results of the Stamper-Woodruff assays indicated that the majority of lymphocyte binding to HEV of CONALT was mediated by L-selectin-PNAd interactions, it was possible that $\alpha_4\beta_7$ -MAdCAM-1 interactions might be important in the tight binding second step of the rolling process as well as in retention of lymphocytes.

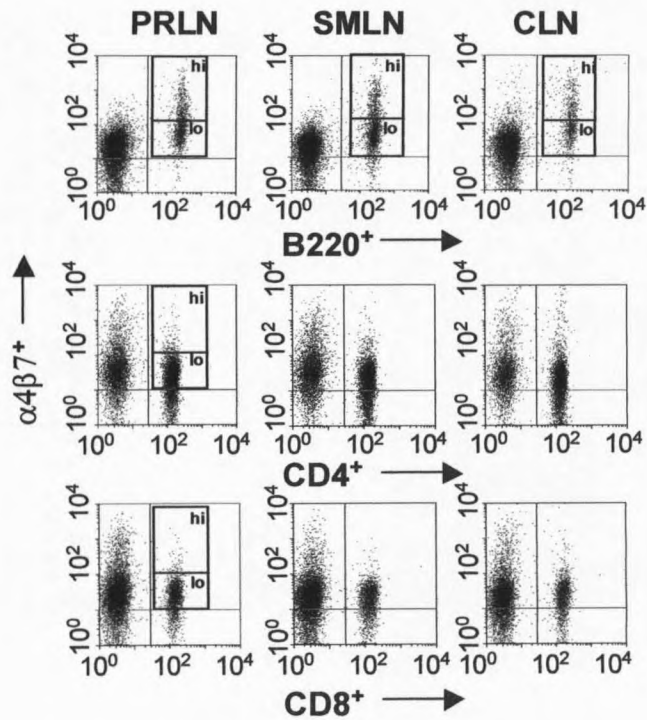


Figure 10. CONALT lymphocytes express primarily $\alpha_4\beta_7^{\text{low}}$. Plots depict expression of $\alpha_4\beta_7$ by B220⁺, CD4⁺, and CD8⁺ lymphocytes, and $\alpha_4\beta_7^{\text{low}}$ and $\alpha_4\beta_7^{\text{high}}$ populations are indicated for CONALT B lymphocytes and PRLN T lymphocytes. Plots are representative of three experiments.

Therefore, lymphocytes that bound to HEV via L-selectin-PNAd interactions might require $\alpha_4\beta_7$ to extravasate into the lymph node and/or remain there. In order to test this possibility we performed *in vivo* lymphocyte homing experiments using CONALT lymphocytes isolated from C57BL/6-GFP mice, which constitutively express enhanced GFP on all nucleated cell types (170). Use of these mice provided a ready source of GFP-expressing lymphocytes, thus reducing the number of donor mice required in these experiments, since cell mortality would not occur as a result of chemical labeling. Since the number of lymphocytes recovered from SMLN, PRLN, or CLN alone would be prohibitively small and require a vast number of donor mice, we combined lymphocytes from all CONALT for use in these experiments.

Studies of short term lymphocyte homing (4 hours post injection; Table 8) revealed that in the presence of nonspecific isotype control Ab, CONALT lymphocytes were collected in the spleen and a high percentage migrated to MLN, which expressed both PNAd and MAdCAM-1. The percentage of GFP⁺/total CONALT lymphocytes found in SMLN was significantly higher than the percentage of GFP⁺ CONALT lymphocytes found to have homed to the PP ($p = 0.02$), suggesting preferential trafficking to CONALT, rather than to the mucosal inductive sites. Fewer GFP⁺ lymphocytes trafficked to CLN, perhaps indicating selective trafficking of CLN B and T cells, which comprised a much lower percentage of the overall CONALT lymphocyte population, back to the CLN. Because of low cell yields from CLN, it was impractical to perform homing experiments with cells from this LN alone. Still, these results show a clear bias for CONALT lymphocytes to traffic selectively to the CONALT, rather than the other

mucosal inductive sites, NALT and PP.

Table 8. Percentages of control Ab treated, donor GFP⁺ lymphocytes^a found in mucosal and peripheral tissue 4 hours and 24 hours after injection.

Tissue	4 hours	24 hours
NALT	0.23 ± .07	0.37 ± .22
SMLN	0.52 ^b ± .17	0.77 ^c ± .13
PRLN	0.38 ± .24	0.73 ^c ± .15
CLN	0.30 ± .08	0.50 ± .35
PLN	0.50 ± .21	1.00 ± .30
SPL	1.22 ± .41	0.67 ± .15
MLN	0.46 ± .20	0.83 ± .20
PP	0.24 ± .05	0.30 ± .06

^aMean values of the percentages of GFP⁺ lymphocytes/total lymphocytes in tissues from five experiments.

^bPercentage of GFP⁺ lymphocytes is significantly higher than percentage of lymphocytes in PP. $p = 0.02$

^cPercentages of GFP⁺ lymphocytes are significantly higher than percentage of lymphocytes in NALT and PP. $p = 0.03$

In addition, these data reveal that the anti-L-selectin mAb MEL 14 significantly blocked nearly all lymphocyte homing to SMLN, PRLN, and CLN ($p < 0.001$; Fig 11 A), while the anti- β_7 mAb FIB 30 had no impact upon homing, correlating with the results observed in Figure 7. Interestingly, though Stamper-Woodruff assays indicate that $\alpha_4\beta_7$ may play a role in lymphocyte binding to HEV in CLN and PRLN, FIB 30 mAb showed

no blocking of short term lymphocyte homing in these tissues, though it did block homing to the PP, MLN, and NALT. Results from our *ex vivo* binding studies revealed that $\alpha_4\beta_7$ interactions with diffuse VCAM-1 expression in PRLN played an important role in lymphocyte binding. However, the sectioning of the node for the Stamper-Woodruff assay might provide access to the diffuse VCAM-1, allowing the cells to bind around, but not actually to, the HEV.

Analysis of long term lymphocyte retention of CONALT lymphocytes (Table 8) after 24 hours again revealed that a significantly higher percentage of CONALT GFP⁺ lymphocytes were retained in SMLN, and PRLN rather than in NALT and PP ($p = 0.03$). As observed following short-term lymphocyte homing, a smaller percentage of lymphocytes was retained in CLN when compared to the other nodes. Correlating with the data shown in Figure 7, MEL 14 significantly blocked the majority of lymphocyte retention in CONALT (Fig 11 B; $p < 0.001$), while FIB 30 mAb had little to no effect on lymphocyte retention. These results also showed that treatment with FIB 30 mAb significantly reduced the percentage of GFP⁺ lymphocytes retained within CLN ($p = 0.03$) indicating a possible role for $\alpha_4\beta_7$ binding to the diffuse MAdCAM-1 expressed in the follicles of this tissue. Surprisingly, however, treatment with FIB 30 mAb did not reduce lymphocyte retention in the PRLN, where it appears likely that $\alpha_4\beta_7$ can bind to diffuse VCAM-1. The ability FIB 30 mAb to block lymphocyte retention in PP, which also expresses diffuse MAdCAM-1, was reduced as well. These results suggest FIB 30 might have limited ability to remain bound to $\alpha_4\beta_7$ and retain its activity through 24 hours. In contrast, the MEL 14 mAb is retained on the surface of the lymphocytes

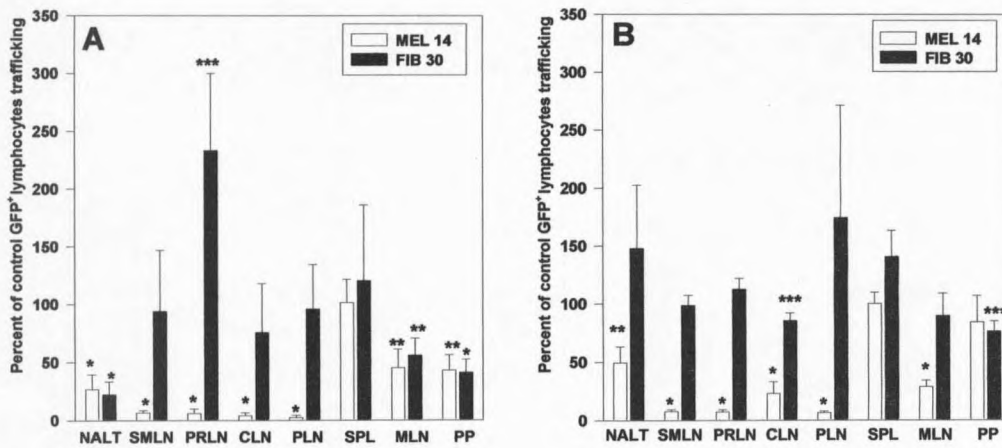


Figure 11. Treatment with an anti-L-selectin Ab reduces lymphocyte homing and retention in CONALT. CONALT lymphocytes were derived from GFP⁺ donor mice and treated with either MEL 14 or FIB 30 mAb, and then injected into recipient C57BL/6 mice. The results of five experiments (mean $\pm$ SEM) are depicted as the percentage of GFP⁺ lymphocytes that trafficked to each tissue in the presence of IgG2a control antibody at (A) 4 hours and (B) 24 hours post-injection. * $p < 0.001$ ** $p \leq 0.01$ *** $p = 0.03$.

throughout 24 hours and continues to prevent lymphocyte trafficking at this time. With the results from our *ex vivo* lymphocyte binding assays, these data indicate the primary importance of L-selectin for trafficking to the mucosal LN of the head and neck, though the CLN appears to rely on some mucosal addressin-homing receptor interactions.

Implications of variable expression of MAdCAM-1 among CONALT

It has been demonstrated that the CONALT, mucosal inductive sites for the upper respiratory tract and oral cavity, express primarily a peripheral addressin phenotype much like the NALT (189). However, there is variability in the percentage of HEV co-expressing MAdCAM-1 and PNAd between the CLN, SMLN, and PRLN, with the CLN expressing the most mucosal addressin phenotype, while the PRLN expresses almost exclusively peripheral addressin. Though naive lymphocyte binding and trafficking in these LN is mediated primarily by PNAd-L-selectin interactions, some MAdCAM-1- $\alpha_4\beta_7$ mediated lymphocyte binding occurs in the more mucosal CLN, which also expresses intense staining of extravascular MAdCAM-1, and little to no staining of diffuse VCAM-1. In contrast, the more peripheral PRLN appears to express high levels of extravascular VCAM-1 that can bind $\alpha_4\beta_7$, but does not express diffuse MAdCAM-1. In addition, the results of this study show that > 90% of lymphocytes in the CONALT express L-selectin. In contrast, while the majority of B lymphocytes expressed $\alpha_4\beta_7$, less than half of the T lymphocytes expressed this integrin. Also, the majority of lymphocyte trafficking to the CONALT is mediated by L-selectin. Collectively, these results provide evidence for the primary role of peripheral addressin-homing receptor interactions in lymphocyte

trafficking to CONALT and suggests a role for MAdCAM-1- $\alpha_4\beta_7$ interactions in retention in CLN, but not PRLN or SMLN.

Since the CLN drain the SMLN, and both drain the NALT and the nasal submucosa (60, 86, 87), it might be expected that these LN would share a similar addressin phenotype with each other and with the NALT. Instead, more than double the percentage of HEV in the CLN co-expressed MAdCAM-1 (24%) when compared with the percentage of co-expressing HEV in the SMLN (10%). In addition, both of these LN expressed significantly fewer MAdCAM-1 positive HEV compared to the NALT, where 60% of the HEV expressed both MAdCAM-1 and PNAd (189). Finally, lymphocyte homing studies indicated that CONALT lymphocytes selectively traffic back to CONALT, rather than to the NALT. However, it is also possible that these LN, while they share common pathways of lymphocyte drainage, may not necessarily require the same addressin profiles on their HEV to allow these tissues to mediate cell trafficking from distal mucosal tissues. For local immunity, Ag will drain from the nasal submucosa, the NALT, the oral cavity, or the skin of the head and neck to the various CONALT, whose unique addressin profiles might then support the trafficking and retention of selective lymphocyte subsets to each LN where B and T cells can then activate and assume a memory phenotype. It is also possible that the expression of PNAd by nearly all HEV in the CLN and SMLN, and PNAd-L-selectin mediation of almost all naive lymphocyte binding may serve an important function in these LN. Expression of PNAd by the LN that drain and collect Ag from the nasal passages might allow for the trafficking of naive lymphocytes, almost all of which express L-selectin. Thus, the

chances that a naive lymphocyte will encounter and react to nasally-introduced Ags are greatly increased.

Though little initial lymphocyte binding is mediated through MAdCAM-1- $\alpha_4\beta_7$ interactions, MAdCAM-1 might indeed be necessary for trafficking of some lymphocyte subsets into the CONALT. Because 5-24% of HEV in CONALT co-express MAdCAM-1 and PNAd, it seems likely that MAdCAM-1 provides the glycoprotein backbone for the carbohydrate PNAd expression (101, 119), thereby binding L-selectin⁺ lymphocytes. Results from our Stamper-Woodruff assays showed that in the CLN, which expressed a more mucosal phenotype with 30% of its HEV expressing MAdCAM-1, treatment with FIB 30 mAb and MECA 367 mAb significantly reduced lymphocyte binding. $\alpha_4\beta_7$ interactions with diffuse MAdCAM-1 expression might mediate lymphocyte retention in CLN since the number of lymphocytes capable of homing was reduced by treatment with the FIB 30 mAb.

The "more mucosal" addressin profile expressed by CLN may allow it to perform a unique role as an inductive site in the head and neck. Functionally, while CLN is thought to drain most of the LN of the head and neck (60), the deep CLN have been shown to play an important role in the induction of immunity in the central nervous system, as Ags injected into the brain subsequently drain to the CLN, and removal of the CLN ablates experimental autoimmune encephalomyelitis (88, 89, 94). Located deep within the muscles of the neck, the CLN serve ideally as central nodes, and most likely play an important role in the induction of immunity in the CMIS. Consequently, the expression of MAdCAM-1 on its HEV might allow for selective trafficking of $\alpha_4\beta_7^{\text{high}}$ lymphocytes

from other mucosal sites (149, 151) that might react to antigen that has drained from the central nervous system.

In contrast to CLN and SMLN, the PRLN appear to function as more of a peripheral type of LN. Though located in close proximity to the parotid gland, the PRLN drain the skin of the head and neck (60, 78) and induce primarily systemic IgG responses (78). Our results strengthen these observations, as the PRLN predominantly display PNAd and bind naive lymphocytes primarily through PNAd-L-selectin interactions. Surprisingly, in the PRLN, treatment with FIB 30 mAb reduced naive lymphocyte binding by approximately 40%, but the effect of MECA 367 mAb was negligible. Subsequent treatment of PRLN HEV with MVCAM.A mAb reduced binding by approximately 50%. It has been shown that both $\alpha_4\beta_7$ and $\alpha_4\beta_1$ bind to VCAM-1 expressed by dendritic cells and macrophages (111, 147, 185). Since blocking of β_1 had no effect on binding, it seems likely that the extravascular VCAM-1 in the PRLN mediates *ex vivo* lymphocyte binding in close proximity to, but not on, the HEV through interactions with $\alpha_4\beta_7$. This result was supported by the data from our *in vivo* homing studies, which revealed that treatment with the FIB 30 anti- β_7 mAb had no effect on lymphocyte trafficking to this node, while treatment with an anti-L-selectin mAb almost completely blocked homing. Therefore, the PNAd expressed on the HEV of the PRLN may result in the trafficking of primarily L-selectin⁺ lymphocytes to this node, while high extravascular expression of VCAM-1 contributes to the retention of lymphocytes.

Finally, these results support the notion that MAdCAM-1- $\alpha_4\beta_7$ interactions may be considered to be predominantly intestinal rather than mucosal. Though the HEV of the

CONALT express MAdCAM-1, most cell binding, as well as lymphocyte homing and retention, was mediated through PNAd-L-selectin interactions. Indeed, MAdCAM-1- $\alpha_4\beta_7$ interactions do not play a major role in homing of lymphocytes in the lung or pulmonary tissues (124, 152-154), nor are they necessary for naive lymphocyte binding to NALT (189). The PNAd expressed on the HEV of the CONALT, the mediation of binding through PNAd-L-selectin interactions, and the high percentage of L-selectin⁺ lymphocytes in these tissues provides further evidence that peripheral addressins and homing receptors are important in lymphocyte trafficking to and from the nasal passages and the salivary glands. This also suggests that pathways of lymphocyte homing to these tissues differ greatly from those observed in the gut. Collectively, this would imply that not all mucosal tissues phenotypically or functionally behave as intestinal tissues, and the variability among mucosal tissues to effect distal mucosal immunity may be dictated by the residing addressin phenotype.

CHAPTER 5

MAdCAM-1 EXPRESSION IS INCREASED ON HEV AND DENDRITIC CELLS OF CT IMMUNIZED NALT

Introduction

Nasal-associated lymphoid tissue (NALT) induces strong immune responses following nasal immunization. Since i.n. immunization represents an attractive route for vaccination (20, 21, 27, 28, 36), it is important to determine the mechanism for the trafficking of lymphocytes to and from this site. As a mucosal inductive site, NALT has often been compared with the intestinal PP (26). However, our previous work (189) has shown that unlike the PP, where MAdCAM-1- $\alpha_4\beta_7$ interactions mediate lymphocyte homing to this tissue, lymphocyte trafficking to naive NALT is mediated primarily by PNA^d - L-selectin interactions.

Changes in addressin expression might occur during an immune response in NALT. Studies have shown that MAdCAM-1 is up-regulated in inflamed intestinal tissue (112, 148, 193), inflamed pancreas (194), and in CNS during chronic experimental allergic encephalitis (195, 196). In addition, expression of the MAdCAM-1 ligand, $\alpha_4\beta_7$, may be necessary for intestinal memory cells to traffic to sites of inflammation (149-151, 197). In contrast, memory lymphocyte homing to peripheral sites (157-159) as well as the lung (198) appears to be mediated by PNA^d-L-selectin interactions. It is unknown, however, whether lymphocyte homing to the immunized NALT will continue to be mediated primarily through peripheral addressin - homing receptor interactions, or if

mucosal immunization will result in an upregulation and corresponding increase in functionality of MAdCAM-1.

Recently, it has been determined that non-HEV-associated MAdCAM-1 is up-regulated in immunized PP (145). This diffuse expression of addressins has also been noted in PLN, where non-vascular VCAM-1 has been observed in germinal centers (147). More importantly, diffuse MAdCAM-1 and VCAM-1 found in germinal centers has proven to be functional, as lymphocytes bind specifically via expression of $\alpha_4\beta_7$ or $\alpha_4\beta_1$. Expression of diffuse MAdCAM-1 in GC and in the SED of PP has been shown to correlate with the presence of DC in these areas (145).

All DC can be identified by their expression of CD11c (199). Recently it has been reported that these DC can be subdivided into three groups based upon expression of various cell surface antigens, and dynamics within the lymphoid tissue (200), though little is known about their location or roles within the lymph node. It has also been reported that two subsets of dendritic cells can be found in the PP (57, 201). These subsets can be classified as sub-epithelial dome (SED) dendritic cells, characterized by CD11c⁺/NLDC-145⁻ phenotype, and interfollicular region (IFR) dendritic cells characterized by a CD11c⁺/NLDC-145⁺ phenotype. The SED layer of epithelial cells is thought to play a specialized role in the presentation of antigens that are captured from the intestinal LP. Given that NALT shares structural similarities with the PP, including a layer of M cells, it is likely that these two subsets of dendritic cells will be identified in the NALT as well, though it has yet to be determined if the diffuse MAdCAM-1 expressed in NALT also associates with DC localization.

In the following studies, we show that the expression of MAdCAM-1 by HEV of the NALT is up-regulated by immunization with CT, which induces a potent Th-2 type response in mucosal tissues (172, 202). The functionality of the addressin is increased as well, since blocking with anti-MAdCAM-1 antibodies reduces the ability of lymphocytes to bind to immunized NALT. Finally, we have shown that the expression of non-HEV-associated MAdCAM-1 co-localizes with N418⁺ DC in the SED of immunized NALT, providing further evidence for the role of addressin expression by antigen-presenting cells in mucosal inductive sites.

Results and Discussion

The percentage of HEV expressing MAdCAM-1 is increased in CT immunized NALT

Since MAdCAM-1 has been shown to be up-regulated in mucosal sites following immunization (112, 148, 184) we questioned whether the inductive site for the nasal passages, the NALT, which in a non-immune state expresses primarily PNAd, would show a similar response after CT immunization. Mice were nasally immunized with 5 µg CT, then boosted 7 and 14 days post initial immunization with 2.5 µg CT. NALT were removed at various days post-immunization, stained for expression of PNAd and MAdCAM-1 via immunofluorescent or immunohistochemical staining, and HEV expressing MAdCAM-1 were enumerated.

Our results show that by 10 days post-immunization, a significant increase in the number of MAdCAM-1 expressing HEV occurred in NALT (Figure 12, Table 9). Whereas naive NALT co-expresses MAdCAM-1 and PNAd on 60% of its HEV (189),

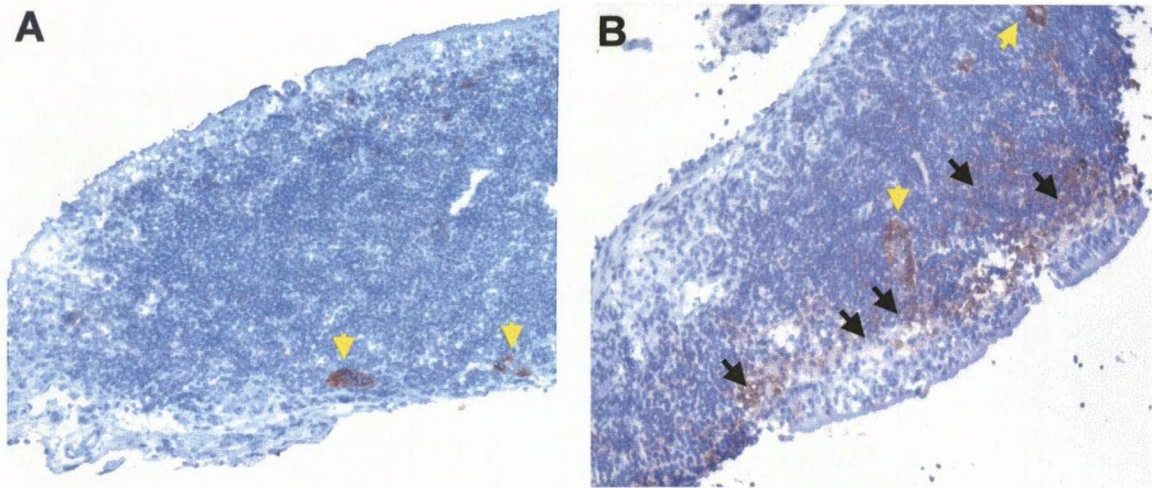


Figure 12. MAdCAM-1 expression is increased on HEV (yellow arrowheads) and in stromal cell areas (black arrows) in 10 day CT-immunized NALT. Non-immune (A) and 10 day CT-immunized (B) NALT sections were stained with MECA 367, and color was visualized by AEC substrate and counterstain with hematoxylin.

Table 9. Increase in the number of HEV expressing MAdCAM-1 in CT immunized NALT^a

Days post CT immunization	Percentage of HEV expressing MAdCAM-1	number of NALT analyzed ^b
0	57.8 ± 2.5	16
1	66.0 ± 1.9	4
3	71.2 ± 10.6	3
5	73.3 ± 7.2	3
10	78.9 ± 6.2 ^c	12
14	73.2 ± 1.8	2
21	91.6 ± 8.4	2

^aMean values are expressed as the percentage of HEV expressing MAdCAM-1, either with PNAd or alone/total number of HEV/node

^b 7 - 10 HEV are found per NALT

^c $p < 0.05$

the number of MAdCAM-1 positive HEV in 10 day post-immunized NALT increased to nearly 80% ($p < 0.05$). The number of HEV expressing MAdCAM-1 continued to increase, and by day 21, an average of 90% of HEV were stained by the MECA 367 mAb. The majority (>99%) of HEV counted in these studies co-expressed MAdCAM-1 with PNAd; very few HEV expressed MAdCAM-1 alone (data not shown).

Increased MAdCAM-1 expressed by immunized NALT HEV is functional

We next performed a Stamper-Woodruff *ex vivo* assay in order to determine if the MAdCAM-1 expressed by the HEV in immunized NALT had an increased function in the binding of lymphocytes to this tissue. MLN lymphocytes were rotated over frozen NALT sections in the presence of isotype control antibodies, anti-L-selectin mAb MEL 14, anti- β_7 mAb FIB 30, anti-PNAd mAb MECA 79, or anti-MAdCAM-1 antibody MECA 367. Comparison of *ex vivo* assays performed on immunized NALT with the results obtained from our studies on normal NALT revealed that MECA 367 and FIB 30 showed increased blocking of lymphocyte binding to HEV, while the ability of MECA 79 and MEL14 to block binding was reduced (Figure 13). In these experiments, both MECA 79 and MEL 14 treatments completely blocked lymphocyte binding to naive NALT, while MECA 367 reduced binding by approximately 40%. In immunized NALT, treatment with MECA 367 reduced binding to 50% of control. In addition, the MEL 14 mAb, which blocks 95% of cell binding in normal NALT, blocked approximately 85% of binding in immune NALT. The most striking difference between normal and immune NALT, however, was the effect of the FIB 30 antibody, which did not block lymphocyte

binding to naive NALT at all, but reduced binding to immunized NALT by 40% (Figure 13).

When the number of cells binding to HEV was compared between normal and immunized tissue, we found that almost 24 times more lymphocytes bound to immunized NALT HEV than normal NALT HEV in the presence of MEL 14 (Figure 14). The number of cells binding to tissue in the presence of MECA 79 in immunized tissue was two-fold higher than that of cells binding in normal tissue. In addition, the number of lymphocytes binding to immunized NALT with treatment of FIB 30 was decreased by one-fold from the number of lymphocytes binding to normal NALT, indicating additional blocking by this antibody. As evidenced by the increased ability of FIB 30 to block lymphocyte binding and the decrease in the ability of MEL 14 and MECA 79 to block binding to immunized NALT, the MAdCAM-1 expressed by HEV in immunized NALT tissue is functional.

Diffuse MAdCAM-1 staining correlates with N418⁺ DC cell staining

In addition to an increase in the number of HEV expressing MAdCAM-1 on the HEV of CT immunized NALT, we also observed an increase in the intensity of staining of diffuse MAdCAM-1 both in follicular regions, as well as in the SED region of immunized NALT (Figures 12 and 15). Since it has been reported that MAdCAM-1 may be expressed by DC in the follicle of the PP (145), we investigated whether diffuse expression of MAdCAM-1 correlated with co-localization of DC subsets.

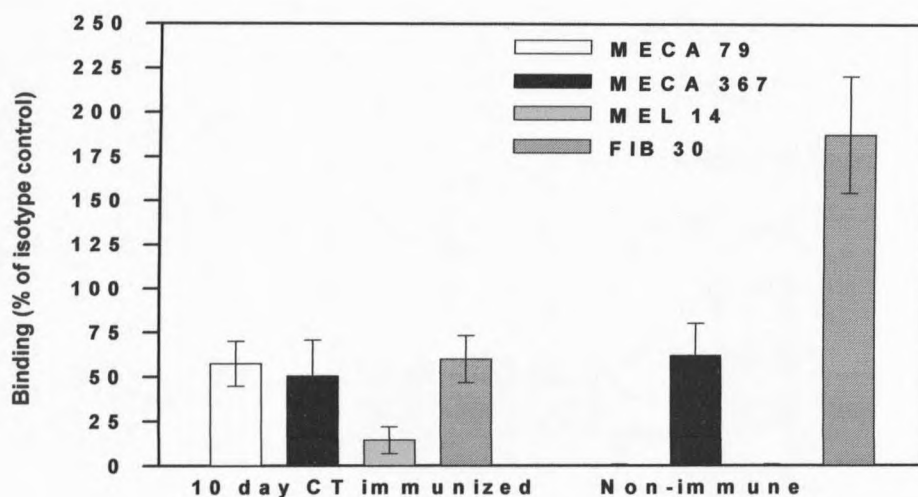


Figure 13: MEL 14 and MECA 79 mAb have decreased blocking ability in 10 day CT-immunized NALT. MLN lymphocytes ($10^7/\text{ml}$) were treated with $50\mu\text{g}/\text{ml}$ of anti-L-selectin mAb MEL14, anti- β_7 mAb FIB 30, anti-MAdCAM-1 mAb MECA 367 or anti-PNAd mAb MECA 79, or purified isotype-matched negative control mAb. The average number of lymphocytes bound/HEV in each node was determined, and results are expressed as the percentage of cells bound in the presence of isotype-matched control Ab. Results are the mean of three experiments $\pm$ SEM.

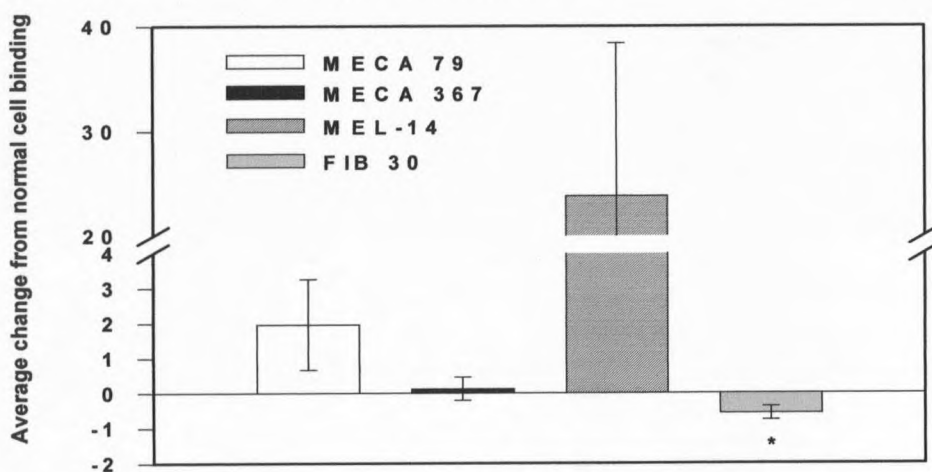


Figure 14: The ability of MEL 14 and MECA 79 mAbs to block naive lymphocyte binding to immunized NALT HEV is decreased. The average number of cells binding to HEV in normal NALT in the presence of FIB 30, MEL 14, MECA 79, or MECA 367 was set as a standard, while the number of cells binding in the presence of the same mAb in immunized NALT is expressed as x times the standard number of cell binding. More cells bind in the presence of MEL 14 and MECA 79 mAbs, while fewer cells bind in the presence of FIB 30 mAb. Results are the mean of three experiments $\pm$ SEM.

In order to identify DC subsets that were present in NALT, we performed immunoperoxidase stains using mAb N418, which identifies CD11c⁺ DC located in both SED and interfollicular (IFR) regions, as well as mAb NLDC-145, which binds to IFR DC only. Our results showed that both SED N418⁺ and IF NLDC-145⁺ DC were present in NALT (Figure 16). More importantly, N418 staining of SED DC appeared to correlate with expression of diffuse MAdCAM-1 in the SED region of the NALT (Figure 15).

To further determine if N418⁺ SED DC were indeed expressing MAdCAM-1, we next performed a double immunofluorescent stain using N418 and MECA 367. Results from these experiments revealed that in non-immune NALT, N418⁺ DC and diffuse MAdCAM-1 staining was observed with some co-localization of MECA 367 and N418 Abs (Figure 17 left panel). These results indicate that some diffuse MAdCAM-1 present in the SED region of non-immune NALT was expressed by DC while some cells expressing MAdCAM-1 did not stain with the N418 Ab. Staining of CT immunized NALT tissue 10 days post-immunization showed an increase in staining of MAdCAM-1 and definite co-localization of N418 and MECA 367 staining (Figure 17 right panel), indicating that some immunized SED DC expressed MAdCAM-1. However, some diffuse MAdCAM-1 staining was again observed independent of N418⁺ cells, suggesting that expression of diffuse MAdCAM-1 in the SED region of the NALT may not be entirely dependent upon DC. Co-localization of diffuse MAdCAM-1 and DC markers was not observed in GC.

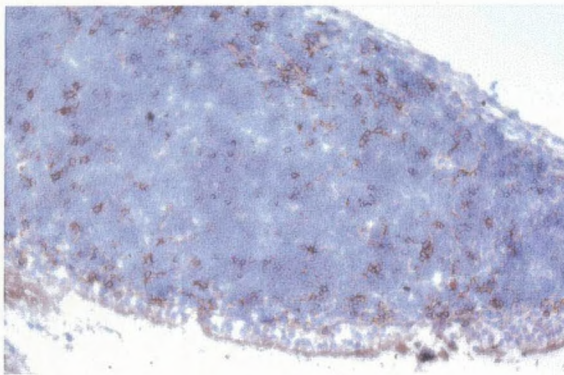
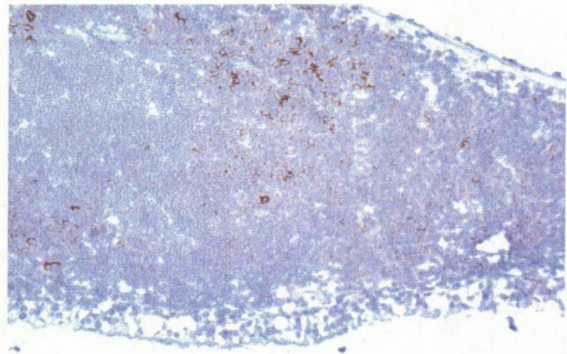
**N418****NLDC-145**

Figure 15: Two subsets of dendritic cells are present in immunized NALT. 10 day CT-immunized NALT was treated with N418 mAb, which recognizes DC in SED and GC regions, and NLDC-145, which binds to DC in GC regions. Ab binding was visualized with AEC substrate and counterstained with hematoxylin.

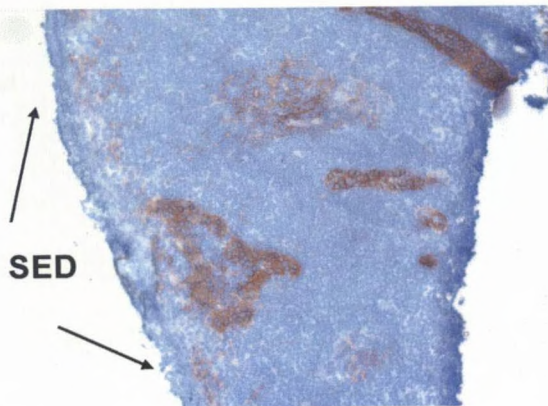
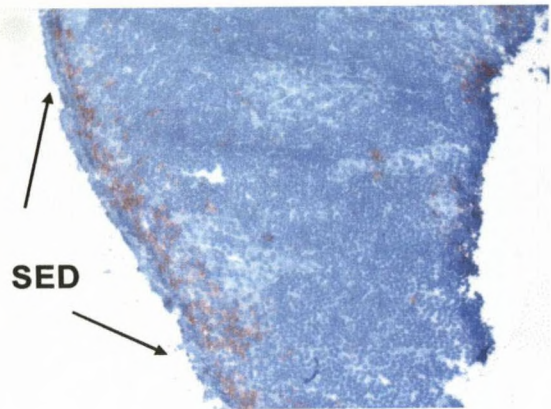
**MAdCAM-1****N418**

Figure 16: Expression of MAdCAM-1 and SED DC appear in subepithelial dome region (SED) of 10 day CT-immunized NALT. Anti-MAdCAM-1 mAb MECA 367 binds to HEV, SED region, and GC, while N418 mAb binds to DC in the SED region. Ab binding is visualized by AEC substrate and hematoxylin counterstain.

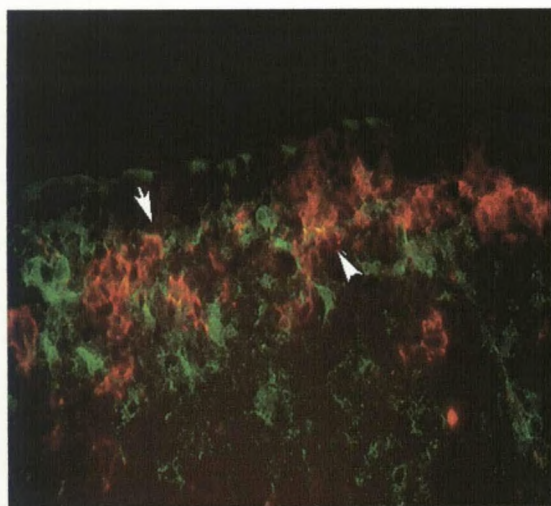
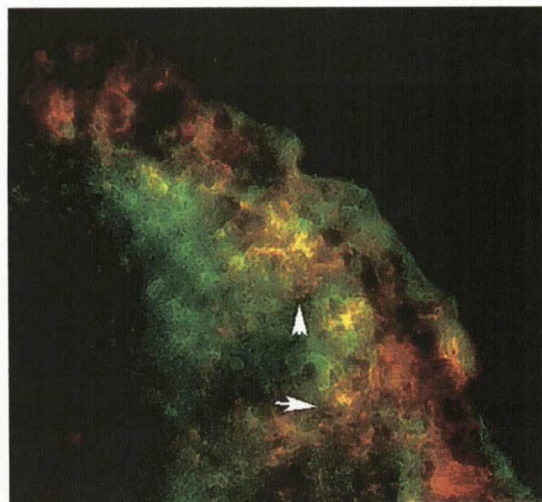
**Normal****10 day CT immunized**

Figure 17. N418⁺ DC co-localize with MAdCAM-1 staining in 10 day CT immunized NALT. NALT were stained with biotinylated MECA 367 mAb followed by SA-FITC (green) and N418 mAb followed by PE labeled anti-hamster secondary Ab. Co-localization of Abs is indicated by white arrowheads

MAdCAM-1 expression on HEV and DC is dynamic

These studies have shown that although PNAd is predominantly expressed on HEV of naive NALT and naive lymphocyte binding to this tissue is primarily mediated by L-selectin interactions (84), mucosal immunization induces an increase in the number of NALT HEV expressing MAdCAM-1. The MAdCAM-1 expression on HEV has an increased function, as *ex vivo* lymphocyte binding to NALT immunized HEV was decreased by treatment with anti-MAdCAM-1 and anti- β_7 mAbs. We also observed intense staining of non-HEV associated diffuse MAdCAM-1. In addition, we have identified co-localization of diffuse MAdCAM-1 expression with N418⁺ DC in the SED region of the NALT.

MAdCAM-1 is upregulated on inflamed intestinal tissue in murine models of colitis (112, 148, 193), as well as in pancreas in mouse diabetic models (194), and in CNS during experimental autoimmune encephalitis (195, 196). This increase in expression was noted on flat-walled endothelial venules located within intestinal lamina propria and stomach, in pancreatic islets, and on epithelial cells in the choroid plexus rather than on HEV located in an organized lymphoid structure. The MAdCAM-1 expressed in these inflamed tissues plays an important role in the recruitment of memory T lymphocytes to these sites, as treatment with MECA 367 and anti- β_7 mAb inhibits trafficking and reduces inflammation (148, 203). It is possible then, that the increase in NALT HEV expressing MAdCAM-1 following CT immunization might provide a mechanism for the recruitment of antigen-specific memory T and B lymphocytes to the NALT. However, all HEV expressing MAdCAM-1 also expressed PNAd, indicating that

there may still be a role for peripheral addressin-L-selectin interactions in memory lymphocyte trafficking.

Blocking of lymphocyte binding by MEL 14 varied greatly between normal and immunized NALT. In the immunized NALT, nearly 24 times more lymphocytes bound to HEV in the presence of this antibody than on naive NALT. In addition, there was a subtle increase in the ability of the FIB 30 and MECA 367 Ab to block lymphocyte binding to immunized versus normal NALT. Therefore, it is likely that some lymphocyte binding to immunized NALT is mediated through $\alpha_4\beta_7$ -MAdCAM-1 interactions. However, the majority of lymphocyte binding to HEV of immunized NALT appeared mediated by PNAd-L-selectin interactions. Together, these data suggest that though the increased MAdCAM-1 expression on HEV has some function, there is still a significant role for peripheral addressin-homing receptor interactions in lymphocyte trafficking to immunized NALT.

We also identified an increase in the diffuse expression of MAdCAM-1 in the follicular and SED regions of the NALT after CT immunization. Double immunofluorescent staining revealed that in immunized and naive NALT, some MAdCAM-1 expression co-localized with N418⁺ staining of DC in the SED region. Expression of MAdCAM-1, and co-localization of MECA 367 and N418 mAb were more pronounced in immunized NALT. Diffuse MAdCAM-1 in normal NALT, as well as some MAdCAM-1 in immunized NALT were expressed by reticular cells other than N418⁺ DC. This is in direct contrast to the results reported by Szabo, *et al* (145), who stated that DC in the GC of inflamed PP and PLN expressed MAdCAM-1. However, no

co-localization antibody staining protocols were performed in that case, and our results clearly show that most cells expressing MAdCAM-1 in NALT are not N418⁺ DC.

Our results suggest that SED region DC do indeed express MAdCAM-1, and expression is upregulated upon immunization with CT. However, in both naive and immunized NALT sections, we observed cells expressing MAdCAM-1 that did not stain with the DC cell marker CD11c. It is possible that macrophages in the SED region of NALT might be expressing MAdCAM-1 as well. We did not observe any co-localization of DC cell markers and MAdCAM-1 in the GC of NALT. It has been reported that in the white pulp of spleen, DC tend to aggregate near reticular cells expressing MAdCAM-1 (204). Therefore, in the GC DC might simply be proximal to, but not expressing MAdCAM-1. In addition, diffuse MAdCAM-1 positive cells in the GC might be expressed by DC, but the intensity of staining of the N418 mAb might be too dim to observe co-localization. In order to address the issue of MAdCAM-1 expression of cell types other than DC, we will continue double immunofluorescent staining studies using MECA 367 and NLDC 145 mAbs, as well as mAbs against macrophages.

Finally, it remains to be determined if the upregulation of MAdCAM-1 in immunized NALT occurs during responses to antigen other than CT. CT has been shown induces the expression of TNF- α in mixed monocyte and lymphocyte cultures (205), which stimulates upregulation of MAdCAM-1 (193, 206). It is possible, then, that the ability of CT to induce TNF- α is the reason for the upregulation of HEV-associated and diffuse MAdCAM-1 staining, and immunization with other Ags might result in a different phenotype of addressin expressed by the NALT. In order to answer this

question, we will analyze mice that have been i.n. immunized with other antigens, specifically Th-1 type antigens, such as adenovirus (207).

These studies have provided important information as to how lymphocytes may be recruited to the NALT during an immune response. Unlike the naive NALT, where the majority of lymphocyte trafficking takes place via peripheral addressin-homing receptor interactions, nasal immunized NALT appears to upregulate expression of MAdCAM-1 on its HEV and on reticular cells in the GC and SED regions as well. More importantly, the increase in MAdCAM-1 expression on HEV correlates with increased function, as lymphocytes rely more upon $\alpha_4\beta_7$ -MAdCAM-1 interactions to bind to immunized NALT tissue. In addition, our results show that DC in the SED region express some MAdCAM-1, indicating a possible role for these cells in the retention of lymphocytes during immune response in NALT. Together, these experiments provide evidence for the role of MAdCAM-1 in the development of a Th-2 type response in the NALT.

CHAPTER 6

DICHOTOMY OF HOMING RECEPTOR DEPENDENCE BY MUCOSAL
EFFECTOR B CELLS: α_E VS. L-SELECTINIntroduction

Recent mucosal vaccination strategies have focused on the ability of i.n. immunization to effect immune responses throughout the CMIS. This route of immunization is attractive due to the ease of administration and the ability to induce immunity at the upper respiratory tract, the distal reproductive tract, and the intestinal LP (27, 30, 34). In contrast, oral immunization induces the expected robust intestinal response and response in the RT, but little to no response in the upper respiratory tract (19). Though the homing receptor-addressin pairs that allow for memory lymphocyte trafficking to the GALT have been well defined (149, 151), the mechanisms that allow for memory lymphocyte homing from the inductive sites to the NP and other mucosal effector sites are unknown.

Memory T and B lymphocyte homing to the gut subsequent oral immunization is mediated through $\alpha_4\beta_7$ interaction with MAdCAM-1 (101, 102, 154, 179). Memory T cells are retained in the lamina propria and intraepithelial compartment of the intestine via the expression of $\alpha_E\beta_7$ integrin and its interaction with E-cadherin. Similar expression has not been observed on B lymphocytes (208). However, in the NALT and the related lymphoid tissues of the head and neck, naive lymphocyte homing is mediated primarily through PNAd and its interaction with L-selectin (84, 189). In addition,

lymphocyte homing to the upper airways in sheep is mediated through peripheral homing receptor addressin interactions (153), and lymphocyte homing to the RT appears to be mediated by homing receptors other than $\alpha_4\beta_7$ (173, 209, 210). These results combined suggest that there is "compartmentalization" of the CMIS.

L-selectin is expressed by nearly all naive T and B lymphocytes, and it is required for trafficking of lymphocytes to lymphoid tissues throughout the body. L-selectin interactions with PNAd carbohydrate expressed by MAdCAM-1 on HEV of PP, allows for the trafficking of naive lymphocytes to this tissue. However, in studies conducted in L-Sel^{-/-} mice (155, 156), cellularity of PP was unaffected by the loss of L-selectin, suggesting a compensatory homing mechanism via $\alpha_4\beta_7$ (155). In contrast, PLN were severely reduced in size and cell number. Immunization studies revealed that L-Sel^{-/-} mice have reduced peripheral DTH responses, as well as delayed graft rejection in the skin (157-159). Though responses of L-Sel^{-/-} mice to peripheral immunization have been well defined, it remains to be determined what effect the loss of L-selectin will have on mucosal immune responses.

Since the NALT relies on L-selectin-PNAd homing interactions rather than $\alpha_4\beta_7$ -MAdCAM-1 interactions, we hypothesized that loss of L-selectin might lead to a reduction in the mucosal immune response after i.n. immunization. We determined the effect of L-selectin deficiency on mucosal effector immune responses in NP, RT, and iLP following i.n. immunization with CT, a potent mucosal adjuvant for producing mostly Th2-dependent Ab responses (172, 202). Comparison of CT-B-specific Ab titers from L-Sel^{+/+} and L-Sel^{-/-} mice revealed that Ag-specific immune responses are abated in NP and

RT, but not iLP. Further investigation shows that compensation for loss of L-selectin in the iLP can be contributed to a subset of mucosal effector cells unique to the intestine and provides additional evidence for the compartmentalization of the CMIS.

Results and Discussion

Vaginal, but not fecal or serum, CT-B-specific IgA titers are reduced in CT-immunized L-Sel^{-/-} mice

The comparison of CT-B-specific mucosal and serum responses in L-Sel^{+/+} and L-Sel^{-/-} mice subsequent to i.n. immunization with CT revealed that loss of L-selectin has a significant impact upon immune responses in the RT, resulting in a nearly 32-fold reduction in vaginal IgA titers in L-Sel^{-/-} mice, and complete abatement of IgG response when compared to L-Sel^{+/+} mice (Fig. 18; $p < 0.001$). In contrast, fecal and serum CT-B-specific IgA titers were not significantly different between L-Sel^{+/+} mice and L-Sel^{-/-} mice. Surprisingly, serum CT-B-specific IgG titer was significantly lower in L-Sel^{-/-} mice than in L-Sel^{+/+} mice by 16-fold ($p < 0.001$), suggesting that the lack of L-selectin resulted in a weaker systemic IgG response. IgG titers in fecal samples were not detectable in L-Sel^{+/+} nor L-Sel^{-/-} mice.

In order to determine the effect of loss of L-selectin on effector immune responses in the nasal passages, we performed B cell ELISPOT assays 16 days post-immunization. Our results indicated that the number of IgA and IgG CT-B-specific antibody forming cells (AFC) in NP was significantly reduced (Fig. 18 C and D) in L-Sel^{-/-} mice. Our ELISPOT data also corroborated the results observed by ELISA, as there was no significant difference in the number of CT-B-specific AFC between L-Sel^{+/+} and L-Sel^{-/-}

iLP, but the antigen specific response in RT was completely abated in L-Sel^{-/-} mice. Collectively, these results suggest the importance of L-selectin for the development of effector immunity for some mucosal tissues as evident in the RT but not the iLP. What remains unclear, however, is how the loss of L-selectin selectively diminishes CT-B-specific mucosal responses in non-intestinal mucosal effector tissues and how intestinal mucosal effector responses remain intact. Therefore, we analyzed the specific mucosal effector lymphocytes that populate the NP, RT and the iLP.

The iLP contains a unique subset of effector B lymphocytes

To determine homing receptor expression on effector B lymphocytes from NP, RT, and iLP, three and four color FACS staining for B220, L-selectin, and β_7 was performed on the cells from 16 day i.n. immunized L-Sel^{+/+} and L-Sel^{-/-} mice. Three distinct populations of B lymphocytes were found in the mucosal effector tissues (Fig. 19A): L-selectin^{high}/ β_7 ^{low} and L-selectin^{low}/ β_7 ^{low} were found in all effector sites, and an L-selectin^{low}/ β_7 ^{high} phenotype displayed by 30% of iLP lymphocytes. Few lymphocytes could be recovered from L-Sel^{-/-} NP or RT (Fig 19B), suggesting that L-selectin is required for lymphocyte trafficking to these effector tissues.

Further analysis of the L-selectin^{low}/ β_7 ^{high} and L-selectin^{low}/ β_7 ^{low} B220⁺ subsets with DATK 32 mAb, which recognizes the $\alpha_4\beta_7$ heterodimer, and with anti- α_E (CD103) M290 mAb (Fig. 19 C and D) revealed that in iLP the β_7 ^{high} subset also expressed α_E ^{high} (Fig. 19 C). The β_7 ^{low} populations found in the NP and RT did not express α_E . However, the α_E negative populations in these tissues and in the iLP were positive for the $\alpha_4\beta_7$

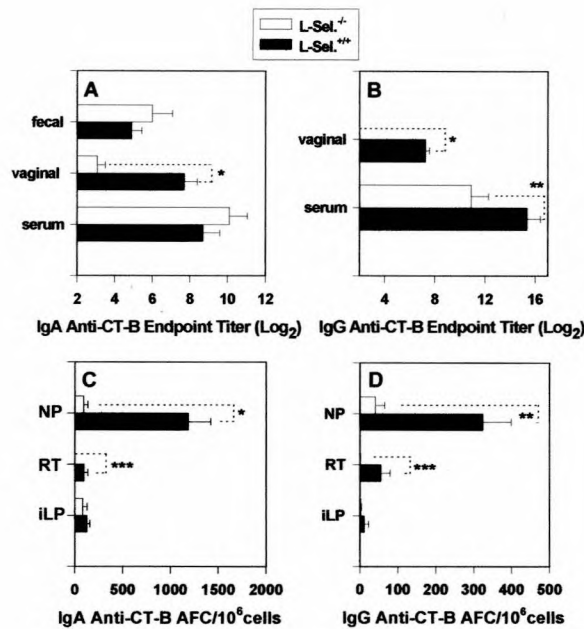


Figure 18. Vaginal and nasal, but not serum or fecal, CT-B-specific Ab responses are reduced in L-Sel^{+/-} mice. Fecal, vaginal, and serum samples were taken from L-Sel^{+/+} and L-Sel^{+/-} mice (5/group) 16 days post primary immunization, and IgA (A) and IgG (B) CT-B-specific Ab titers were determined by ELISA. Vaginal IgA and IgG titers were significantly decreased in L-Sel^{+/-} mice when compared with L-Sel^{+/+} mice. IgA (C) and IgG (D) CT-B-specific AFC/10⁶ lymphocytes were determined by ELISPOT. CT-B-specific AFC were significantly reduced in L-Sel^{+/-} mice. Results are the mean of three experiments $\pm$ SEM. * $p < 0.001$, ** $p < 0.01$, *** $p \leq 0.05$

heterodimer (Fig 19 D) while the $\beta_7^{\text{high}}/\alpha_E^{\text{high}}$ population in the iLP expressed lower levels of $\alpha_4\beta_7$. $\alpha_E\beta_7$ does not bind to MAdCAM-1, and does not appear essential for iLP homing (135); therefore, expression of $\alpha_E\beta_7$ may contribute to retention of B cells at this site. Alternatively, the $\alpha_E\beta_7^+$ population represents a resident iLP B cell population that may have been stimulated by CT immunization. Recent studies also suggest that an as yet unidentified endothelial cell ligand expressed in intestine may mediate $\alpha_E\beta_7$ binding (143), suggesting that the $\alpha_E\beta_7^{\text{high}}$ population may have migrated to the iLP through selective homing interactions independent of L-selectin.

In contrast, staining in the RT and NP revealed that the β_7^{low} population expressed only $\alpha_4\beta_7^{\text{low}}$. This lymphocyte population may be dependent on L-selectin for trafficking to effector sites, where activation of the lymphocyte then causes rapid down-regulation of L-selectin. It is well-known that L-selectin is expressed on nearly all naive lymphocytes, but it is rapidly down-regulated following lymphocyte activation (211, 212). L-selectin mediates memory lymphocyte trafficking to peripheral sites such as inflamed skin (154). Further investigation of L-selectin and β_7 staining on lymphocytes isolated from blood at 16 days post-immunization revealed that these cells are L-selectin^{high}/ β_7^{low} , possibly contributing to the theory that the L-selectin^{low}/ β_7^{low} effector subsets might have downregulated L-selectin upon entry into the mucosal tissues. It is also possible that the L-selectin^{low}/ β_7^{low} subset may have trafficked to the effector sites prior to 16 days post-immunization. The absence of a β_7^{high} population in RT and NP suggests that memory lymphocyte trafficking to these sites is dependent on L-selectin for the initial rolling interaction along endothelial cells in the effector sites. It is also

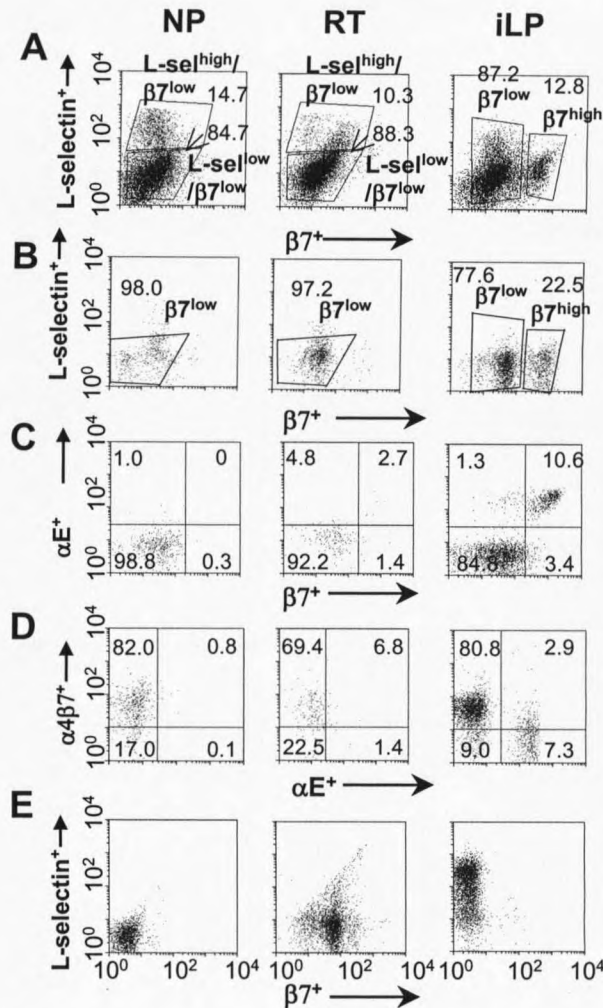


Figure 19. Three distinct homing receptor phenotypes are displayed by lymphocytes in mucosal effector sites. (A) Staining of L-Sel^{+/+} NP, RT, and iLP with anti-L-selectin MEL-14 mAb and anti- β_7 FIB 504 mAb revealed that all three effector tissues contained L-selectin^{high}/ β_7 ^{low} and L-selectin^{low}/ β_7 ^{low} populations, while the iLP contained a unique L-selectin^{low}/ β_7 ^{high} population. (B) L-Sel^{-/-} effector sites contain β_7 ^{high} and β_7 ^{low} populations as well. (C) Analysis of FIB 504 (β_7) staining of B220⁺ L-Sel^{+/+} NP, RT, and iLP lymphocytes, and comparison with M290 (α_E) mAb staining and (D) α_E vs. DATK 32 ($\alpha_4\beta_7$ heterodimer) staining. The β_7 ^{high} population in the iLP expresses high levels of α_E , while the β_7 ^{low} populations in NP, RT, and iLP are positive for $\alpha_4\beta_7$ only. (E) Samples of negative control splenocyte staining (left panel), positive FIB 504 only staining (middle panel) and positive MEL 14 staining (right panel). Results are representative of three experiments.

possible that homing of the β_7^{low} subset might be mediated through $\alpha_4\beta_7$ binding to MAdCAM-1 that could be up regulated in response to immunization. Though FACS analyses have clearly identified three distinct populations of lymphocytes in effector sites, it is unclear which of these populations is responsible for the majority of CT-B-specific and total IgG and IgA production in the various effector sites.

The L-selectin^{low}/ β_7^{low} B lymphocyte subset provides CT-B-specific response in NP and RT

Cell sorting experiments were performed in order to determine which subset of lymphocytes provides CT-specific and total IgA and IgG SFC in effector tissue. NP and RT B220⁺ lymphocytes were sorted for L-selectin^{high}/ β_7^{low} versus L-selectin^{low}/ β_7^{low} (see Fig. 19 A for examples of sorted populations), and SFC responses were enumerated by ELISPOT. For i.n. CT-immunized L-Sel^{+/+} NP and RT (Fig. 20 A, B), the L-selectin^{low}/ β_7^{low} subset of B lymphocytes contained the majority of both IgG and IgA CT-B-specific and total SFC. In the NP, the L-selectin^{high} population accounted for less than 10% of specific and total SFC. This population, however, did not provide SFC in RT (Fig. 3 A). Additional sorting experiments revealed that the lymphocytes contained within these effector populations could be classified as IgD^{low} memory cells (data not shown). Since the lymphocyte yields from NP and RT in L-Sel^{-/-} mice were poor, similar cell sorting experiments could not be conducted on these tissues.

The L-selectin^{low}/ β_7^{high} ($\alpha_E\beta_7^{\text{high}}$) subset produces CT-B-specific and total IgA SFC in iLP

In contrast to the NP and RT, the iLP contains a β_7^{high} ($\alpha_E\beta_7^+$) cell population and

very few L-selectin^{high} lymphocytes. From the results of NP and RT sorting, the contribution of the L-selectin^{high} population to SFC in iLP would be minimal at best. Therefore, sorting experiments were conducted with the β_7^{high} and β_7^{low} populations (see Fig. 19A for examples of sorted populations). Since lymphocyte trafficking to the iLP appeared independent of L-selectin, sorted populations from both L-Sel^{+/+} and L-Sel^{-/-} mice were obtained. The β_7^{high} lymphocyte population provided the majority of IgA CT-B-specific and total SFC in both L-Sel^{-/-} and L-Sel^{+/+} mice (Fig. 20 C), confirming the hypothesis that this subset does provide an L-selectin-independent mechanism for inducing immunity in the intestine following i.n. immunization.

Unexpectedly, upon analysis of the contribution of the β_7^{low} lymphocyte subset to SFC response, these studies revealed that the number of total IgA SFC in the β_7^{low} lymphocyte population of L-Sel^{-/-} mice was reduced (~300 SFC) when compared to the number of SFC obtained from the β_7^{low} population in L-Sel^{+/+} mice (~2300 SFC). The number of total IgA SFC produced in the β_7^{low} population in L-Sel^{+/+} mice equaled more than half of the number of SFC found within the β_7^{high} lymphocyte population (~2300 SFC versus ~4000 SFC). Although there is a subset of β_7^{low} lymphocytes present in the gut, it was found not to be specific for CT. This population may be dependent upon L-selectin for trafficking and may have been induced via the i.n. route of immunization, resulting in a reduced total IgA response in the L-Sel^{-/-} mice. It also resembles the L-selectin^{low}/ β_7^{low} lymphocyte subset observed in NP and RT.

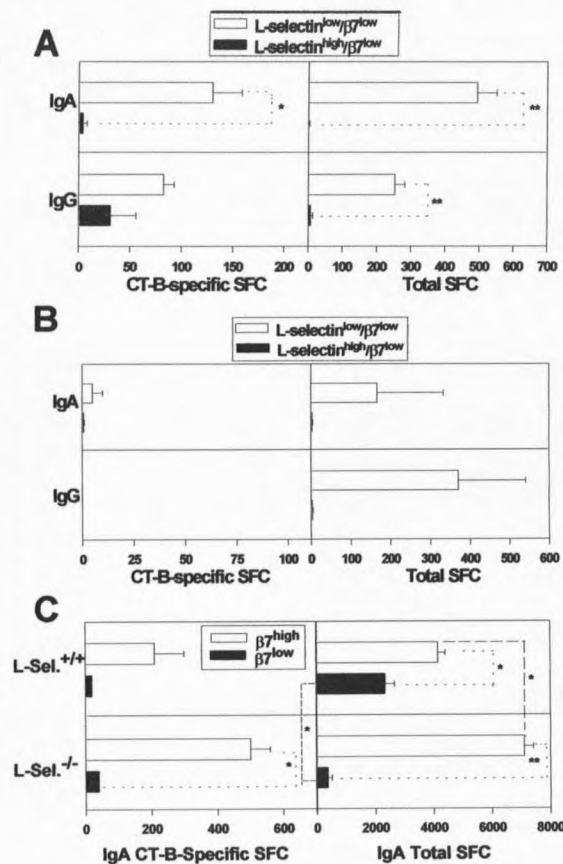


Figure 20. Specific effector populations produce CT-B-specific and total Ab. (A) The L-selectin^{low}/β₇^{low} population of lymphocytes in the NP provide the majority of CT-B-specific and total IgG- and IgA-producing lymphocytes. An insufficient number of NP lymphocytes was obtained from L-Sel^{-/-} mice. Results are representative of four experiments. * $p < 0.02$, ** $p = 0.001$. (B) Only the L-selectin^{low}/β₇^{low} lymphocyte population provides Ab response in RT. No SFC were observed in the L-selectin^{high}/β₇^{low} lymphocyte population. An insufficient number of RT lymphocytes was obtained from L-Sel^{-/-} mice. Results are the mean of two experiments $\pm$ SEM. (C) The β₇^{high} lymphocyte population provides CT-specific response in iLP of L-Sel^{-/-} mice. The β₇^{high} population contained the majority of CT-B-specific IgA SFC in L-Sel^{+/+} and L-Sel^{-/-} mice and contained a significantly greater number of total IgA SFC as well (* $p \leq 0.02$). The number of IgA SFC in the β₇^{low} population was significantly less (* $p \leq 0.02$) in L-Sel^{-/-} mice than L-Sel^{+/+} mice. IgG results were negligible and are not included. Data are mean of three experiments $\pm$ SEM. ** $p < 0.005$ Significance was determined by a paired student's t test.

This study has identified important differences in intestinal versus non-intestinal mucosal sites. We have identified a subset of $\alpha_E\beta_7^+$ B220⁺ effector lymphocytes in the iLP that are not dependent upon the expression of L-selectin to traffic to the iLP. We have also identified a subset of L-selectin^{low}/ β_7 ^{low}/ $\alpha_4\beta_7^+$ lymphocytes that provides the majority of CT-B-specific and total immune response in the NP and RT. Surprisingly, this subset provides a significant contribution to total IgA response in the iLP as well. This subset might represent lymphocytes that are Ag stimulated in the nasal inductive site and subsequently traffic to distal mucosal sites, while the $\alpha_E\beta_7^+$ population may represent a resident intestinal lymphocyte population stimulated by CT immunization, or a gut-specific population that has homed from the NP.

It is important to determine if the subsets of lymphocytes induced in this experiment are strictly Th 2-type, or if immunization with a Th 1-type antigen might induce different responses. Preliminary studies in our laboratory indicate that i.n. immunization with attenuated adenovirus induces L-selectin^{low}/ β_7 ^{low}/ $\alpha_4\beta_7^+$ populations in both NP and iLP, while both L-selectin^{low}/ β_7 ^{low}/ $\alpha_4\beta_7^+$ and $\alpha_E\beta_7^{\text{high}}$ populations are induced in iLP, indicating that these populations may be stimulated via i.n. immunization, regardless of the type of Ag. More importantly, it remains to be determined if a loss of L-selectin will result in reduced effector NP and RT responses after oral immunization. Ongoing studies in our laboratory are addressing this question, but preliminary results show that effector responses in orally immunized L-Sel^{-/-} mice differ vastly from those following i.n. immunization. These data support the idea that non-intestinal mucosal sites function separately from the intestine. In conclusion, this study provides evidence

for separation of the CMIS into "intestinal" versus "non-intestinal" effector sites and suggests a novel requirement for peripheral addressin-homing receptor interactions in mucosal effector sites. This evidence provides further support for the notion that the CMIS is, in fact, compartmentalized.

CHAPTER 7

NON-INTESTINAL EFFECTOR IMMUNE RESPONSES ARE NOT REDUCED IN L-SELECTIN^{-/-} MICE FOLLOWING ORAL IMMUNIZATIONIntroduction

The effect of the loss of L-selectin on peripheral T cell and humoral B cell responses has been well characterized. Specifically, L-Sel^{-/-} mice have a delayed rejection of skin grafts, reduction in the ability of T cells to traffic to sites of inflammation, and a reduction in initial delayed-type hypersensitivity response (157-159). However, though humoral Ab production is initially decreased after subcutaneous (s.c.) immunization in L-Sel^{-/-} mice, intraperitoneal (i.p.) immunization actually results in an increase in IgM and some isotypes of IgG (165). These results suggest that immunization via a mucosal-type route may stimulate immunity by mechanisms other than L-selectin.

Several studies have clearly shown that $\alpha_4\beta_7$ expression by B and T lymphocytes results in preferential trafficking to the iLP and that $\alpha_4\beta_7^{\text{high}}$ lymphocytes provide protective immunity against mucosal pathogens such as rotavirus (149-151). In addition, since naive lymphocyte homing to the mucosal inductive site, the PP, is mediated via $\alpha_4\beta_7$ - MAdCAM-1 interactions, the cellularity of PP is not affected in L-Sel^{-/-} mice (155, 156). These results suggest that the predominance of $\alpha_4\beta_7$ - MAdCAM-1 interactions in both naive and memory B and T cell responses in the GALT compensate for the loss of L-selectin.

Ab responses at mucosal effector sites of L-Sel^{-/-} mice are less well understood.

Our previous study (Chapter 6; (213) determined that immune responses in the NP and RT, but not the iLP are abated following i.n. immunization of L-Sel^{-/-} mice with CT. In addition, we found that L-selectin^{low}/β7^{low} (α₄β₇⁺) B lymphocytes provide the effector immune response in NP and RT, while L-selectin^{low}/β7^{low} (α₄β₇⁺) and L-selectin^{low}/β7^{high} (α_E⁺) B lymphocyte populations were present in the iLP. In fact, the L-selectin^{low}/β7^{high} (α_E⁺) provides the majority of CT-B-specific response in the iLP, and is unaffected by loss of L-selectin. In contrast, cell number and CT-B-specific AFC are reduced in the L-selectin^{low}/β7^{low} lymphocyte population, indicating that L-selectin may be necessary for the trafficking of this B lymphocyte population to effector sites.

It is also possible that nasal inductive immune responses were affected in L-Sel^{-/-} mice. The NALT and CONALT rely primarily on L-selectin-PNAd interactions to mediate naive lymphocyte trafficking; therefore, the loss of AFC response in L-Sel^{-/-} NP and RT might be indicative of the inability to induce an immune response. It seems likely that induction of immune response in the PP, where α₄β₇ binding occurs, might not be affected by loss of L-selectin, and subsequent effector immune responses will remain unchanged from L-Sel^{+/+} mice.

To test this hypothesis, we orally immunized mice with CT, and examined immune responses in the NP, RT, and iLP at 16 days post-immunization. Our results showed that oral immunization of L-Sel^{-/-} mice results in effector immune responses that are indistinguishable from responses in L-Sel^{+/+} mice. Identification of lymphocyte homing receptors expressed by B lymphocytes after oral immunization revealed L-selectin^{low}/β7^{low} (α₄β₇⁺) and L-selectin^{low}/β7^{high} (α_E⁺) subpopulations identical to those

observed after i.n. immunization were found in iLP, while the NP and RT contained only L-selectin^{low}/β7^{low} (α₄β₇⁺) B lymphocytes. Sorting experiments revealed that, unlike after i.n. immunization, loss of L-selectin did not affect numbers of AFC in the L-selectin^{low}/β7^{low} (α₄β₇⁺) population. Our results also showed that oral immunization induced enhanced AFC response in L-Sel^{-/-}, but not L-Sel^{+/+} CONALT. Together, these data show that route of immunization affects B cell immune responses in L-Sel^{-/-} mice, and further defines the CMIS as “intestinal” vs. “non-intestinal”.

Results and Discussion

Oral immunization of L-Sel^{-/-} mice induces AFC in NP, RT, and iLP

In order to determine the effect of loss of L-selectin on mucosal effector immunity following oral immunization, we orally immunized mice with 10 μg of CT on days 0, 7, and 14. Mice were sacrificed at day 16 post-primary immunization, and AFC responses determined by ELISPOT. Our results showed that unlike nasal immunization, oral immunization with CT induces effector immune responses in the iLP, as well as the NP and RT of L-Sel^{-/-} mice. As expected, robust CT-B-specific and total IgA AFC were observed in L-Sel^{+/+} and L-Sel^{-/-} iLP (Figure 21). These results showed that oral immunization is not as effective as i.n. immunization at stimulating RT immunity, since CT-B-specific responses in this site were negligible. Surprisingly, oral immunization induced significantly greater CT-B-specific IgA AFC in L-Sel^{-/-} NP ($p \leq 0.05$), though the total number of AFC were fewer than 200 (~144). However, a greater number of total IgG AFC were induced in L-Sel^{-/-} NP (~1068) than in L-Sel^{+/+} NP (~153), though

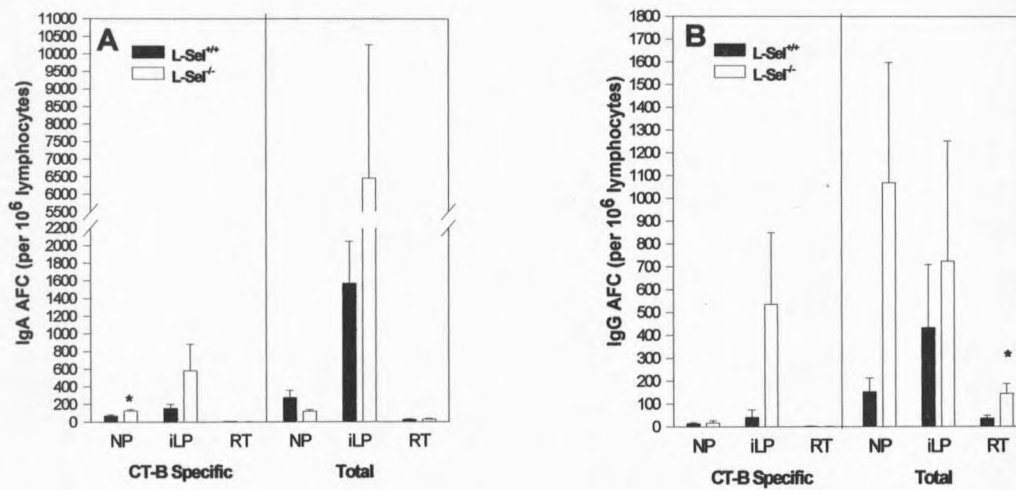


Figure 21: Oral CT immunization induces AFC in NP and RT, as well as iLP. ELISPOT analyses were performed at 16 days postprimary immunization and CT-B-specific and total IgA (A) and IgG (B) AFC were determined. Results are the mean of three experiments $\pm$ SEM. * $p \leq 0.05$

this number was not significantly different from the AFC observed in L-Sel^{+/+} NP. Numbers of CT-B-specific and total IgG AFC did not differ between L-Sel^{+/+} and L-Sel^{-/-} iLP. However, total IgG AFC were significantly increased in L-Sel^{-/-} RT ($p \leq 0.05$). These results show that loss of L-selectin does not affect effector immune responses following oral immunization with CT, and may even increase response in L-Sel^{-/-} NP and RT. Therefore, we next investigated whether oral immunization induces a unique subset of lymphocytes that express a higher level of $\alpha_4\beta_7$ or α_E that might compensate for the lack of L-selectin.

Effector B lymphocyte subsets induced by oral immunization are similar to those observed after nasal immunization

We have previously shown that nasal immunization with CT induces L-selectin^{low}/ β_7 ^{low} ($\alpha_4\beta_7^+$) and L-selectin^{high}/ β_7 ^{low} ($\alpha_4\beta_7^+$) lymphocyte populations in NP, RT, and iLP, and L-selectin^{low}/ β_7 ^{high} (α_E^+) B lymphocytes in the iLP only. To determine if similar populations of lymphocytes exist in the mucosal effector sites following oral immunization, we observed the expression of $\alpha_4\beta_7$, L-selectin, and α_E on B lymphocytes isolated from mucosal effector sites at 16 days post oral immunization. Since all β_7 ^{high} lymphocytes express α_E (Figure 19; 203(134)), we used the M290 (anti- α_E) mAb, and the anti- $\alpha_4\beta_7$ DATK 32 mAb instead of FIB 504 mAb in these studies to determine if the same B lymphocyte subpopulations were induced subsequent to oral CT immunization. Our results showed that the three lymphocyte populations obtained from 16 day orally CT immunized mice (Figure 22) are similar to those observed with i.n. CT immunized mice (Figure 19). L-selectin^{low}/ $\alpha_4\beta_7^+$ and L-selectin^{high}/ $\alpha_4\beta_7^+$ B lymphocyte populations

were observed in L-Sel^{+/+} NP, RT, and iLP, while iLP again contained a unique population of L-selectin^{low}/α_E⁺ B lymphocytes. Interestingly, in L-Sel^{+/+} NP, 58% of B lymphocytes were found to expressed L-selectin^{high} (Figure 22 A), a much greater percentage that was found in i.n. CT immunized L-Sel NP (15%, Figure 19). However, only 10% of L-Sel^{+/+} RT B lymphocytes expressed L-selectin^{high} (Figure 22 B). While L-Sel^{-/-} lymphocytes did not express L-selectin, staining profiles of α₄β₇ and α_E on NP, RT, and iLP were similar to those observed in L-Sel^{+/+} mice. As was observed in i.n. CT immunized mice, L-Sel^{-/-} iLP contained a higher percentage of α_E⁺ B lymphocytes (20%), than did L-Sel^{+/+} B lymphocytes (9%) (Figure 22 F and I). Since these results show that effector immune responses in orally immunized mice are not the result of up-regulation of α₄β₇ or α_E, we next performed immunofluorescent sorting experiments to determine the contributions of these lymphocyte subsets to effector site immunity.

Ability of the L-selectin^{low}/α₄β₇⁺ subset to induce immunity is not affected in orally immunized L-Sel^{-/-} mice.

Our previous studies showed that the majority of immune response in the NP and was contained within the L-selectin^{low}β₇^{low}(α₄β₇⁺) B lymphocyte subset, and that AFC cells from this subset could not be recovered in L-Sel^{-/-} mice. In contrast the L-selectin^{low}β₇^{high}(α_E⁺) B lymphocyte subset provided CT-B-specific and total immunity in the iLP, and AFC responses in this subset were not reduced in L-Sel^{-/-} mice (Figure 20). Sorting for L-selectin^{low}/α₄β₇⁺ and L-selectin^{high}/α₄β₇⁺ B lymphocyte subsets in the L-Sel^{+/+} NP and RT lymphocyte populations revealed that as in i.n. immunized mice, the L-selectin^{low}/α₄β₇⁺ subset provided the majority of CT-B-specific and total AFC in both L-

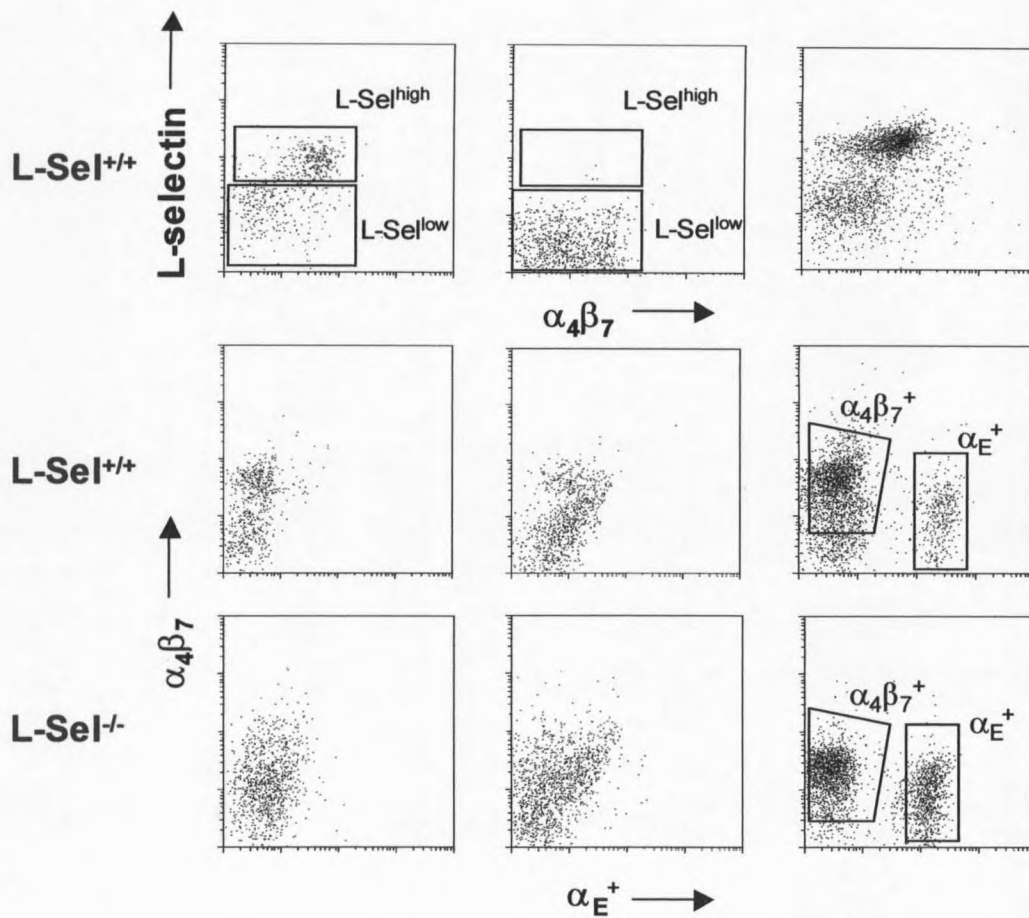


Figure 22: Three distinct lymphocyte populations are observed on lymphocytes in mucosal effector sites 16 days postprimary oral immunization. Lymphocytes were stained with anti L-selectin mAb MEL 14, anti $\alpha_4\beta_7$ mAb DATK 32, anti α_E mAb M290, and anti B220 mAb. Staining of L-selectin vs. $\alpha_4\beta_7$ on L-Sel^{+/+} B lymphocytes (top panels), staining of $\alpha_4\beta_7$ vs. α_E on L-Sel^{+/+} B lymphocytes (middle panels) and L-Sel^{-/-} B lymphocytes (bottom panels) is shown. All three effector tissues contained L-sel^{high}/ $\alpha_4\beta_7^+$ and L-sel^{low}/ $\alpha_4\beta_7^+$ lymphocyte subsets, while only the iLP contained an α_E^+ B lymphocyte population. Sample sort gates are shown.

Sel^{+/+} NP (Fig 23 A and B), though the L-selectin^{high}/α₄β₇⁺ population did provide some CT-B-specific and total IgA AFC in NP. Surprisingly, the L-selectin^{high}/α₄β₇⁺ subset contributed the majority of the total IgA AFC in L-Sel^{+/+} RT (Figure 23 C) $p = 0.03$). In orally CT immunized L-Sel^{-/-} NP, unlike i.n. CT immunized L-Sel^{-/-} NP, we were able to obtain enough lymphocytes for use in cell sorting experiments, indicating that trafficking to the NP was occurring since the number of AFC produced in L-Sel^{+/+} and L-Sel^{-/-} NP and was similar. We were also able to isolate sufficient lymphocytes for homing experiments from L-Sel^{-/-} RT. Since these lymphocytes express low or no L-selectin, we classified the B lymphocytes isolated from L-Sel^{-/-} NP and RT as L-selectin^{low}/α₄β₇⁺ (see Figure 22 G and H for examples of sorted populations). This L-selectin^{low}/α₄β₇⁺ contained IgG and IgA CT-B-specific and total AFC in both the NP and RT. Moreover, AFC responses in this subpopulation in L-Sel^{-/-} NP and RT were not significantly different from those observed in the L-selectin^{low}/α₄β₇⁺ subpopulations of B lymphocytes isolated from L-Sel^{+/+} NP and RT.

Since the iLP contains a population of L-selectin^{low}/α_E⁺ B lymphocytes that is not found in NP or RT, we compared the contribution of this subset with the α₄β₇⁺ subset (Figure 23 E and F). As was observed in nasally immunized mice, we found that the L-selectin^{low}/α_E⁺ subset contributes to CT-B-specific and total IgA AFC, though in the L-Sel^{-/-} iLP the α₄β₇⁺ subset contained a significantly higher number of total IgA SFC ($p = 0.01$). In addition, the L-selectin^{low}/α_E⁺ subset did not contribute at all to the production of total IgG SFC in the iLP, as the α₄β₇⁺ subset contained a significantly higher number of total IgG SFC in L-Sel^{+/+} iLP ($p < 0.1$). These results suggest that there may be

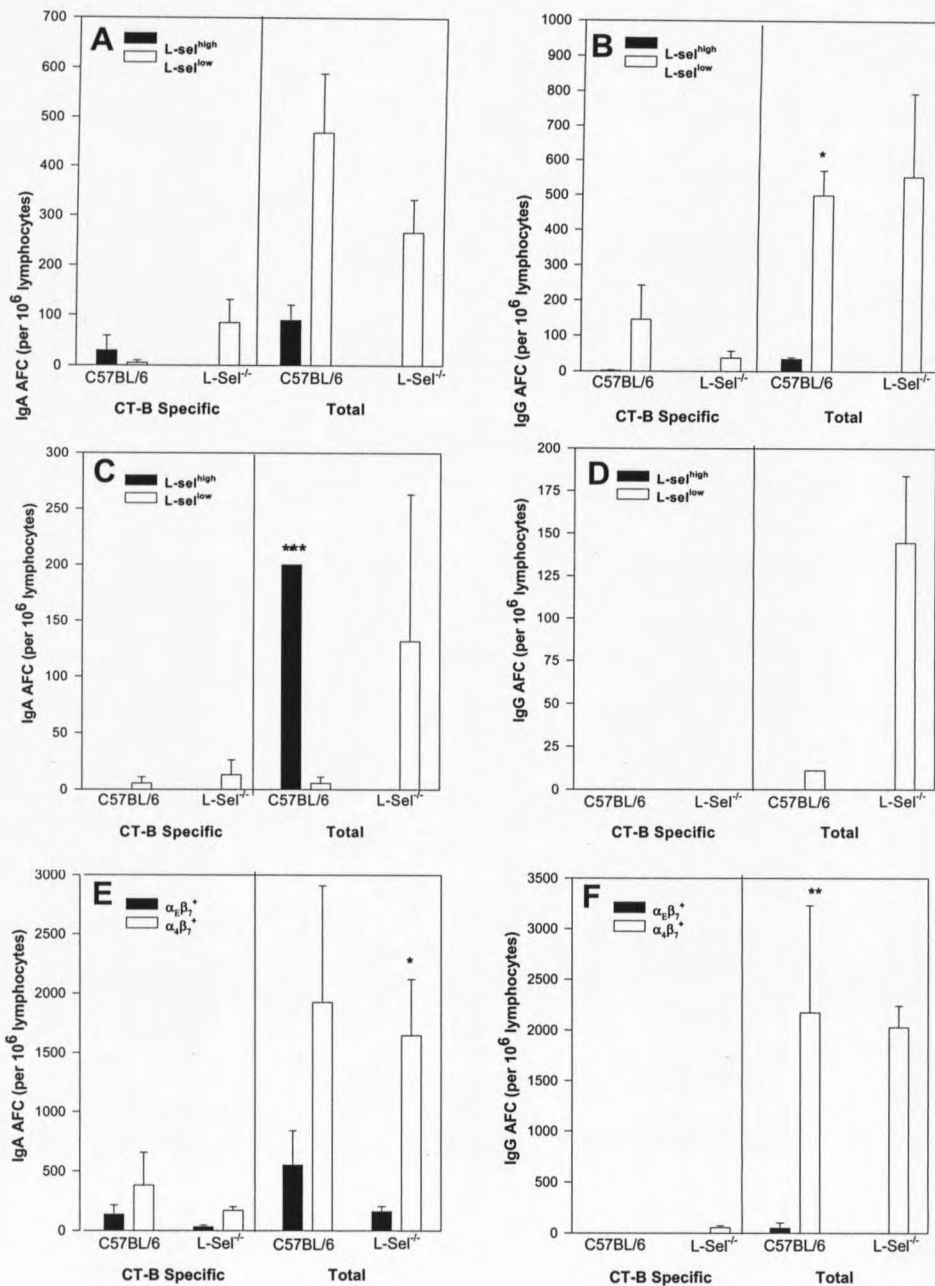


Figure 23: The L-selectin^{low}/ $\alpha_4\beta_7^+$ B lymphocyte subset contains the majority of CT-B-specific and total AFC in orally immunized L-Sel^{+/+} and L-Sel^{-/-} mice. NP IgA (A), IgG (B), RT IgA (C), IgG (D), and iLP IgA (E) and IgG (F) AFC/10⁶ lymphocytes are shown. B220⁺ lymphocytes were gated and L-Sel^{high}/ $\alpha_4\beta_7^+$ (for L-Sel^{+/+}) and L-Sel^{low}/ $\alpha_4\beta_7^+$ (for L-Sel^{+/+} and L-Sel^{-/-}) populations sorted from the NP and RT, while $\alpha_4\beta_7^+$ and α_E^+ populations were sorted from iLP. Results are the mean of four experiments $\pm$ SEM are depicted. * $p \leq 0.01$, ** $p < 0.1$, *** $p = 0.03$.

segregation of B lymphocytes that produce IgA and IgG in the iLP, as the L-selectin^{low}/α_E⁺ subset contained IgA, but not IgG AFC. Furthermore, these results show that AFC found in the α₄β₇⁺ (β₇^{low}) subset after oral immunization are not reduced as they are after nasal immunization with CT. Clearly, though the same subsets of lymphocytes are induced in oral immunization as are induced after i.n. immunization, some mechanism is compensating for the loss of L-selectin in the induction of immune responses in the NP and RT. Therefore, we next investigated Ab responses in the inductive sites for the NP, the NALT and CONALT.

LN cellularity is reduced in L-Sel^{-/-} CONALT

The size and cellularity of L-Sel^{-/-} SMLN, PRLN, and CLN is markedly reduced in normal, non-immune animals. Lymphocyte counts/mouse obtained after dissection of animals for ELISPOT revealed that the number of lymphocytes in orally immunized L-Sel^{-/-} CONALT remains far less than L-Sel^{+/+} CONALT (Table 10). The number of lymphocytes recovered from L-Sel^{-/-} CONALT ranged as low as 3-10% of the number of lymphocytes recovered from L-Sel^{+/+} CONALT. These results indicate that lymphocytes generated from the induction of immune response in the PP are not trafficking to L-Sel^{-/-} CONALT.

AFC are increased in CONALT after CT oral immunization

Since the number of lymphocytes recovered from L-Sel^{-/-} CONALT was severely reduced, we next investigated whether inductive immune responses still exist at these sites, as well as in the NALT and PP. ELISPOT analysis revealed that there was not a

significant difference in the number of CT-B-specific and total IgA and IgG AFC in L-Sel^{-/-} and L-Sel^{+/+} mice. In addition, while high numbers of IgA and IgG AFC were observed in the PP, CT-B-specific AFC in L-Sel^{+/+} and L-Sel^{-/-} NALT were negligible, and only L-Sel^{-/-} NALT contained total IgA or IgG AFC (Figure 24 A and B). These results indicate that while loss of L-selectin does not significantly affect AFC response in PP, responses generated in PP do not efficiently disseminate to the NALT.

Table 10: Number of lymphocytes/mouse obtained from L-Sel^{+/+} and L-Sel^{-/-} CONALT at 16 days post-oral CT immunization

	SMLN	PRLN	CLN
L-Sel ^{+/+}	5.4 x 10 ⁶	2.0 x 10 ⁶	1.6 x 10 ⁶
	6.8 x 10 ⁶	2.9 x 10 ⁶	2.8 x 10 ⁶
	2.9 x 10 ⁶	930,000	1.6 x 10 ⁶
L-Sel ^{-/-}	1.4 x 10 ⁶	106,000	340,000
	1.0 x 10 ⁶	100,000	360,000
	1.0 x 10 ⁶	440,000	480,000

LN were removed from mice, combined in groups of like LN, and disrupted via dounce homogenization. Results were derived as the total number of LN lymphocytes/number of mice/experimental group. Results from three experiments at 16 days post-oral CT immunization are shown.

Our ELISPOT analyses also showed that CT-B-specific AFC were not induced in L-Sel^{+/+} CONALT (Figure 24 C and D). Surprisingly, CT-B-specific AFC were increased 10-fold in L-Sel^{-/-} SMLN ($p < 0.001$); nearly 100-fold in L-Sel^{-/-} PRLN, and 62-fold in L-Sel^{-/-} CLN ($p \leq 0.01$; Figure 24 C). Total IgA AFC were even more strikingly increased as L-Sel^{-/-} SMLN AFC increased 30-fold ($p < 0.001$); PRLN AFC increased 34-fold ($p \leq 0.01$); and CLN AFC increased nearly 400-fold ($p < 0.001$).

While both L-Sel^{+/+} and L-Sel^{-/-} CT-B-specific IgG AFC were limited, L-Sel^{-/-} CLN contained a significantly greater number of CT-B-specific AFC ($p = 0.04$; Figure 24 D). In addition, total IgG AFC were significantly increased 8-fold in L-Sel^{-/-} SMLN ($p < 0.001$); 19-fold in PRLN ($p \leq 0.01$); and 45-fold in CLN ($p < 0.001$). Though lymphocytes are not trafficking to these sites, it is apparent that robust Ab responses are still induced, and loss of L-selectin appears to increase AFC in the CONALT.

Oral CT immunization stimulates unexpected immune responses in L-Sel^{-/-} mice

Our results clearly show that loss of immune responses in the NP and RT at 16 days post-i.n. CT immunization is related to the route of immunization, since oral immunization induces robust intestinal effector responses to CT, but also induces AFC in the non-intestinal NP and RT. Since response to immunization in the iLP appears to rely on $\alpha_4\beta_7$ expression by B and T cells (149-151), we hypothesized that oral immunization would stimulate an $\alpha_4\beta_7$ subset that could traffic to effector immune sites in the absence of L-selectin. However, our flow cytometry analysis revealed that the subpopulations of B lymphocytes induced in orally immunized mice were similar to those observed after i.n. CT immunization. L-selectin^{high}/ $\alpha_4\beta_7^+$ and L-selectin^{low}/ $\alpha_4\beta_7^+$ populations were found in NP, RT, and iLP, while only iLP contained a L-selectin^{low}/ α_E^+ population of B lymphocytes. We also showed a retention of function by these populations. The number of lymphocytes recovered from L-Sel^{-/-} NP and RT after i.n. immunization was very small, indicating that lymphocytes were not trafficking to these sites. In contrast, after oral immunization, comparable numbers of lymphocytes were obtained from L-Sel^{+/+} and

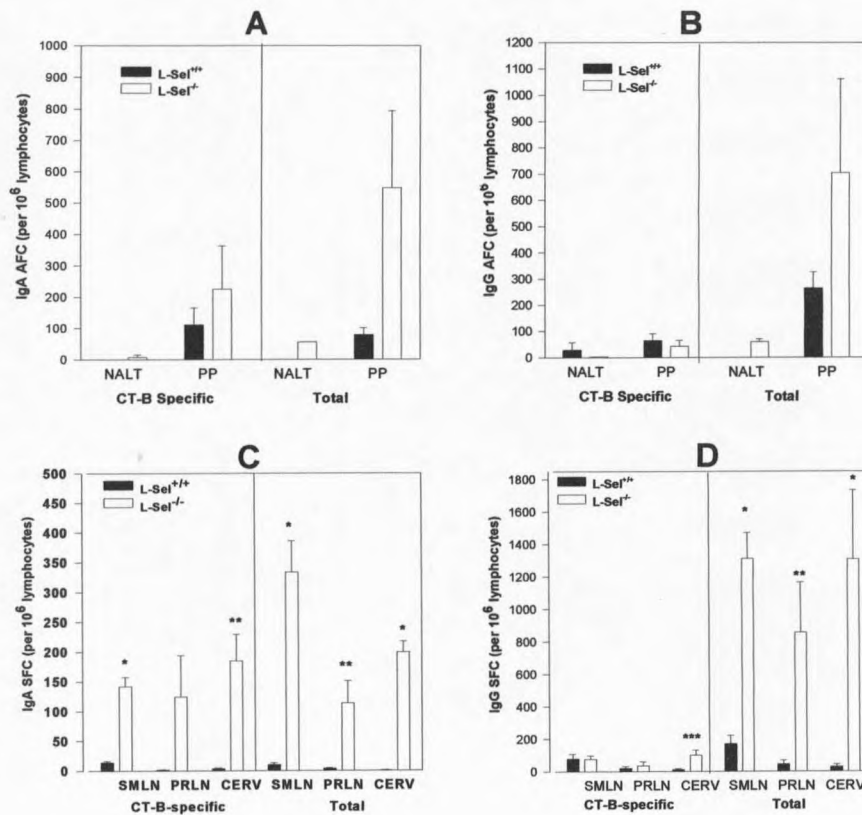


Figure 24: CT-B-specific and total IgA AFC, and total IgG AFC are increased in L-Sel^{-/-} CONALT. B cell ELISPOT assays were performed on NALT and PP (A and B), SMLN, PRLN, and CLN (C and D) isolated from orally CT immunized mice at 16 days post-primary immunization. The mean of three experiments $\pm$ SEM is shown. * $p < 0.001$, ** $p \leq 0.01$, *** $p = 0.04$

L-Sel^{-/-} NP and RT, indicating that lymphocytes are indeed trafficking to these sites. In addition, the same L-selectin^{low}/α₄β₇⁺ lymphocyte population that contained the majority of CT-B-specific and total AFC in nasally immunized mice also provided the majority of AFC in L-Sel^{+/+} and L-Sel^{-/-} orally immunized NP and RT. This population played an important role in the iLP, as well, contributing the greatest number of CT-B-specific and total IgA and IgG AFC in this site.

Some CT-B-specific and total IgA AFC were found in the α_E⁺ iLP L-Sel^{+/+} and L-Sel^{-/-} B lymphocyte population as well, though the α_E⁺ population did not contribute to IgG AFC. This result is intriguing because it suggests a separation of IgA and IgG producing cells according to lymphocyte homing receptor expression (135). α_Eβ₇ is believed to play an important role in lymphocyte retention in the intestine where it binds to E cadherin expressed on the basolateral surface of epithelial cells, and possibly to a unique glycoprotein expressed by intestinal cells (138, 143); therefore, the expression of α_E integrin by IgA plasma cells might provide a mechanism for the selective retention of IgA-secreting cells in the gut, where the majority of immune response is S-IgA.

It is unclear exactly how immune responses are being induced in the non-intestinal mucosal effector sites following oral immunization. It is possible that the α₄β₇ expressed by the effector cells isolated from these sites might mediate homing to NP and RT. However, the same lymphocyte subsets are induced following i.n. immunization, with a resulting severe reduction of AFC in L-Sel^{-/-} NP and RT. It seems likely, then, that lymphocyte stimulation in a different inductive site might account for the restoration of immune response in the orally immunized L-Sel^{-/-} mice. Our results show that while a

specific and total IgA and IgG AFC are induced in PP, L-Sel^{+/+} NALT and CONALT contain few AFC. This suggests that lymphocytes are draining from the PP and eventually migrating to the effector sites directly.

The loss of L-selectin results in a striking increase in the number of CT-B-specific and total IgA, as well as total IgG AFC in CONALT after oral immunization. This result is even more surprising considering the reduced cellularity of L-Sel^{-/-} CONALT. Though lymphocyte trafficking to these sites is markedly reduced, stimulation of resident B cell populations must be occurring. Similar results have been observed in L-Sel^{-/-} PLN after peripheral immunization, including an increase in the number of germinal centers found in L-Sel^{-/-} LN (165). It is unclear, however, exactly how stimulation of resident B cell populations is occurring in the L-Sel^{-/-} CONALT. The nature of the CT antigen, a potent mucosal adjuvant, might allow for the non-specific stimulation of B cells in L-Sel^{-/-} CONALT, though this phenomenon should occur in L-Sel^{+/+} CONALT as well. This suggests that lack of L-selectin may in fact reduce the ability of B lymphocytes to migrate out of the CONALT via the afferent lymphatics. Recent work has demonstrated that this might be the case, as a mannose receptor found lymphatic endothelium appears to bind L-selectin (214); therefore, L-Sel^{-/-} lymphocytes might be unable to traffic from LN via the efferent lymphatics.

Finally, these results indicate important differences in the NALT and PP. Loss of L-selectin might affect the lymphocyte populations trafficking to NALT, resulting in a reduced immune response. In contrast, loss of L-selectin in PP can be compensated by $\alpha_4\beta_7$ binding to MAdCAM-1 (155), therefore more lymphocytes can traffic through this

LN in the absence of L-selectin, potentially inducing the immune response. In conclusion, these results clearly show that the CMIS is divided along "intestinal" vs. "non-intestinal" lines, L-selectin deficiency does not reduce effector immune responses as it does in i.n. immunized mice.

CHAPTER 8

EFFECTOR IMMUNE RESPONSES IN NON-INTESTINAL MUCOSAL EFFECTOR SITES IN L-SELECTIN^{-/-} MICE ARE DELAYED, NOT ELIMINATEDIntroduction

We have previously shown that L-selectin is required to induce immune responses at the non-intestinal effector sites subsequent to i.n. immunization (Chapter 6; 213). This evidence provides support for the hypothesis that CMIS may be compartmentalized. We found that immune responses in the NP and RT were reliant upon a L-selectin^{low}/β7^{low} (α₄β₇⁺) population of B lymphocytes, and that loss of L-selectin reduced the ability of this population to effect immune responses. In contrast, the iLP contains both L-selectin^{low}/β7^{low} (α₄β₇⁺) and L-selectin^{low}/β7^{high} (α_E⁺) B lymphocyte populations, and the α_E⁺ population provided the majority of Ag-specific AFC in iLP, though the L-selectin^{low}/β7^{low} (α₄β₇⁺) population played a role in total AFC production in this site. AFC responses in orally immunized L-Sel^{-/-} mice were not reduced, though B lymphocytes displaying L-selectin^{low}/α₄β₇⁺ and α_E⁺ profiles similar to those identified after i.n. immunization were found (Chapter 7). These studies indicated that iLP is unique from the NP and RT, and that loss of L-selectin abated immune responses at 16 days post i.n. but not oral, immunization. Obviously, the induction of mucosal immunity after oral immunization does not appear to rely on L-selectin, as it does after nasal immunization. It is not clear why this is the case, since no unique subsets of lymphocyte homing receptors are induced after oral immunization. Therefore, it is possible that the

loss of non-intestinal immune responses in i.n. immunized L-Sel^{-/-} mice might be the result of a delay in the induction of Ab responses in the inductive sites or a delay in release of lymphocytes to effector sites.

Loss of T cell responses at peripheral sites in L-Sel^{-/-} mice has been well documented (157, 159, 164), but humoral immune responses in L-Sel^{-/-} mice are less well understood. Enhanced humoral IgG and IgM responses were observed in L-Sel^{-/-} mice following i.p. immunization. In contrast, L-Sel^{-/-} mice immunized subcutaneously showed reduced IgG and IgM humoral responses to a T cell dependent Ag at day 7 post-immunization; however, these responses were restored by day 14 post-immunization. In addition, secondary memory responses to Ag in L-Sel^{-/-} mice were greater than those observed in L-Sel^{+/+} mice (165). These results suggest that B lymphocyte responses after peripheral, but not mucosal (i.p.) immunization might be delayed, but not eliminated in L-Sel^{-/-} mice. It is unknown if this trend will be maintained in mucosal inductive and effector sites.

In the following studies we determined the effect of loss of L-selectin on inductive immune responses in the NALT and CONALT at 16 days post-primary immunization and found that L-Sel^{-/-} mice did not show a decrease in AFC in these sites, indicating that reduced immune responses in L-Sel^{-/-} NP and RT were not the result of a loss of inductive site immunity. We next investigated immune responses in effector sites at 6 weeks post-primary immunization, and found that effector immune responses in L-Sel^{-/-} NP and RT were restored by this time point. However, the restoration of AFC in these sites was not the result of increased $\alpha_4\beta_7$ or $\alpha_E\beta_7$ expression by these lymphocytes.

Finally, these studies showed a striking increase in CT-B-specific and total AFC in L-Sel^{-/-} CONALT, indicating that loss of L-selectin affects the production of Ab in some inductive sites. These results provide important insight into the role of L-selectin over long term Ab response in both inductive and effector sites.

Results and Discussion

Loss of effector response in i.n. immunized L-Sel^{-/-} mice is not due to loss of inductive site Ab production

Our previous work (213) has shown that NP and RT IgA and IgG AFC were severely compromised in L-Sel^{-/-} mice following i.n. CT immunization. To further elucidate the mechanism for this loss of Ab production, we investigated the immune response of L-Sel^{-/-} and L-Sel^{+/+} NALT and PP, inductive sites for NP and iLP, respectively. We found that at 16 days post-CT immunization, there was no significant difference in NALT response between L-Sel^{+/+} and L-Sel^{-/-} mice (Figure 25). Although the presence of total IgG and IgA AFC was decreased in L-Sel^{-/-} NALT, these differences were not statistically significant. The number of CT-B-specific AFC found in PP was low, yet increases in total IgA and IgG AFC were observed in PP after i.n. immunization. These data indicate that loss of effector site immunity in L-Sel^{-/-} mice after i.n. immunization must be caused by a deficiency in lymphocyte homing after activated B cells drain from the NALT.

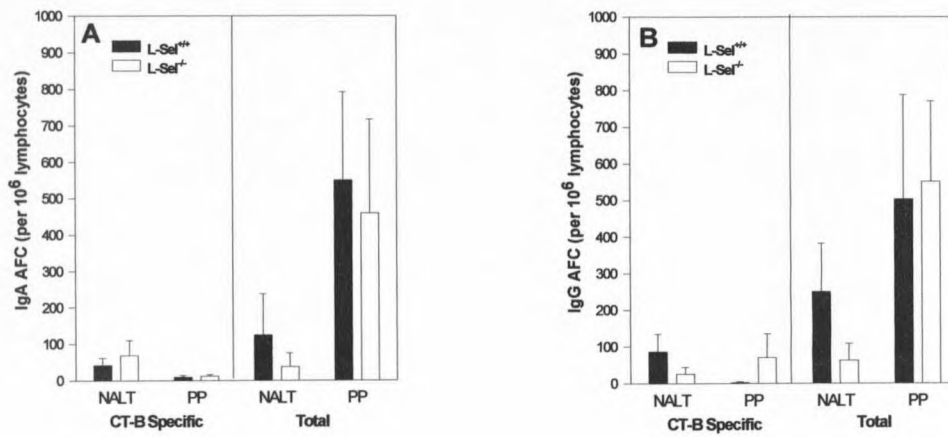


Figure 25. Inductive immune responses are found in L-Sel^{-/-} NALT and PP 16 days post-primary i.n CT. immunization. CT-B-specific and total IgA (A) and IgG (B) AFC were determined by ELISPOT. Results are the mean of three experiments $\pm$ SEM.

Total IgA response is increased in L-Sel^{-/-} CONALT in i.n. immunized mice

We next investigated AFC production in the LN that drain the NALT and NP, the CONALT. Enumeration of the number of lymphocytes obtained from L-Sel^{-/-} CONALT indicated that these LN were much smaller than L-Sel^{+/+} CONALT. In fact, the number of lymphocytes/LN/mouse in L-Sel^{-/-} mice averaged from 10-25% of the lymphocytes/LN/mouse obtained from L-Sel^{+/+} mice (Table 11). These results indicated that L-selectin deficiency appears to reduce the number of lymphocytes found in the CONALT, and may account for the reduction in immune response in the NP and RT.

Table 11. Number of lymphocytes/mouse obtained from L-Sel^{+/+} and L-Sel^{-/-} CONALT at 16 days post-i.n. CT immunization^a

	SMLN	PRLN	CLN
L-Sel ^{+/+}	17 x 10 ⁶ 17.3 x 10 ⁶ 18.8 x 10 ⁶	2.3 x 10 ⁶ 3.1 x 10 ⁶ 2.0 x 10 ⁶	3.5 x 10 ⁶ 6.3 x 10 ⁶ 4.8 x 10 ⁶
L-Sel ^{-/-}	840,000 2.3 x 10 ⁶ 625,000	1.2 x 10 ⁶ 33,000 162,500	1 x 10 ⁶ 560,000 950,000

^aLN were removed from mice combined in groups of like LN, and disrupted via dounce homogenization. Results were derived as the total number of node lymphocytes/number of mice per group. Results from 3 experiments at 16 days post i.n. CT immunization are shown.

Surprisingly, reduction in the total number of lymphocytes present in L-Sel^{-/-} CONALT did not correlate with a reduction in Ag-specific and total AFC responses. We observed no significant decrease in the number of AFC produced in L-Sel^{-/-} CONALT vs.

L-Sel^{+/+} CONALT (Figure 26). In fact, there was a significant increase in the number of total IgA AFC in the L-Sel^{-/-} CONALT. L-Sel^{-/-} SMLN produced an average of 3.5X more AFC than L-Sel^{+/+} SMLN ($p < 0.01$), while L-Sel^{-/-} CLN produced 2.4 X more IgA AFC than did L-Sel^{+/+} CLN ($p < 0.01$). The most striking difference in the L-Sel^{-/-} mice was observed in the PRLN. In L-Sel^{+/+} mice this LN produced very little IgA response following i.n. immunization (~ 42 AFC/ 10^6 lymphocytes), while in L-Sel^{-/-} mice this number was increased by nearly 13-fold (536 AFC/ 10^6 lymphocytes; $p < 0.001$). In addition, CT-B-specific response was increased in L-Sel^{-/-} PRLN as well (140 AFC/ 10^6 lymphocytes; $p \leq 0.02$).

Since CONALT express varying levels of MAdCAM-1 (84), it is possible that expression of $\alpha_4\beta_7$ and subsequent binding to MAdCAM-1 might have compensated for the loss of L-selectin in these LN, we next studied the expression of $\alpha_4\beta_7$ and L-selectin on L-Sel^{+/+} and L-Sel^{-/-} NALT and CONALT. Our results showed that L-Sel^{-/-} mice did not display increased expression of $\alpha_4\beta_7$ when compared with L-Sel^{+/+} mice (Figure 27). In addition, nearly half of the B220⁺ lymphocytes in L-Sel^{+/+} SMLN, PRLN, and CLN expressed L-selectin, suggesting a role for this homing receptor in the induction of immune responses in these LN. However, the increased IgA responses in L-Sel^{-/-} CONALT refutes this hypothesis.

Immune responses are restored in L-Sel^{-/-} NP and RT by 6 weeks post-immunization

Kinetic studies of fecal, vaginal, and serum Ab responses revealed that CT-B-specific IgG and IgA responses may be reduced in L-Sel^{-/-} RT, but can be restored by day

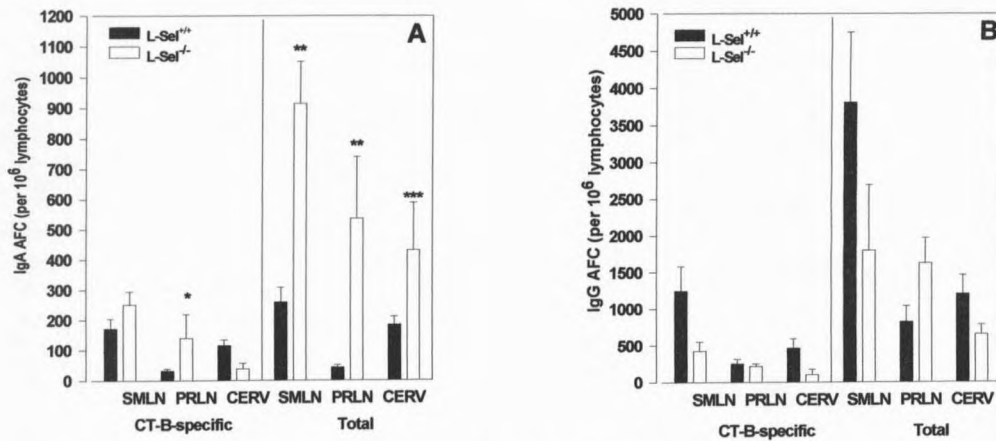


Figure 26. Total IgA AFC are increased in L-Sel^{-/-} CONALT at 16 days post i.n. CT immunization. CT-B-specific and total IgA (A) and IgG (B) AFC were determined by ELISPOT. Results are the mean of three experiments $\pm$ SEM. * $p \leq 0.02$, ** $p < 0.001$ *** $p < 0.01$

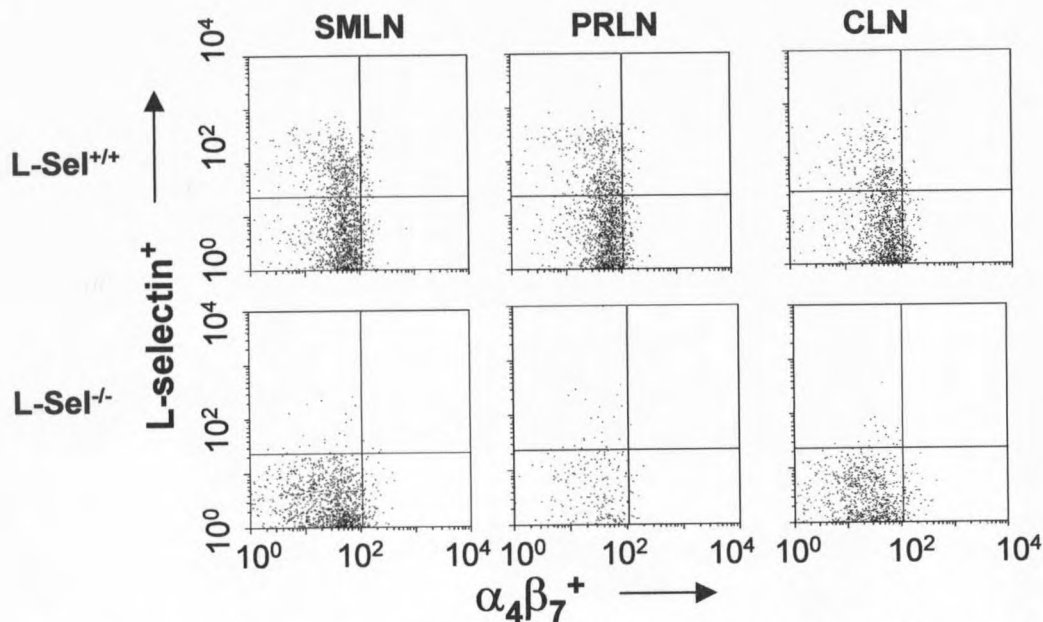


Figure 27. $\alpha_4\beta_7$ expression is not increased on L-Sel^{-/-} CONALT 16 days post CT i.n. immunization. Lymphocytes were stained with anti L-selectin mAb MEL 14, anti $\alpha_4\beta_7$ mAb DATK 32, and anti-B220 mAb. L-selectin and $\alpha_4\beta_7$ staining profiles are shown for B220⁺ lymphocytes. Results are representative of three experiments.

21 post immunization (Figure 28). In both L-Sel^{+/+} and L-Sel^{-/-} mice, serum IgG and IgA Ab responses peak by day 21 post-immunization, and then decline (Figure 28 A and B). The same response is observed in RT, where L-Sel^{-/-} and L-Sel^{+/+} responses peak first at day 21 and 25 post immunization, and then decline. However, L-Sel^{-/-} IgA response in RT surpasses L-Sel^{+/+} RT IgA response at approximately day 30 post-immunization, and continues to remain more robust as far as 6 weeks post immunization (Figure 28 C). Finally, while fecal IgA responses are similar until day 26 post immunization, the L-Sel^{-/-} response is severely reduced by day 30 post-immunization (Figure 28 D).

ELISPOT experiments revealed that by 6 weeks post immunization immune responses in the NP and RT have been restored (Figure 29 A). In addition, total IgG and IgA responses in L-Sel^{-/-} RT are not significantly different from those observed in L-Sel^{+/+} RT, though the number of Ag-specific AFC is negligible in these tissues. There was no significant difference in the number of Ag-specific or total IgA and IgG AFC between L-Sel^{+/+} and L-Sel^{-/-} mice. However, by 6 weeks post-immunization, iLP CT-B-specific responses in both L-Sel^{+/+} and L-Sel^{-/-} mice were reduced to < 200 AFC/10⁶ lymphocytes. These data indicate that a lack of L-selectin does not permanently affect the effector cell response to i.n. immunization in the NP and RT. Furthermore, L-selectin deficiency may actually enhance effector immune responses in the NP at 6 weeks post immunization.

L-selectin^{low} and $\alpha_4\beta_7$ ^{low} lymphocytes are present in effector sites at 6 weeks post immunization

In order to determine if homing receptor profiles on effector lymphocytes change by 6 weeks post immunization, we performed flow cytometry analysis on L-Sel^{+/+} and L-

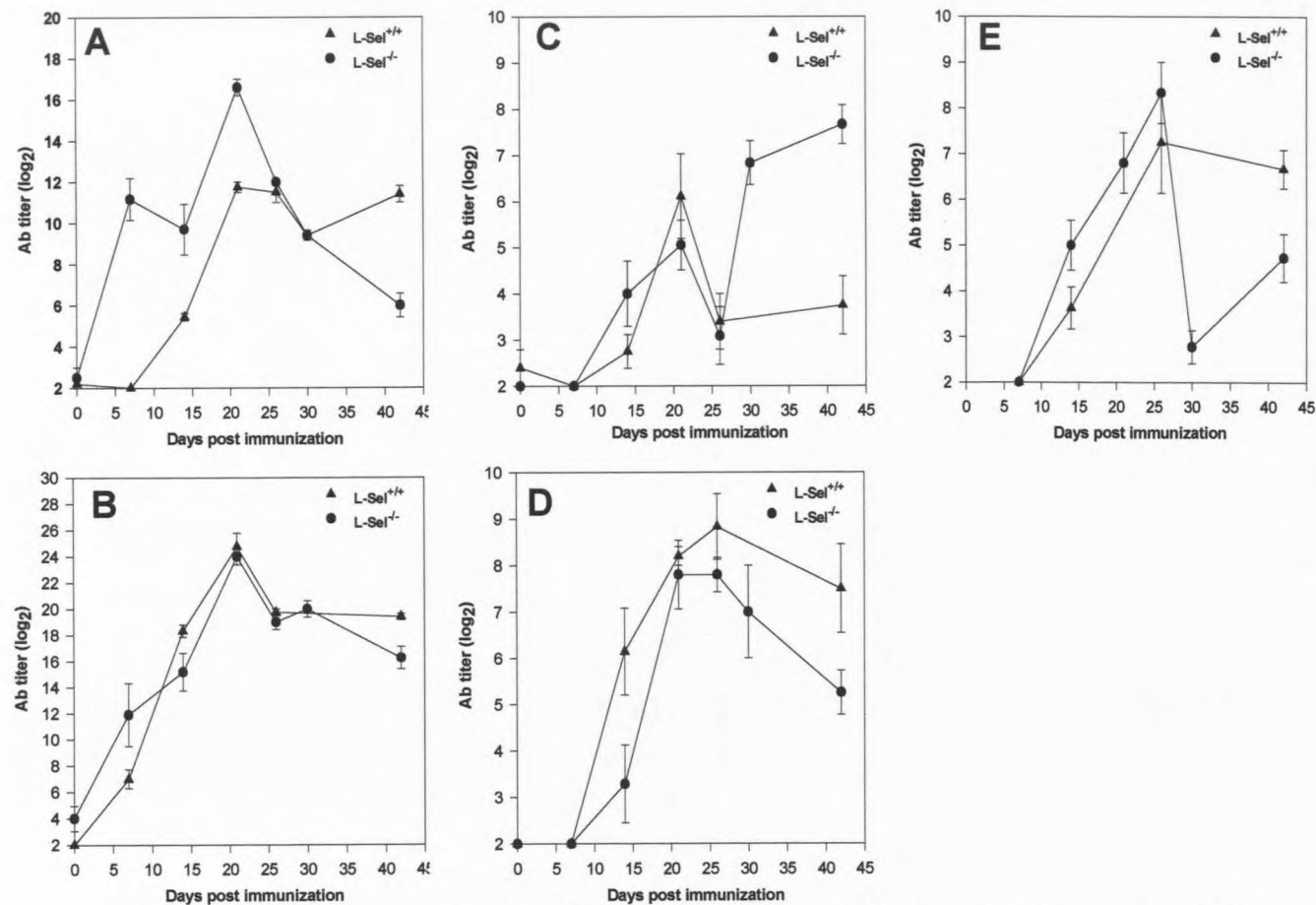


Figure 28. Ab responses in L-Sel^{+/+} and L-Sel^{-/-} mice as determined by ELISA from days 0-42 post i.n. CT immunization. Serum IgA (A) and IgG (B), vaginal IgA (C) and IgG (D), and fecal IgA (E) are shown

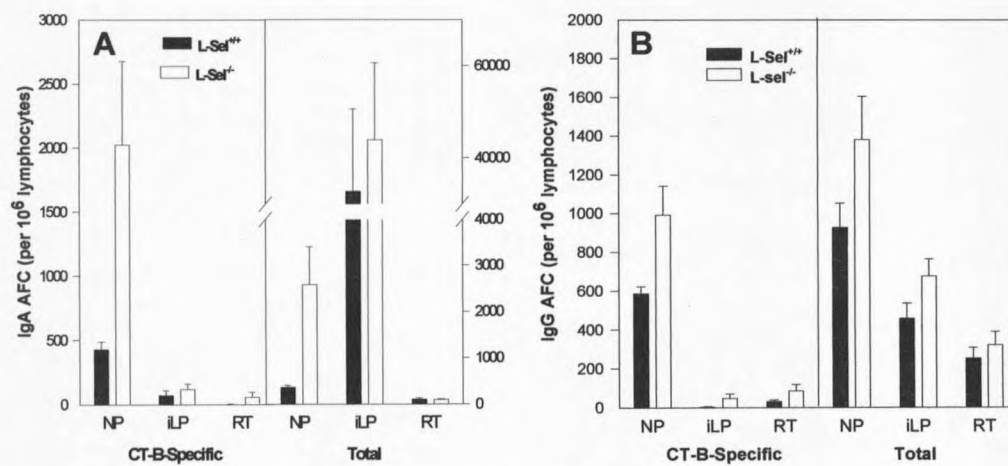


Figure 29. AFC responses are restored in $L\text{-Sel}^{-/-}$ NP and RT by 6 weeks post i.n. CT immunization. CT-B-specific and total IgA (A) and IgG (B) AFC were determined by ELISPOT. Results are the mean of three experiments $\pm$ SEM.

Sel^{-/-} NP, RT, and iLP. In RT and iLP, we found that the majority of B220⁺ lymphocytes are L-selectin^{low} and $\alpha_4\beta_7$ ^{low}, which is the same homing receptor phenotype expressed by effector lymphocytes at day 16 post immunization (Figure 30 A). However, in L-Sel^{+/+} NP, approximately 21% of B lymphocytes appear L-selectin^{high}. In addition, we found two populations of $\alpha_4\beta_7$ ^{low} B220⁺ lymphocytes in the effector tissues. In the LP, the majority of B lymphocytes expressed $\alpha_4\beta_7$ at levels approximating those observed in 16 dpi immunized effector sites (Chapter 6, Figure 19). However, in the NP approximately 50% of B lymphocytes expressed a lower level of $\alpha_4\beta_7$. In the RT, this population was increased to 68% of B lymphocytes. Similar populations were observed in L-Sel^{-/-} mice as well (Figure 30 B). These results indicate that although CT-specific and total immune responses are restored in L-Sel^{-/-} mice 6 weeks after immunization, this change is not due to increased expression of the mucosal homing receptors $\alpha_4\beta_7$.

In 16 day i.n. immunized NALT, iLP contains a unique α_E ⁺ B220⁺ population that is not found in RT or NP. In 6 week i.n. immunized mice, this population is still present, however, in L-Sel^{-/-} mice the percentage of B lymphocytes expressing α_E is decreased to approximately 6% (Figure 30 D), whereas in L-Sel^{-/-} mice at 16 dpi this population consists of 23% of the total B220⁺ cells (Chapter 6; Figure 19). In contrast, 25% of L-Sel^{+/+} iLP B lymphocytes expressed α_E (Figure 30 C), compared to 13% at 16 days post immunization. In addition, most α_E ⁺ lymphocytes expressed a lower level of $\alpha_4\beta_7$ than α_E ⁻ lymphocytes. Finally no α_E ⁺ lymphocytes were observed in L-Sel^{+/+} and L-Sel^{-/-} NP and RT, though $\alpha_E\beta_7$ expression was increased slightly in these tissues (α_E ^{low}). In addition, as is most obviously seen in the NP, the $\alpha_E\beta_7$ ^{low} population also expressed a

slightly lower level of $\alpha_4\beta_7$. These results indicate that restoration of the immune response in L-Sel^{-/-} NP and RT at 6 weeks post immunization is not the result of an up-regulation of $\alpha_4\beta_7$ or $\alpha_E\beta_7$ in these tissues. In fact, the majority of the B220⁺ lymphocytes in NP and RT maintain an L-selectin^{low}/ $\alpha_4\beta_7$ ⁺/ α_E ⁻ phenotype similar to that observed in 16 d.p.i. immunized mice, while iLP contains L-selectin^{low}/ $\alpha_4\beta_7$ ⁺/ α_E ⁻ and L-selectin^{low}/ $\alpha_4\beta_7$ ^{low}/ α_E ⁺ lymphocyte populations.

Together, these results suggest that although L-Sel^{-/-} NP and RT effector Ab responses are restored in 6 week immunized mice, this response is not due to an increase in $\alpha_4\beta_7$ or $\alpha_E\beta_7$ expression by B lymphocytes in these tissues. Though L-selectin does not appear to be necessary for the induction of immunity at 6 weeks post-immunization, it is possible that compensatory homing by $\alpha_4\beta_7$ interactions might be responsible for the restoration of immune response in the non-intestinal effector sites at this time point. In order to more fully understand the mechanisms that allow for the recovery of Ag specific immune response in L-Sel^{-/-} effector sites, we next investigated Ab responses in the inductive NALT and CONALT.

CT-B-specific and total immune responses are increased in L-Sel^{-/-}CONALT

Investigation of responses in inductive sites at 6 week post immunization revealed that CT-B-specific, as total IgG and IgA responses were maintained in both L-Sel^{+/+} and L-Sel^{-/-} NALT (Figure 31 A and B). An increase in the number of AFC produced in these tissues was also observed when compared to 16 day immunized NALT. In contrast, CT-B-specific AFC were absent from PP at this time point, though strong total IgA and

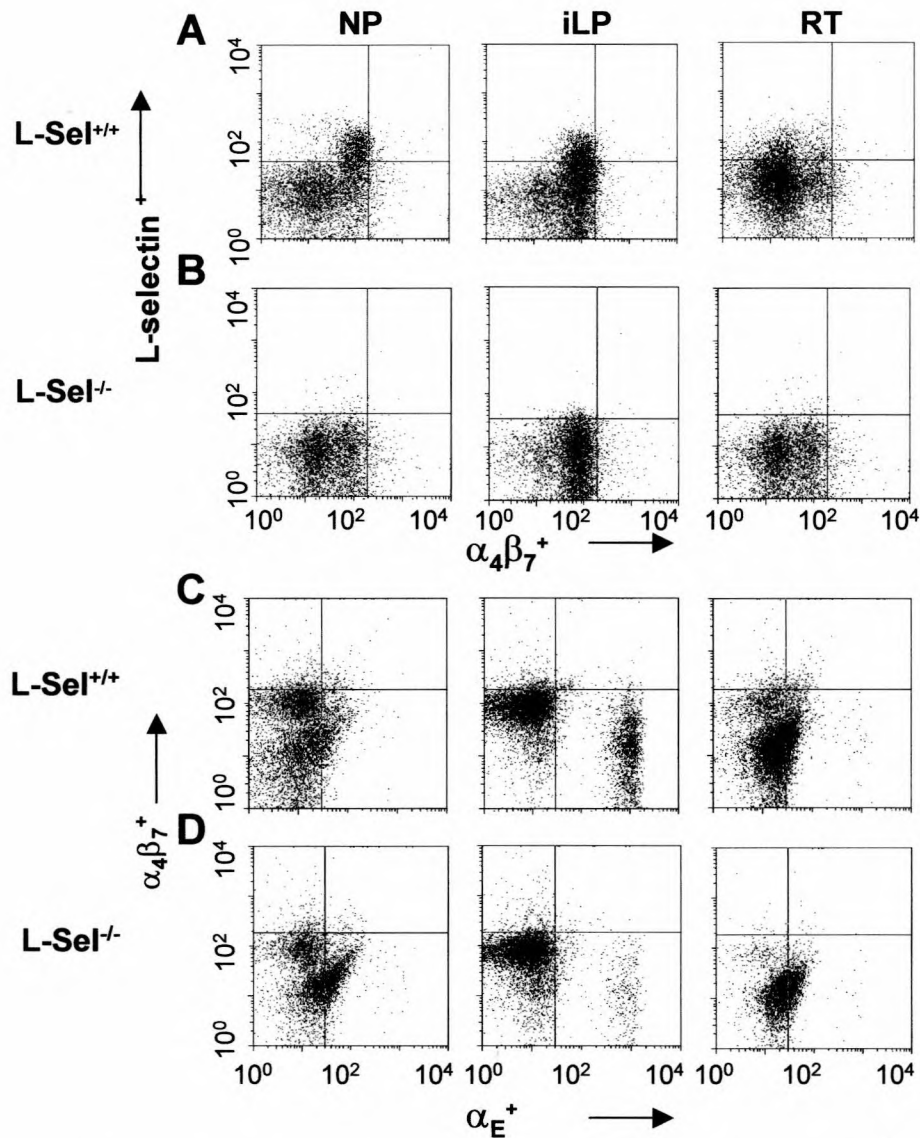


Figure 30. $\alpha_4\beta_7^+$, but not $\alpha_4\beta_7^{\text{high}}$ expression is observed in L-Sel^{+/+} and L-Sel^{-/-} NP, iLP, and RT 6 weeks post i.n. CT immunization. Lymphocytes were treated with anti-L-selectin mAb MEL 14, anti- $\alpha_4\beta_7$ mAb DATK 32, anti- α_E mAb M290, and anti-B220 mAb. Staining profiles of L-selectin vs. $\alpha_4\beta_7$ are shown for L-Sel^{+/+} (A) and L-Sel^{-/-} B lymphocytes (B). $\alpha_4\beta_7$ vs. α_E staining profiles are shown for L-Sel^{+/+} (C) and L-Sel^{-/-} B lymphocytes (D). No significant changes in staining profiles are observed between L-Sel^{+/+} and L-Sel^{-/-} effector tissues. Results are representative of three experiments.

IgG AFC responses still remained in both L-Sel^{+/+} and L-Sel^{-/-} PP.

We have previously shown that L-selectin deficiency does not affect immune responses in CONALT at 16 days post i.n. immunization, and may in fact increase total IgA response in these tissues (Figure 31 C and D). In order to determine if the CT-B-specific Ab responses in CONALT at 16 d.p.i. were maintained until 6 weeks post immunization, we performed B cell ELISPOT analysis on CONALT at this time point. Our results showed that increased total IgA responses in CONALT were maintained in 6-week immunized animals. L-Sel^{-/-} SMLN contained 8.2X more total IgA AFC than L-Sel^{+/+}, while L-Sel^{-/-} SMLN and CLN contained 6.7X more AFC than L-Sel^{+/+} CLN. Again, the most striking difference between IgA AFC in L-Sel^{+/+} and L-Sel^{-/-} mice was observed in the PRLN, where L-Sel^{-/-} AFC responses were 41X greater than L-Sel^{+/+} AFC responses (average number of AFC of 19 vs. 782; $p < 0.01$). In addition, CT-B-specific IgA responses were increased in L-Sel^{-/-} CONALT as well, with AFC responses of 13.7, 41, and 4 times greater than L-Sel^{+/+} SMLN, PRLN, and CLN, respectively. Studies of IgG AFC responses in L-Sel^{-/-} CONALT also revealed a significant increase in CT-B-specific and total response in SMLN and PRLN. Again, response in PRLN showed the greatest change in L-Sel^{-/-} mice, with a 40-fold increase in CT-B-specific and 10-fold increase in total IgG response ($p < 0.001$); while total IgG response in SMLN increased 3-fold, ($p < 0.01$). However, a significant increase in the number of CT-B-specific or total IgG in the CLN was not observed. Finally, the magnitude of all CT-B-specific and total AFC responses in CONALT was as great or greater than those observed at 16 days post primary immunization, indicating that Ab responses in L-Sel^{-/-} CONALT

are long-lived.

The enhanced immune response in L-Sel^{-/-} CONALT did not appear to be the result of an influx of lymphocytes to these tissues at 6 weeks post-immunization. As was noted at 16 days post-immunization, L-Sel^{-/-} CONALT were much smaller, and contained far fewer lymphocytes than did L-Sel^{+/+} CONALT, however, the number of lymphocytes recovered both L-Sel^{+/+} and L-Sel^{-/-} CONALT was less than the number recovered at 16 days post immunization. These results indicated that trafficking to these LN, but not response to CT, might indeed be disrupted by the loss of L-selectin

An $\alpha_E\beta_7$ lymphocyte population may provide increased AFC in L-Sel^{-/-} CONALT

In order to determine if the significant increase in CONALT AFC production in L-Sel^{-/-} CONALT was due to unique populations of B lymphocytes, we performed a FACS analysis for α_E , $\alpha_4\beta_7$, and L-selectin expression on CONALT lymphocytes. Our results showed that at 6 weeks post immunization, levels of L-selectin on SMLN, PRLN, and CLN were similar to those observed on lymphocytes from these LN at 16 days post immunization, that is, nearly 50% of B lymphocytes expressed L-selectin (Figure 32 A). Expression of $\alpha_4\beta_7$ on lymphocytes isolated from 6 week immunized L-Sel^{+/+} CONALT revealed no change in expression from L-Sel^{+/+} CONALT at 16 days post-immunization (mean fluorescence ~ 100). However, some lymphocytes isolated from L-Sel^{-/-} CONALT at 6 weeks post immunization appeared to express lower levels of $\alpha_4\beta_7$, similar to the results observed in effector sites at this time point. In addition, very few B220⁺ lymphocytes were recovered from L-Sel^{-/-} CLN, possibly indicating that the majority of lymphocytes in L-Sel^{-/-} CLN at this time point are CD4⁺CD8⁺ T cells (Pascual lab,

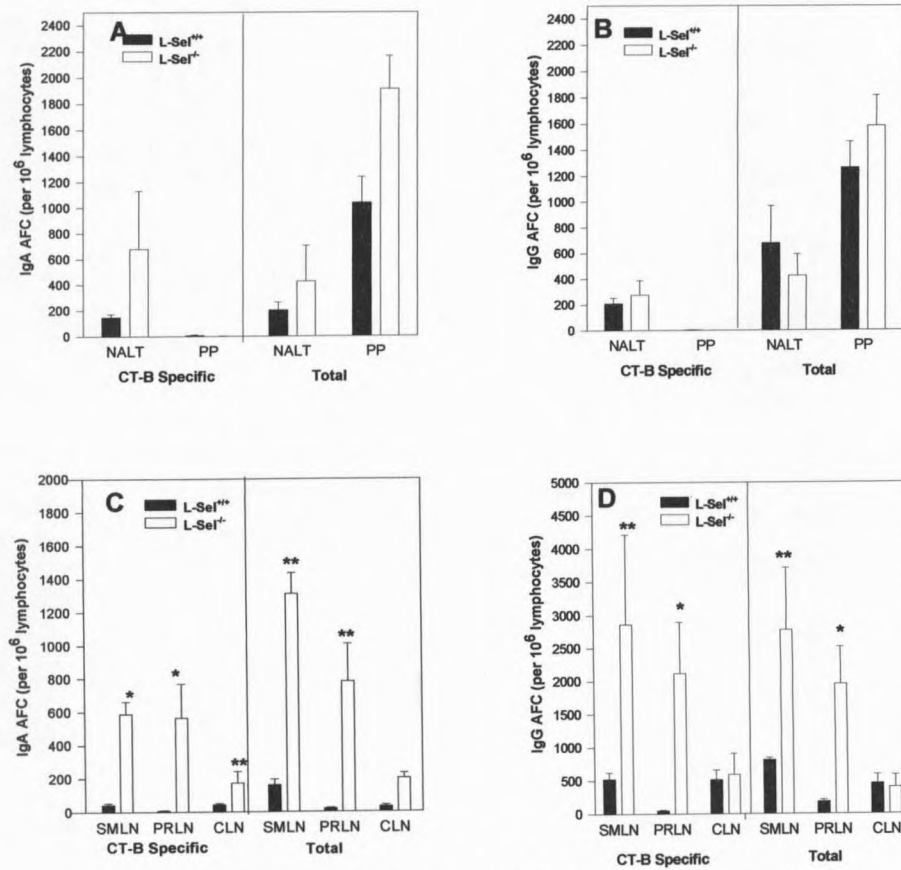


Figure 31. CT-B-specific and total AFC are increased in 6 week i.n. CT immunized L-Sel^{-/-} CONALT. ELISPOT analysis was performed to determine CT-B-specific IgA (A) and IgG (B) in L-Sel^{+/+} and L-Sel^{-/-} NALT and PP, and CT-B-specific IgA (C) and IgG (D) in L-Sel^{+/+} and L-Sel^{-/-} CONALT. Results are the mean of three experiments $\pm$ SEM. * $p < 0.001$, ** $p \leq 0.01$.

Table 12. Number of lymphocytes/mouse obtained from L-Sel^{+/+} and L-Sel^{-/-} CONALT at 6 weeks post-i.n. CT immunization^a

	SMLN	PRLN	CLN
L-Sel ^{+/+}	7.4 x 10 ⁶	2.0 x 10 ⁶	1 x 10 ⁶
	6.4 x 10 ⁶	960,000	386,000
	9 x 10 ⁶	3.2 x 10 ⁶	3.4 x 10 ⁶
L-Sel ^{-/-}	780,000	90,000	180,000
	700,000	76,000	124,000
	340,000	280,000	40,000

^aLN were removed from mice combined in groups of like LN, and disrupted via dounce homogenization. Results were derived as the total number of node lymphocytes/number of mice per group. Results from 3 experiments at 6 weeks post i.n. CT immunization are shown.

unpublished observations). Finally, observation of the expression of $\alpha_E\beta_7$ integrin on L-Sel^{+/+} and L-Sel^{-/-} CONALT revealed that 7% of SMLN B lymphocytes, 15% of PRLN B lymphocytes, and 7% of CLN B lymphocytes expressed α_E in L-Sel^{-/-} mice (Figure 32 B). In contrast, 5% or fewer CONALT B lymphocytes from L-Sel^{+/+} mice at the same time point expressed α_E .

Together, these results indicate that although the number of lymphocytes recovered from L-Sel^{-/-} CONALT is still less than lymphocytes recovered from CONALT of L-Sel^{+/+} mice, these mice are capable of mounting an enhanced, sustained antibody response subsequent i.n. immunization. This response is not reliant upon L-selectin expression, though 50% of lymphocytes in L-Sel^{+/+} CONALT express this homing receptor. In addition, L-Sel^{-/-} CONALT B lymphocytes might actually lose some expression of $\alpha_4\beta_7$, but increase expression of α_E . Clearly, immune response in CONALT is not dependant upon L-selectin interactions, and lack of L-selectin may actually enhance antibody response in these tissues.

Our results have clearly shown that loss of NP and RT immunity in L-Sel^{-/-} mice at 16 days post-i.n. CT immunization is the result of a delay of Ab response in these sites, rather than the complete abatement of AFC. Kinetic studies indicate that Ab responses are restored in L-Sel^{-/-} NP and RT by around 21-26 days post immunization, and that these responses may even be increased in L-Sel^{-/-} mice.

Our previous studies showed that Ag-specific responses in L-Sel^{-/-} NP and RT were significantly reduced, while responses in L-Sel^{-/-} iLP were not significantly different from those in L-Sel^{+/+} LP. In order to determine if loss of response in the non-intestinal

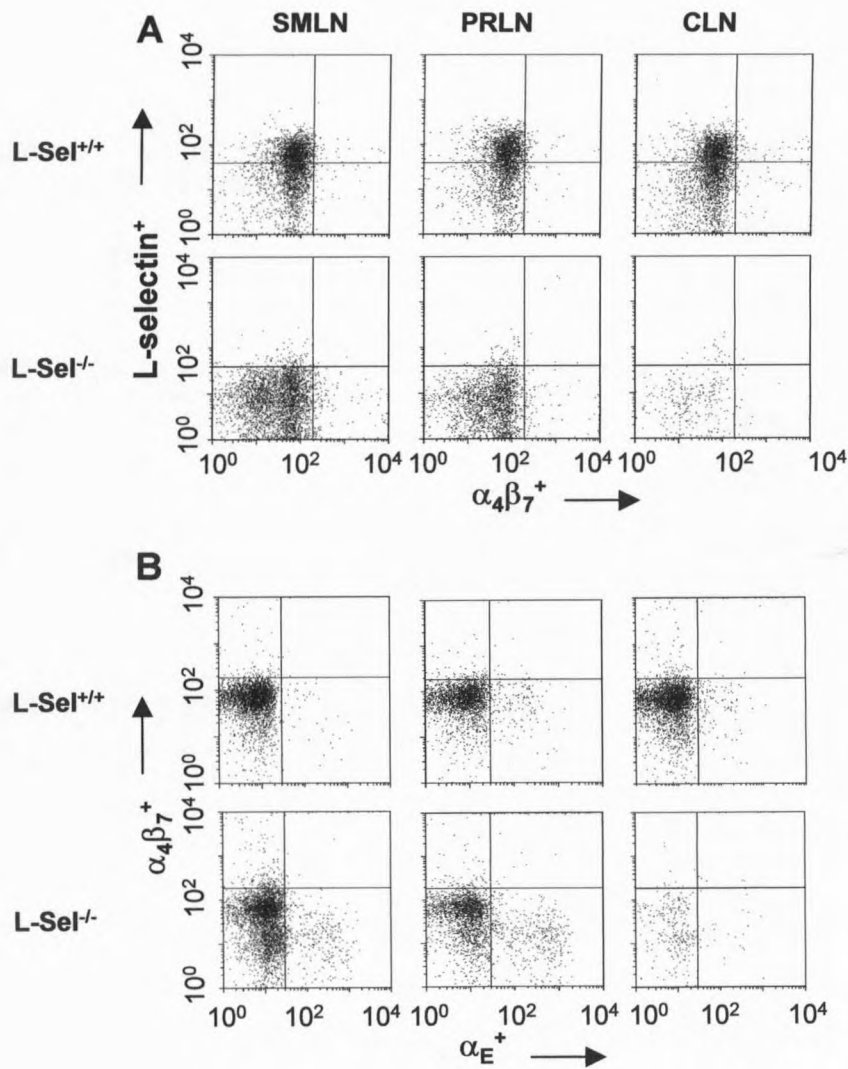


Figure 32. $\alpha_4\beta_7$ expression is not increased, but $\alpha_E\beta_7$ is increased on L-Sel^{-/-} CONALT 6 weeks days post CT i.n. immunization. Lymphocytes were stained with anti L-selectin mAb MEL 14, anti $\alpha_4\beta_7$ mAb DATK 32, and anti-B220 mAb. L-selectin vs. $\alpha_4\beta_7$ staining profiles (A) and α_E vs. $\alpha_4\beta_7$ staining profiles (B) are shown for B220⁺ L-Sel^{+/+} and L-Sel^{-/-} lymphocytes. Results are representative of three experiments.

effector immune tissues was due to a lack of inductive site immunity we performed B cell ELISPOT analyses on L-Sel^{+/+} and L-Sel^{-/-} NALT lymphocytes, and compared these responses to those found in PP. Our results showed that L-selectin deficiency has no effect on AFC response in NALT, or in the PP. Though L-selectin deficiency has little effect on lymphocyte trafficking to PP (155) maintenance of Ab response in L-Sel^{-/-} NALT is surprising, given that the majority of lymphocyte binding to the naive NALT is mediated by L-selectin-PNAd interactions (189). However, we have also shown that CT immunization results in an increase in expression of functional MAdCAM-1 in NALT (Chapter 5), therefore it is possible that MAdCAM-1- $\alpha_4\beta_7$ interactions might compensate for the loss of L-selectin in immunized NALT. In addition, since immunized L-Sel^{-/-} NALT remain smaller in size than immunized L-Sel^{+/+} NALT, it is possible that the immune response in this site might be the result of stimulation of existing lymphocyte populations.

In order to further elucidate the inductive immune response in i.n. immunized L-Sel^{-/-} mice we also observed AFC response in the draining CONALT. As was observed in NALT, loss of L-selectin had no effect on induction of immunity in these sites. In fact, total IgA response was increased in L-Sel^{-/-} CONALT. Again, these results were surprising, given that the majority of lymphocyte binding to these LN is mediated through L-selectin-PNAd interactions as well (84). Indeed, cell numbers were significantly reduced in CONALT at 16 days post primary immunization, indicating a loss of lymphocyte trafficking to these sites. Again, it is possible that MAdCAM-1 might be up-regulated in these LN following CT immunization. Also, CT draining to these sites

might stimulate resident lymphocyte populations.

More importantly, these AFC responses indicate a role for the PRLN in immune response to i.n. introduced antigens. This lymph node has the "most peripheral" phenotype of the CONALT, (Chapter 4; 84) and has previously been shown to produce immune response to Ags that drain from the skin of the head and neck, rather than from the nasal mucosa (60, 78). However, these results indicate a role for this tissue in immune response to i.n. immunization, especially in L-selectin deficient mice, indicating possible draining of the nasal mucosa, or selective trafficking of Ag-specific B lymphocytes.

Our previous studies (Chapter 6; 213) showed that L-Sel^{-/-} NP and RT AFC were negligible in 16 day post i.n. immune mice, and that immune response in these sites in L-Sel^{+/+} and L-Sel^{-/-} mice was primarily contained in a L-selectin^{low}/α₄β₇⁺ population of B lymphocytes. In contrast, AFC in iLP were produced by L-selectin^{low}/α₄β₇⁺ (double low) B lymphocytes, as well as a unique subset of L-selectin^{low}/α₄β₇^{low}/α_E⁺ B lymphocytes. Immune responses in the double low populations were decreased at this time point, and lymphocyte trafficking to L-Sel^{-/-} NP and RT appeared affected, as fewer numbers of lymphocytes were recovered from these sites. In L-Sel^{-/-} iLP, the double low population also showed a decreased total IgA response.

However, in 6 week post-immune L-Sel^{-/-} mice, the numbers of lymphocytes recovered from L-Sel^{+/+} and L-Sel^{-/-} NP and RT were comparable, indicating lymphocyte trafficking in the absence of L-selectin might be occurring in these sites. In order to determine if the restoration of responses at non-intestinal mucosal effector sites was due

to the influx of AFC that express higher levels of $\alpha_4\beta_7$ or $\alpha_E\beta_7$ we investigated the profiles of B lymphocytes in NP and RT at 6 weeks post-immunization. We found that subsets of lymphocytes in NP, RT, as well as iLP remained the same, with the double low population of B lymphocytes dominant in all three tissues, and the α_E^+ population remaining in the iLP. However, the percentage of B lymphocytes expressing α_E was decreased in L-Sel^{-/-} iLP. These results indicate that lymphocyte trafficking to the non-intestinal L-Sel^{-/-} mucosal effector sites is most likely mediated in part through $\alpha_4\beta_7$ binding interactions with MAdCAM-1. It is possible that immunization with CT might induce TNF- α driven up-regulation of MAdCAM-1 in effector sites (193, 205, 206). In future studies, we will undertake immunohistochemical staining studies to determine the expression of MAdCAM-1 in NP and RT, as well as determine the effect of nasal immunization of antigens other than CT on mucosal immune responses in L-Sel^{-/-} mice at this time point.

We also observed immune responses in the mucosal inductive sites at 6 weeks post-immunization. Again, numbers of AFC found in L-Sel^{-/-} NALT and PP were not significantly lower than L-Sel^{+/+} NALT and PP. However, by 6 weeks post-immunization with CT, NALT maintains a robust Ag-specific immune response, while this response is abated in both L-Sel^{+/+} and L-Sel^{-/-} PP. These results indicate that long-term stimulation of immune responses in the local, but not distal immune inductive sites occurs following i.n. immunization.

We found that CT-B-specific and total AFC responses were significantly increased in the CONALT at 6 weeks post-i.n. CT immunization. Again, however, the

number of lymphocytes recovered from 6 week immunized L-Sel^{-/-} CONALT was less than the number recovered from L-Sel^{+/+} CONALT, indicating that lymphocyte trafficking in these sites was impaired due to L-selectin deficiency. Therefore, it seems likely that increased immune responses in these LN might be due to stimulation of existing B lymphocyte populations, or selective trafficking of Ag-responsive lymphocytes. Previous studies have shown that i.p. and s.c. immunization induces elevated humoral immune responses in L-Sel^{-/-} PLN though these LN, like the CONALT, remained smaller in L-Sel^{-/-} mice. However GC in the L-Sel^{-/-} PLN were found to be markedly larger and more organized than GC in L-Sel^{+/+} PLN. (165) Though little evidence was observed for lymphocyte trafficking in these LN, the increased size of the germinal centers in L-Sel^{-/-} PLN indicates possible expansion of existing B cell populations.

It is possible, then, that L-selectin deficiency might affect the ability of lymphocytes to migrate out of LN by way of efferent lymphatics as well as traffic via HEV binding. In fact, it has been shown that while activated B lymphocytes shed L-selectin (211), re-expression of L-selectin is important for the trafficking of memory B lymphocytes (163). Also, recent studies have shown that L-selectin is important in the binding of lymphocytes to efferent lymphatics in the lymph node, and may play a role in the migration of memory lymphocytes from the initial site of stimulation (214). Finally, L-Sel^{-/-} mice retain increased numbers of memory T cells in their PLN, though these nodes remain smaller than their L-Sel^{+/+} counterparts (215). Therefore, increased immune responses in L-Sel^{-/-} NALT might be the result of an inability of B lymphocytes

to traffic from the LN, and the delay in response in L-selectin-deficient effector sites might be a decreased ability of memory lymphocytes to move out of the inductive sites. Future histochemical studies will address the issue of the retention of naive, activated, and memory B lymphocytes in L-Sel^{+/+} and L-Sel^{-/-} CONALT.

Finally, studies of lymphocyte homing receptor profiles on L-Sel^{-/-} CONALT indicated while there was not an increase in $\alpha_4\beta_7$ expression, there may be an increased number of $\alpha_E\beta_7$ -expressing lymphocytes. This result is particularly intriguing in context of the decrease in $\alpha_E\beta_7^+$ lymphocytes in the L-Sel^{-/-} iLP, indicating that this population might actually migrate from the intestine to other mucosal sites. $\alpha_E\beta_7$ has not previously been shown to mediate T lymphocyte trafficking, rather it appears to play a role in lymphocyte retention in the iLP (135, 142). However, recently, a novel ligand for $\alpha_E\beta_7$ has been observed (143), and little is known about the role of $\alpha_E\beta_7$ on B, rather than T lymphocytes.

In conclusion, our results indicate that restoration of the non-intestinal mucosal immune response in L-selectin deficient mice might be a function of delay in lymphocyte trafficking from the mucosal inductive tissues. In addition, these data indicate a continued important role of the double low B lymphocyte subset in Ab response the NP and RT, and suggest a novel role for $\alpha_E\beta_7$ expression on B lymphocytes. Finally, these results further define the role of L-selectin in the induction and dissemination of antibody responses following i.n. immunization.

CHAPTER 9

SUMMARY

There appears to be a definite compartmentalization of intestinal vs. non-intestinal effector sites in the CMIS. That is, i.n. immunization preferentially induces effector immune responses in the NP and RT, while oral immunization induces iLP immune response. It has been well defined that MAdCAM-1- $\alpha_4\beta_7$ interactions mediate lymphocyte trafficking to the GALT; however, preliminary results indicated that lymphocyte homing after i.n. immunization was not dependent upon MAdCAM-1- $\alpha_4\beta_7$ interactions. We hypothesized that peripheral homing receptor-addressin interactions are important for the induction of mucosal effector immunity following i.n. immunization.

We first investigated the addressins expressed by HEV of NALT, a unique mucosal inductive site for the upper airways, and compared our results with those observed in the intestinal inductive site, the PP. Our results, as described in Chapter 3, clearly show that all NALT HEV express PNAd, while approximately 60% of NALT HEV co-express MAdCAM-1. This result differs greatly from the PP, where all HEV express MAdCAM-1. Functional assays showed that naive lymphocyte binding to NALT HEV is mediated primarily by L-selectin-PNAd interactions, rather than $\alpha_4\beta_7$ -MAdCAM-1 interactions as is the case in the PP (Figure 33). In fact, lymphocyte binding to the NALT more closely resembles lymphocyte binding to the PLN. In addition, 85-90% of lymphocytes express L-selectin, compared to 50-70% of

lymphocytes in the PP. Together, these results indicated that NALT is a unique mucosal inductive site that was dependent upon peripheral L-selectin-PNAd, rather than intestinal $\alpha_4\beta_7$ -MAdCAM-1 interactions.

As shown in Chapter 4, further studies of addressin expression on the draining LN of the head and neck, the CONALT, revealed that these LN, too, express a primarily peripheral addressin phenotype (summarized in Figure 33). However, expression of PNAd and co-expression of MAdCAM-1 differ among these LN, and differ from the NALT, as well. The “most mucosal” of these LN, the CLN co-express MAdCAM-1 on 24% of HEV, compared to the 60% of HEV that co-express MAdCAM-1 in NALT. Fewer (15%) of SMLN HEV express MAdCAM-1, while the PRLN most resembles a PLN; nearly all (94%) of its HEV express PNAd only. Naive lymphocyte binding to these tissues, as in the NALT, is mediated primarily by PNAd-L-selectin interactions, with some dependence on MAdCAM-1- $\alpha_4\beta_7$ interactions, depending on the percentage of HEV that express MAdCAM-1. *In vivo* homing studies confirmed that L-selectin interactions are necessary for lymphocyte trafficking to these LN. Together with the results from our NALT studies, these experiments clearly support our hypothesis that lymphocyte homing to the nasal inductive sites is mediated primarily through peripheral, rather than intestinal, homing receptor – addressin interactions.

Since our previous studies determined the phenotype and function of addressins expressed by the HEV of naive NALT, we next investigated the changes in addressin expression that occur after immunization with a potent mucosal adjuvant, CT. In Chapter 5, we describe an increase in the number of HEV that express MAdCAM-1 after

immunization with CT. Up to 90% of NALT HEV may express MAdCAM-1 at 21 days post-primary CT immunization. In addition, *ex vivo* binding assays performed on 10 day CT-i.n. immunized NALT indicated that the increased MAdCAM-1 is functional and plays a role in the binding of naive lymphocytes to immunized NALT, suggesting a role for MAdCAM-1- $\alpha_4\beta_7$, as well as PNAd-L-selectin interactions during an immune response. These studies also showed that SED region DC might co-express MAdCAM-1 during an immune response, possibly contributing to lymphocyte retention in the NALT. Therefore, we have shown that while MAdCAM-1- $\alpha_4\beta_7$ interactions play a negligible role in the binding of lymphocytes to non-immunized NALT, this intestinal homing receptor-addressin pair may play an important role in lymphocyte trafficking during an immune response.

Next, we investigated the role of L-selectin in the induction of NP, iLP, and RT immunity subsequent i.n. CT immunization. In Chapter 6 we describe our findings that L-Sel^{-/-} mice show decreased AFC responses in the NP and RT, but not the iLP. Identification of homing receptor expression and subsequent sorting studies revealed that immune response in the NP and RT is dependent solely upon a L-selectin^{low}/ β_7 ^{low}($\alpha_4\beta_7^+$) subset of B lymphocytes that is severely reduced in L-Sel^{-/-} mice. In contrast, Ab responses in iLP are mediated by the L-selectin^{low}/ β_7 ^{low}($\alpha_4\beta_7^+$) lymphocyte, as well as a unique L-selectin^{low}/ β_7 ^{high}(α_E^+) subset of B lymphocytes. These results clearly show that a compartmentalization of effector immune responses into intestinal vs. non intestinal, and that L-selectin is important in the induction of effector immunity in NP and RT following i.n. immunization.

In Chapter 7, we investigated the effect of the route of immunization on immune responses in L-Sel^{-/-} mice. ELISPOT data revealed that, in direct contrast to i.n. immunization, oral immunization results in Ab responses in all effector sites, including the NP and RT. These results suggested that up-regulation of $\alpha_4\beta_7$ on lymphocytes subsequent oral immunization might compensate for the loss of L-selectin. However, identification of homing receptors expressed by effector site lymphocytes after oral immunization showed that the same L-selectin^{low}/ β_7 ^{low}($\alpha_4\beta_7^+$) B lymphocyte subset is present in NP, RT, and iLP, while the iLP also contains the L-selectin^{low}/ β_7 ^{high}(α_E^+) subset of B lymphocytes. However, the L-selectin^{low}/ β_7 ^{low}($\alpha_4\beta_7^+$) subset was not reduced in L-Sel^{-/-} NP and RT, and AFC responses produced by this subset were the same in L-Sel^{-/-} and L-Sel^{+/+} mice. Studies of the inductive site responses revealed the expected robust Ab response in PP, with a weak response in NALT. However AFC in L-Sel^{-/-} CONALT were much greater than L-Sel^{+/+} CONALT, though fewer lymphocytes were recovered from L-Sel^{-/-} CONALT.

We next investigated whether the loss of NP and RT Ab response in L-Sel^{-/-} mice is the result of a delay, rather than a complete loss of immune response. As shown in Chapter 8, Ab responses in NP and RT are restored by 6 weeks post primary immunization. Again, no increase in $\alpha_4\beta_7$ or α_E was observed on effector site lymphocytes that might compensate for loss of L-selectin. However, studies of AFC response in the inductive NALT and CONALT again revealed a surprising increase in Ab response in L-Sel^{-/-} CONALT, though the number of lymphocytes recovered from these LN remained significantly smaller than L-Sel^{+/+} CONALT. These results indicate that

loss of L-selectin might interfere with the ability of activated lymphocytes to disseminate from LN, resulting in a delay in immune response.

The studies of oral vs. i.n. immunization at 16 days post-CT immunization indicate an important role for the site of induction in the generation of immune responses at effector sites. Though similar homing receptor profiles were observed on the effector lymphocytes, induction of immune response via the PP induced Ab responses in L-*SEL*^{-/-} NP and RT, while induction in the L-*SEL*^{-/-} NALT resulted in abatement of immune responses in the non-intestinal site. $\alpha_4\beta_7$ -MadCAM-1 interactions mediate naive lymphocyte trafficking to PP, while L-selectin-PNAd interactions are required for lymphocyte homing to naive NALT. Therefore, it seems likely that the loss in NP and RT immunity after i.n. immunization is the result of a lack of lymphocyte trafficking to the NALT, and a subsequent inability to mount an Ab response. However, enumeration of AFC in L-*SEL*^{-/-} NALT and CONALT indicated that Ab response is being generated at these sites after i.n. immunization. If this is the case, then what mechanism is responsible for the reduction in Ab response in L-*SEL*^{-/-} non-intestinal mucosal effector sites subsequent to i.n. immunization?

Studies of long term Ab responses in the NP and RT may have provided an answer to this question. By 6 weeks post-i.n. CT immunization, Ab responses have been restored in L-*SEL*^{-/-} NP and RT. Enumeration of AFC in the CONALT revealed that, like orally immunized L-*SEL*^{-/-} CONALT, a significant increase in CT-B-specific and total AFC are observed in L-*SEL*^{-/-} CONALT after i.n. immunization. More importantly, fewer T lymphocytes were recovered from L-*SEL*^{-/-} CONALT after oral and 6 week i.n. CT

immunization than from L-Sel^{+/+} CONALT. This result suggests that lymphocytes are not homing to these sites, therefore, the increased number of AFC in L-Sel^{-/-} CONALT is likely the result of expansion of existing B lymphocyte populations. The increase in the number of AFC in L-Sel^{-/-} CONALT suggests that Ag-specific B lymphocytes might be "trapped" in these tissues, and that the delay in effector site immunity might be the result of the inability of activated B lymphocytes to move from the inductive sites to the effector sites. Restoration of immune response after a substantial increase in L-Sel^{-/-} CONALT AFC might indicate that migration of memory lymphocytes from the inductive sites might occur slowly, and depend on the overproduction of Ag-specific B cells.

However, if increase in L-Sel^{-/-} CONALT AFC is a marker for the induction of effector site immunity in L-Sel^{-/-} non-intestinal sites, oral immunization, but not i.n. immunization induces responses at these sites by 16 days post-immunization, while response after i.n. immunization is delayed. Clearly, induction of activated B cells in the PP takes place in a different microenvironment than induction in the NALT, lymphocyte trafficking in this site is less dependent upon L-selectin. Therefore, it is possible that some specialized lymphocyte subsets might migrate from the PP, and via $\alpha_4\beta_7$ interactions traffic to CONALT where they help to trigger the immune response in these LN.

Though our results indicate an important role for L-selectin in nasal immune response, several questions remain to be answered. First, our model system of immunization induces primarily strong Th2-type responses; therefore additional immunization studies using antigens that stimulate Th1-type immunity need to be

performed. In addition, our results from Chapter 5 indicate that MAdCAM-1- $\alpha_4\beta_7$ might play some role in the induction of immunity following i.n. immunization, and might facilitate lymphocyte trafficking from the PP after oral immunization. Future studies of effector site immunity using β_7 -deficient mice will address this question. Finally, the increase in AFC in L-Sel^{-/-} CONALT needs to be investigated further. It is possible that loss of L-selectin might inhibit the ability of activated lymphocytes to migrate from LN via afferent lymphatics, therefore we will attempt to ascertain the lymphocyte populations present in L-Sel^{-/-} LN via immunohistochemical studies.

In conclusion, our results have indicated an important role for L-selectin – PNAd interactions in the induction of immunity in the NALT and NP, and have provided evidence for compartmentalization of the CMIS into “intestinal” vs. “non-intestinal” mucosal effector sites.

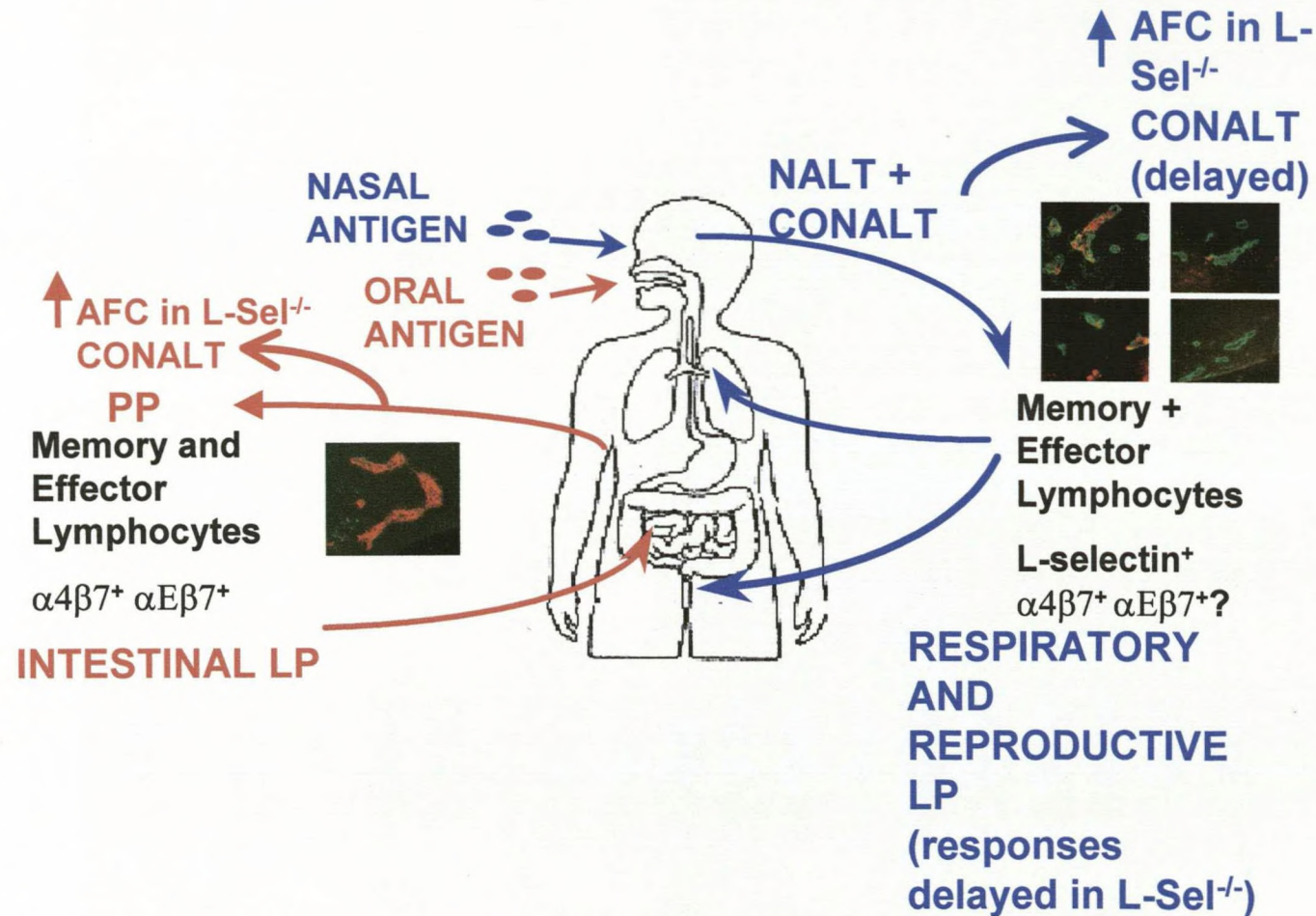


Figure 33. Differences in addressin and homing receptor expression and function contribute to the compartmentalization of the CMIS. The NALT and CONALT express PNAd on the majority of HEV, while PP HEV express primarily MAdCAM-1. Blue (I.n.) and red (oral) color schemes indicate the homing receptors that mediate lymphocyte homing to effector sites.

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APPENDICES

APPENDIX A

LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
CLN	cervical lymph node
CONALT	cranial, oral, and nasal-associated lymphoid tissue
CMIS	common mucosal immune system
CT	cholera toxin
CT-B	cholera toxin B subunit
DC	dendritic cells
ELISA	enzyme-linked immunosorbant assay
ELISPOT	enzyme-linked spot forming assay
GC	germinal center
IEL	intraepithelial lymphocyte
i.n.	intranasal
i.p.	intraperitoneal
ILP	intestinal lamina propria
LP	lamina propria
LN	lymph node
mAb	monoclonal antibody
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MLN	mesenteric lymph node
NALT	nasal-associated lymphoid tissue
NP	nasal passages
pIgR	polymeric immunoglobulin receptor
PNAd	peripheral node addressin
PLN	peripheral lymph node
PRLN	parotid lymph node
RT	reproductive tract
s.c.	subcutaneous
SED	subepithelial dome

sIgA	surface immunoglobulin A
S-IgA	secreted immunoglobulin A
SMLN	submaxillary lymph node
VCAM-1	vascular cell adhesion molecule-1

APPENDIX B

LIST OF PUBLICATIONS

The studies described in this dissertation have been published in the following papers and abstracts:

Peer reviewed publications

Csencsits, K. L., Jutila, M. A., and Pascual, D. W. 1999. Nasal-associated lymphoid tissue (NALT): phenotypic and functional evidence for the primary role of peripheral node addressin in naive lymphocyte adhesion to HEV in a mucosal site. *J. Immunol.* 163:1382-1389.

Csencsits, K. L., Walters, N., and Pascual, D. W., 2001. Cutting Edge: Dichotomy of homing receptor dependence by mucosal effector B cells: α E vs. L-selectin. *J. Immunol.* 167: 2441-2445.

Csencsits, K. L. Jutila, M. A., and Pascual, D.W. 2001. Mucosal addressin (MAdCAM-1) expression and naive lymphocyte binding interactions vary among the cranial, oral, and nasal-associated lymphoid tissues (CONALT). (Submitted).

Published abstracts and presentations

Csencsits, K. L. Jutila, M. A., and Pascual, D. W. 1998. High endothelial venules (HEV) in the inductive nasal tissues co-express MAdCAM-1 and PNAd, with recruitment of naive lymphocytes predominantly mediated by PNAd. *FASEB J.* 12: A580, # 3368. Oral presentation: Experimental Biology 98, San Francisco, CA, April 18-22, 1998

Csencsits, K. L., Jutila, M. A., and Pascual, D. W. 1999. MAdCAM-1 function is increased in initial lymphocyte binding to high endothelial venules (HEV) in murine nasal-associated lymphoid tissue (NALT) immunized with cholera toxin (CT). *Immunol. Let.* 69:147, 35.25. Oral presentation: 10th International Congress of Mucosal Immunology, Amsterdam, The Netherlands, June 27-July 1, 1999.

Pascual, D. W., Jutila, M. A., and **Csencsits, K. L.** 1999. MAdCAM-1 co-localizes with antigen-presenting cells in the mucosal inductive sites following immunization. 10th International Congress of Mucosal Immunology. *Immunol. Let.* 69:118, 29.3.

Csencsits, K. L., and Pascual, D. W. Peripheral addressin - homing receptor interactions dominate in the mucosal salivary gland and associated lymph node. *FASEB J.* 14:A1148, #149.19.

Csencsits, K. L., N. Walters, and D. W. Pascual. Compartmentalization of the mucosal immune system: L-selectin deficiency abates cholera toxin (CT)-specific secretory IgA responses in nasal passages (NP) and reproductive tract (RT), but not intestinal lamina propria (iLP). *FASEB J.* 15: A330 #272.23

Oral Presentation: Experimental Biology 2001, Orlando, FL, March 31-April 4, 2001.

I have also collaborated with members of the laboratory on studies of NALT M cell binding to reovirus protein $\sigma 1$. Details of these experiments can be found in the following articles:

Wu, Y., Boysun, M., **Csencsits, K. L.,** and Pascual, D. W. 2000. Gene transfer facilitated by a cellular targeting molecule, reovirus protein $\sigma 1$. *Gene Therapy* 7: 61-69.

Wu, Y., Wang, X., **Csencsits, K. L.,** Haddad, A., Walters, N., and Pascual, D. W. 2001. M-cell targeted DNA vaccination. *Proc. Natl. Acad. Sci., USA* 98: 9318-9323.

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