Cutting Edge: Dichotomy of Homing Receptor Dependence by Mucosal Effector B Cells: αE Versus L-Selectin

Keri L. Csencsits, Nancy Walters and David W. Pascual

*J Immunol* 2001; 167:2441-2445; doi: 10.4049/jimmunol.167.5.2441

http://www.jimmunol.org/content/167/5/2441

---

**Why The JI?**

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

---

**References**  This article cites 30 articles, 18 of which you can access for free at: http://www.jimmunol.org/content/167/5/2441.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Recent mucosal vaccination strategies have focused on the ability of intranasal (i.n.) immunization to effect immune responses throughout the common mucosal immune system (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 intestinal tract, the distal reproductive tract (RT), and the nasal passages; MAdCAM-1, mucosal addressin cell adhesion molecule-1; PNAd, peripheral node addressin; iLP, intestinal lamina propria; CT, cholera toxin; CT-B, CT B subunit; AFC, Ab-forming cells; L-Sel, L-selectin-deficient.

The common mucosal immune system may be compartmentalized because lymphocyte homing to the upper respiratory tract appears to be mediated by L-selectin interactions rather than α4β7 interactions, as is the case for gut-associated lymphoepithelial tissue. To assess the role of L-selectin in effector B cell immunity, L-selectin-deficient mice were intranasally immunized with cholera toxin (CT), and mucosal immune responses were compared with C57BL/6 mice. The absence of L-selectin correlated with a reduction in CT-specific secretory-IgA responses in nasal passages and reproductive tract, but not intestinal lamina propria. Cell sorting experiments showed that an L-selectin-dependent subset was responsible for CT-specific responses in nasal passages and reproductive tract, whereas an α4β7+ B cell subset was responsible for L-selectin-independent intestinal immunity. This study provides evidence for compartmentalization of the common mucosal immune system into “intestinal” vs “nonintestinal” effector sites. The Journal of Immunology, 2001, 167: 2441–2445.

Keri L. Csencsits, Nancy Walters, and David W. Pascual

Veterinary Molecular Biology, Montana State University, Bozeman, MT 59717

Received for publication May 8, 2001. Accepted for publication July 13, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by U.S. Public Health Service Grant AI-40288 and in part by the Montana Agricultural Station and U.S. Department of Agriculture Formula Funds. This is Montana Agricultural Station Journal Series No. 2000-84.

2 Address correspondence and reprint requests to Dr. David W. Pascual, Veterinary Molecular Biology, Montana State University, Bozeman, MT 59717-3610. E-mail address: dpascual@montana.edu

3 Abbreviations used in this paper: i.n., intranasal; CMIS, common mucosal immune system; RT, reproductive tract; GALT, gut-associated lymphoepithelial tissue; NP, nasal passages; MAdCAM-1, mucosal addressin cell adhesion molecule-1; PNAd, peripheral node addressin; iLP, intestinal lamina propria; CT, cholera toxin; CT-B, CT B subunit; AFC, Ab-forming cells; L-Sel+/−, L-selectin-deficient.

Copyright © 2001 by The American Association of Immunologists 0022-1767/01/$02.00
of mucosal effector cells unique to the intestine, and provides additional evidence for the compartmentalization of the CMIS.

**Materials and Methods**

**Mice**

A breeding colony of L-Sel−/− mice on a B6 background (Refs. 17 and 18; The Jackson Laboratory, Bar Harbor, ME) was maintained. C57BL/6 (L-Sel+/+) mice were purchased from B&K Universal (Kent, WA). Mice were immunized via nasal drip on day 0 with 5 μg CT in 10 μl sterile PBS (List Biological Laboratories, Campbell, CA) and were boosted on days 7 and 14 postprimary immunization with 2.5 μg CT. Mice were sacrificed 16 days after primary immunization. Serum, fecal, and vaginal wash samples were collected on days 0, 7, and 14 postprimary immunization.

**ELISA detection of CT-B-specific IgA and IgG**

A CT-B (List Biological Laboratories)-specific ELISA was performed as previously described (23).

**Lymphocyte isolation and purification**

NP, iLP, and RT lymphocytes were isolated as previously described (15, 23, 24) using collagenase Type IV (Sigma, St. Louis, MO; Ref. 25). Tissues from 5–10 mice per group were used in each experiment. One to three million viable lymphocytes per tissue per mouse from NP and iLP and ~50,000 viable lymphocytes per mouse from RT were recovered.

**B cell ELISPOT for detection of IgG and IgA Ab-forming cells (AFC)**

Standard B cell ELISPOT methods were used as previously described (23).

**Flow cytometry and lymphocyte sorting**

NP, iLP, and RT lymphocytes combined from 15 mice 16 days postimmunization were stained for three- or four-color flow cytometry analysis as follows: cells were stained with anti-β7, FIB 504 mAb supernatant, followed by a biotinylated goat anti-rat IgG (BioSource International, 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-α4β7 heterodimer DATK-32-PE mAb, anti-α4 M290-FTIC mAb, and anti-B220-CyChrome mAb (BD Pharmingen, San Diego, CA) were used for four-color analysis. B220+ lymphocytes were sorted on a FACS Vantage (BD Biosciences, Mountain View, CA) according to β7 and L-selectin staining. β7high and β7low L-selectinlow populations were sorted from L-Sel+/+ and L-Sel−/− iLP, whereas L-selectin+/+ subpopulations were isolated from L-Sel+/+ NP and RT. All cells were sorted at ≥97% purity, counted, and the respective populations used in a B cell ELISPOT.

**Results and Discussion**

**Vagalinal, 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 on 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 with L-Sel+/+ mice (Fig. 1; p < 0.001). In contrast, fecal and serum CT-B-specific IgA titers were not significantly different between L-Sel+/+ 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+/+ or L-Sel−/− mice.

To determine the effect of the loss of L-selectin on effector immune responses in the NP, we performed B cell ELISPOT assays 16 days postimmunization. Our results indicated that the number of IgA and IgG CT-B-specific AFC in NP was significantly reduced in L-Sel−/− mice (Fig. 1, C and D). 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 Ag-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. However, what remains unclear is how the loss of L-selectin selectively diminishes CT-B-specific mucosal responses in nonintestinal 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 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 β7 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. 2A): L-selectin+/+β7high/β7low L-selectin+/+β7low L-selectin−/−β7high/β7low populations were found in all effector sites, and an L-selectin−/−β7high/β7low phenotype was displayed by 13–23% of ILP lymphocytes. Few lymphocytes could be recovered from L-Sel−/− NP or RT (Fig. 2B), suggesting that L-selectin is required for lymphocyte trafficking to these effector tissues.

Further analysis of the L-selectin−/−β7high/β7low and L-selectin+/+β7high/β7low B220+ subsets with DATK 32 mAb, which recognizes the α4β7 heterodimer, and with anti-α4 (CD103) M290 mAb (Fig. 2, C and D) revealed that in iLP, the β7high subset also expressed α4high (Fig. 2C). The β7low populations found in the NP and RT did not express α4. However, the α4-negative populations in these tissues and in the iLP were positive for the α4β7 heterodimer (Fig.
FIGURE 2. Three distinct homing receptor phenotypes are displayed by lymphocytes in mucosal effector sites when examined 16 days postprimary i.n. immunization. A, Staining of L-Sel<sup>high</sup> NP, RT, and iLP with anti-L-selectin MEL-14 mAb and anti-β<sub>7</sub>, FIB 504 mAb revealed that all three effector tissues contained L-selectin<sup>high/β<sub>7</sub>low</sup> and L-selectin<sup>low/β<sub>7</sub>low</sup> populations, whereas the iLP contained a unique L-selectin<sup>low/β<sub>7</sub>high</sup> population. B, L-Sel<sup>−/−</sup> effector sites contain β<sub>7</sub>high and β<sub>7</sub>low populations as well. C, Analysis of FIB 504 (β<sub>7</sub>) staining of B220<sup>+</sup> L-Sel<sup>−/−</sup> NP, RT, and iLP lymphocytes, and comparison with M290 (α<sub>E</sub>) mAb staining and (D) α<sub>E</sub> vs DATK 32 (α<sub>E</sub>β<sub>E</sub>, heterodimer) staining. The β<sub>7</sub>high population in the iLP expresses high levels of α<sub>E</sub>, whereas the β<sub>7</sub>low populations in NP, RT, and iLP are positive for α<sub>E</sub>β<sub>E</sub> only. E, Samples of negative control splenocyte staining (left panel), positive FIB 504 only staining (middle panel), and positive MEL-14 staining (right panel). Percentages of subsets are as indicated. Results are representative of three experiments.

2D), whereas the β<sub>7</sub>high/α<sub>E</sub>high population in the iLP expressed lower levels of α<sub>E</sub>β<sub>E</sub>. α<sub>E</sub>β<sub>E</sub> does not bind to MAdCAM-1 and does not appear essential for iLP homing (26); therefore, expression of α<sub>E</sub>β<sub>E</sub> may contribute to the retention of B cells at this site. Alternatively, the α<sub>E</sub>β<sub>E</sub> low 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 α<sub>E</sub>β<sub>E</sub> binding (27), suggesting that the α<sub>E</sub>β<sub>E</sub> low 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<sub>7</sub>low population expressed only α<sub>E</sub>β<sub>E</sub>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 (28, 29). L-selectin mediates memory lymphocyte trafficking to peripheral sites such as inflamed skin (7). Further investigation of L-selectin and β<sub>7</sub> staining on lymphocytes isolated from blood at 16 days postimmunization revealed that these cells are L-selectin<sup>high/β<sub>7</sub>low</sup> contributing to the notion that the L-selectin<sup>low/β<sub>7</sub>low</sup> effector subsets might have down-regulated L-selectin upon entry into the mucosal tissues. It is also possible that the L-selectin<sup>low/β<sub>7</sub>low</sup> subset may have trafficked to the effector sites before 16 days postimmunization. The absence of a β<sub>7</sub>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 possible that homing of the β<sub>7</sub>low subset might be mediated through α<sub>E</sub>β<sub>E</sub> binding to MAdCAM-1 that could be up-regulated in response to immunization. Although 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<sup>low/β<sub>7</sub>low</sup> B lymphocyte subset provides CT-B-specific response in NP and RT

Cell sorting experiments were performed to determine which subset of lymphocytes provides CT-specific and total IgA and IgG AFC in effector tissue. NP and RT B220<sup>+</sup> lymphocytes were sorted for L-selectin<sup>high/β<sub>7</sub>low</sup> vs L-selectin<sup>low/β<sub>7</sub>low</sup> (see Fig. 2A for examples of sorted populations), and AFC responses were enumerated by ELISPOT. For i.n. CT-immunized L-Sel<sup>−/−</sup> NP and RT (Fig. 3, A and B), the L-selectin<sup>low/β<sub>7</sub>low</sup> subset of B lymphocytes contained the majority of both IgG and IgA CT-B-specific and total AFC. In the NP, the L-selectin<sup>high</sup> population accounted for <10% of specific and total AFC. However, this population did not provide AFC in RT (Fig. 3B). Additional sorting experiments revealed that the lymphocytes contained within these effector populations could be classified as IgD<sup>low</sup> memory cells (data not shown). Because the lymphocyte yields from NP and RT in L-Sel<sup>−/−</sup> mice were poor, similar cell sorting experiments could not be conducted on these tissues.

The L-selectin<sup>low/β<sub>7</sub>low</sup> (α<sub>E</sub>β<sub>E</sub>low) subset produces CT-B-specific and total IgA AFC in iLP

In contrast to the NP and RT, the iLP contains a β<sub>7</sub>high (α<sub>E</sub>β<sub>E</sub>low) cell population and very few L-selectin<sup>high</sup> lymphocytes. Results of NP and RT sorting indicated that the contribution of the L-selectin<sup>high</sup> population to AFC in iLP would be minimal at best. Therefore, sorting experiments were conducted with the β<sub>7</sub>high and β<sub>7</sub>low populations (see Fig. 2A for examples of sorted populations). Because lymphocyte trafficking to the iLP appeared independent of L-selectin, sorted populations from both L-Sel<sup>−/−</sup> and L-Sel<sup>+/+</sup> mice were obtained. The β<sub>7</sub>high lymphocyte population provided the majority of IgA CT-B-specific and total AFC in both L-Sel<sup>−/−</sup> and L-Sel<sup>+/+</sup> mice (Fig. 3C), 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<sub>7</sub>low lymphocyte subset to AFC response, these studies revealed that the number of total IgA AFC in the β<sub>7</sub>low lymphocyte population of L-Sel<sup>−/−</sup> mice was reduced (~300 AFC) when compared with the number of AFC obtained from the β<sub>7</sub>low population in L-Sel<sup>+/+</sup> mice (~2300 AFC). The number of total IgA AFC produced in the
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, whereas the $\alpha_\beta_7^{+}$ population may represent a resident intestinal lymphocyte population stimulated by CT immunization, or a gut-specific population that has homed from the NP.

However, it is important to determine whether the subsets of lymphocytes induced in this experiment are strictly Th2-type, or whether immunization with a Th1-type Ag might induce different responses. Preliminary studies in our laboratory indicate that i.n. immunization with attenuated adeno virus induces L-selectin$^{low}$/ $\beta_7^{low}/\alpha_\beta_7^{+}$ populations in both NP and RT, whereas both L-selectin$^{low}/\beta_7^{low}/\alpha_\beta_7^{+}$ and $\alpha_\beta_7^{+}$ 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 whether a loss of L-selectin will result in reduced effector NP and RT responses after oral immunization.

However, we have recently shown that oral immunization of L-Sel$^{-/-}$ mice with a Salmonella vaccine vector expressing colonization factor Ag I results in attenuated mucosal IgA Abs while maintaining serum IgG responses (30). In fact, a preferential bias in serum IgG2a Abs was noted when compared with similarly immunized L-Sel$^{-/-}$ mice. Ongoing studies in our laboratory are addressing whether the types of Th cells induced impact effector B cell development and what effector B cells are induced subsequent to oral immunization with CT. Collectively, the evidence suggests that several pathways exist for B cell homing to effector tissues. The question remains as to whether these alternative pathways can compensate for deficiencies in any of these mechanisms and whether they are dependent on the types of Th cells induced. In conclusion, this study provides evidence for separation of the CMIS into “intestinal” vs “nonintestinal” 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.

**Acknowledgments**

Special thanks to Dr. E. C. Butcher and Dr. Mark A. Jutila for providing MEGA 79, MECA 367, and FIB 30 mAbs; additional thanks to Dr. Mark A. Jutila, Montana State University, for reviewing this manuscript and for providing insightful comments.

**References**


5. Rott, L. S., J. R. Rosé, D. Bass, M. B. Williams, H. B. Greenberg, and E. C. Butcher. 1997. Expression of mucosal homing receptor $\alpha_\beta_7$ by circulating $\beta_7^{high}$ 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$^{low}/\beta_7^{low}$ lymphocyte subset observed in NP and RT.

This study has identified important differences in intestinal vs nonintestinal mucosal sites. Most importantly, we have identified a subset of $\alpha_\beta_7^{+}$ B220$^{+}$ effector lymphocytes in the iLP that are independent of the expression of L-selectin to traffic to the iLP. In addition, we have identified a subset of L-selectin$^{low}/\beta_7^{low}/\alpha_\beta_7^{+}$ 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$^{low}/\beta_7^{low}$ lymphocyte subset observed in NP and RT.