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# Distal IgA immunity can be sustained by $\alpha_E\beta_7^+$ B cells in L-selectin$^{-/-}$ mice following oral immunization

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## Abstract

Understanding the role of homing receptors could aid vaccine strategies for developing distal mucosal immunity. Infection studies have revealed that immune intestinal B cells use $\alpha_4\beta_7$ homing receptors, but their role in subsequent oral immunization with soluble antigens is unknown. To assess the influence of L-selectin and $\alpha_4\beta_7$ on distal B cells following oral cholera toxin (CT) immunization, L-selectin-deficient (L-Sel$^{-/-}$) IgA anti-CT-B-specific B cells were enhanced 30-, 9.2-, and 3.5-fold in head and neck lymph nodes (HNLNs), nasal-associated lymphoid tissue, and nasal passages (NPs), respectively, vs. L-Sel$^{+/+}$ mice. Cell-sorted intestinal and NP IgA antibody-forming cells (AFCs) were mostly $\alpha_4\beta_7^+$, unlike HNLN L-Sel$^{-/-}$ IgA and IgG anti-CT-B AFCs that were $\alpha_E\beta_7^+$, contrasting with L-Sel$^{+/+}$ HNLN IgA AFCs that were mostly $\alpha_4\beta_7^+$. *In vitro* studies revealed that L-Sel$^{-/-}$ HNLN B cells preferentially expressed $\alpha_E$ following polyclonal stimulation. These studies show that HNLN B cells express $\alpha_E\beta_7$ in the absence of L-Sel to sustain distal IgA responses.

## INTRODUCTION

The mucosal immune system is comprised of inductive tissues where antigen (Ag) is first taken up and processed for recognition by naive lymphocytes. Subsequently, these B and T lymphocytes proliferate and differentiate, ultimately emigrating to mucosal effector tissues or what is referred to as the common mucosal immune system.[1-5] While past interests were in devising oral vaccines for respiratory diseases and adapting the common mucosal immune system to obtain protective immunity,[6,7] studies have shown that intranasal (IN) delivery is feasible with most Ags,[8] as evidenced by recent influenza vaccines.[9] Consequently, mucosal immunization studies have focused mostly on evaluating the site of vaccination, e.g., oral immunization and gastrointestinal tract immunity, and nasal immunization and upper and lower respiratory tract responses.

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DISCLOSURE

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Understanding how immune lymphocytes migrate and which homing receptors are necessary to seed these distal mucosal compartments, specifically the upper respiratory tract, could enable improved vaccine efficacy. In light of this, lymphocyte expression of the mucosal or intestinal homing receptor $\alpha_4\beta_7$ has been accepted as the principal mode of interaction with the mucosal addressin cell adhesion molecule-1 (MAdCAM-1).[10-15] Such an interaction appears to play a secondary role for lymphocytes migrating to non-intestinal mucosal tissues, as the ability to secrete immune antibodies (Abs) in nasal and reproductive tract secretions was significantly delayed in L-selectin-deficient (L-Sel$^{-/-}$) mice following IN immunization with the potent mucosal adjuvant cholera toxin (CT[16,17]). Furthermore, the nasal-associated lymphoid tissue (NALT[18]), as well as the head and neck lymph nodes (HNLNs[19]), primarily expresses peripheral node addressin with variable expression of MAdCAM-1, and the majority of lymphocyte binding in these sites is mediated by L-Sel–peripheral node addressin interactions. These L-Sel–peripheral node addressin interactions have also been shown to be important for lymphocyte homing to the nasal passages (NPs) and lungs in humans and sheep.[20-22] Such results suggest that peripheral homing receptor-addressin interactions play an important role in lymphocyte trafficking to the non-intestinal inductive sites. However, other homing receptor interactions may have an important role in trafficking to these sites, especially after mucosal immunization. For instance, IN immunization with CT results in the stimulation of a novel gut (distal) B-lymphocyte subset expressing $\alpha_E\beta_7$,[16] and this unique subset of cells has also been found in the HNLNs, subsequent to long-term CT immunization.[17] Furthermore, the NALT and HNLN express varied levels of MAdCAM-1, and it is conceivable that some B-lymphocyte trafficking to these sites might occur via $\alpha_4\beta_7$ interactions.

Studies in humans have revealed that L-Sel, as well as $\alpha_4\beta_7$, is expressed by B lymphocytes stimulated via oral immunization,[4,5,23] suggesting a role for L-Sel in the production of immunity in the intestinal effector site. Likewise, L-Sel$^{-/-}$ mice orally immunized with a *Salmonella* vaccine fail to induce gut IgA responses to the *Salmonella*'s passenger Ag, even though peripheral Ab and T-cell responses are induced.[24] Although our previous studies have shown that early induction of non-intestinal immunity is dependent upon L-Sel,[16] it remains to be determined how the effect of the loss of L-Sel will impact immune responses in the gut after soluble Ag delivery. Results from rotavirus infection studies clearly show the dependence on gut $\alpha_4\beta_7^{high}$ B cells to mediate protection,[25] but differences in the routes of Ag composition (live vs. soluble) and deposition may help explain why differences in homing receptors dependency are observed between infections and immunizations with soluble Ags.

In the light of our past studies, we questioned whether oral CT delivery in L-Sel$^{-/-}$ mice would negatively impact the stimulation of gut (or proximal) IgA responses, as observed with our *Salmonella* vaccine studies[24] and with the IN CT immunization study,[16,17] or whether secretory IgA (S-IgA) responses would be delayed, as previously reported for IN CT immunization. Another intriguing aspect noted in our previous IN CT study was the sustained IgA production by $\alpha_E\beta_7^+$ B cells in L-Sel$^{-/-}$ mice at distal mucosal sites typically not evident with IN immunizations.[17] Thus, we also questioned whether distal (upper respiratory tract) immunity will be supported by these $\alpha_E\beta_7^+$ B cells in L-Sel$^{-/-}$ mice following oral CT immunization.

## RESULTS

### Oral CT immunization of L-Sel$^{-/-}$ mice shows sustained IgA Ab responses

Previous work examined the homing receptors and addressin usage that supports upper respiratory tract responses. It has been shown that peripheral node addressin expression either associates with MAdCAM-1 or remains unassociated, suggesting the importance of L-Sel to upper respiratory tract immunity.[18,19] Moreover, the relevance of L-Sel for upper respiratory tract responses shows that mucosal immunity is compromised in L-Sel$^{-/-}$ non-intestinal mucosal tissues following nasal CT-B immunization.[16,17] Thus, we questioned whether oral CT (soluble Ag) immunization would result in IgA responses. L-Sel$^{-/-}$ and L-Sel$^{+/+}$ mice were orally immunized with CT on days 0, 7, and 14, and anti-CT-B titers were measured on day 16 to obtain an early measurement. The results showed that fecal IgA ($P = 0.021$; Figure 1a) and serum IgA ($P = 0.006$; Figure 1c) anti-CT-B responses were elevated when compared to L-Sel$^{+/+}$ mice. The fecal IgA anti-CT-B titers remained elevated for at least 2 additional weeks and, by day 42, responses diminished when compared with L-Sel$^{+/+}$ mice ($P < 0.001$). L-Sel$^{-/-}$ serum IgA responses remained elevated throughout all the experiments and, for the most part, these IgA titers were significantly greater than those observed in L-Sel$^{+/+}$ mice ($P \leqslant 0.006$). Interestingly, nasal washes from L-Sel$^{-/-}$ mice showed a 16-fold increase in CT-B-specific S-IgA titers, suggesting that in the absence of L-Sel, distal immunity could be augmented. The observed elevation in serum IgA anti-CT-B Abs appeared to be due to increased respiratory IgA titers, as evidenced by the elevated S-IgA present in L-Sel$^{-/-}$ nasal washes ($P = 0.010$) on day 42 (Figure 1b). Serum IgG anti-CT-B responses were similar for both groups for each time point examined (Figure 1d).

To assess the source of enhanced mucosal and serum IgA responses, CT-B-specific enzyme-linked immunosorbent spot (ELISPOT) assay was performed on lymphocyte fractions from the intestinal lamina propria (iLP) and NP 16 days post-primary immunization. The number of IgA and IgG CT-B-specific antibody-forming cells (AFCs) in the NP and iLP were not significantly different between L-Sel$^{-/-}$ and L-Sel$^{+/+}$ mice (Figure 2a,b). Except for a slight reduction in the total IgA AFCs in the L-Sel$^{-/-}$ NP (Figure 2a), the total number of IgA and IgG AFCs was also not significantly different (Figure 2a,b). Likewise, there were no significant differences in IgA or IgG CT-B-specific as well as total IgA or IgG AFCs in the NALT and Peyer's patch (PP) (Figure 2c,d). Only the cervical lymph node (CLN) in L-Sel$^{-/-}$ mice showed an increase in Ag-specific IgA AFCs ($P = 0.002$) as well as total IgA ($P < 0.001$) and total IgG ($P = 0.031$; Figure 2e,f). The total IgA AFCs ($P = 0.003$) were also increased in the submaxillary gland lymph node (SMLN) (Figure 2e).

### Upper respiratory tract immune responses are increased in L-Sel$^{-/-}$ mice by 6 weeks post-primary immunization

To determine whether the observed upper respiratory tract is more responsive with time, B-cell ELISPOT experiments were conducted 4 weeks after the last oral CT dose. No significant differences in the number of IgA or IgG anti-CT-B iLP AFCs were observed between L-Sel$^{+/+}$ and L-Sel$^{-/-}$ mice at this time point (Figure 3a,b). As before, distal immunity was notably augmented in L-Sel$^{-/-}$ mice, as evidenced by the increased L-Sel$^{-/-}$ NP IgA CT-B-specific AFC responses, which were significantly greater than responses

obtained with the L-Sel$^{+/+}$ mice by 3.5-fold ($P < 0.001$). This apparently weak distal B-cell response in L-Sel$^{+/+}$ mice was expected and did not deviate from the 16-day responses. In contrast, the L-Sel$^{-/-}$ NP IgA AFC responses were significantly enhanced by 15-fold by 6 weeks ($P < 0.001$).

To determine whether regional inductive mucosal tissues supported these CT-B-specific Ab responses, lymphocytes isolated from NALT, HNLN, and PP were analyzed by B-cell ELISPOT (Figure 3c,d). Specific and total elevations in IgA AFCs were observed in L-Sel$^{-/-}$ NALT by 9.2-fold ($P = 0.014$) and 6.5-fold ($P = 0.008$) and in HNLN by 30-fold ($P < 0.001$) and 6.7-fold ($P < 0.001$), respectively, when compared to L-Sel$^{+/+}$ mice (Figure 3c). L-Sel$^{-/-}$ IgG CT-B-specific AFCs were also significantly increased in the NALT by 2.4-fold ($P = 0.009$) and in the HNLN by 19-fold ($P < 0.001$) when compared to L-Sel$^{+/+}$ mice (Figure 3d). Although the IgG AFC responses in the PP did not significantly differ between L-Sel$^{+/+}$ and L-Sel$^{-/-}$ mice, the L-Sel$^{+/+}$ PP showed slightly greater ($P = 0.025$) CT-B-specific IgA AFC responses when compared to L-Sel$^{-/-}$ mice. Thus, these data show that specific elevations in upper respiratory tract (distal) AFC responses were sustained in L-Sel$^{-/-}$ mice orally immunized with CT, and these responses were greatly augmented when compared to L-Sel$^{+/+}$ mice.

## CT-B-specific $\alpha_E\beta_7^+$ B cells are present in the gut but not in the NP at 6 weeks post-primary immunization

NP and iLP B-cell subsets were evaluated by flow cytometry from orally CT-immunized L-Sel$^{+/+}$ and L-Sel$^{-/-}$ mice on day 42 (Figure 4). The majority of B220$^+$ lymphocytes in L-Sel$^{+/+}$ and L-Sel$^{-/-}$ NP were found to be $\alpha_4\beta_7^{low}$ and, for the most part, not $\alpha_E\beta_7^+$. In contrast, those in the L-Sel$^{+/+}$ and L-Sel$^{-/-}$ iLP were mostly $\alpha_4\beta_7^{low}$, although a minor subset stained $\alpha_E\beta_7^+$. Yet this population was found to be more prominent in the L-Sel$^{-/-}$ iLP than in the L-Sel$^{+/+}$ iLP by approximately 2.4-fold (Figure 4c,d).

To discern whether the $\alpha_E\beta_7^+$ B cells were Ag-specific, B220$^+$ B cells from the L-Sel$^{+/+}$ and L-Sel$^{-/-}$ iLP and NP were sorted for $\beta_7^{low}$ vs. $\alpha_E\beta_7$ (see Figure 4c,d for examples of sorted populations), and the sorted subsets were evaluated in CT-B-specific ELISPOT assay. Not surprisingly, the IgA anti-CT-B activity was mostly found within the $\beta_7^{low}$ subset; however, a significant portion of the IgA anti-CT-B Abs were produced by the $\alpha_E\beta_7^+$ B cells in both L-Sel$^{-/-}$ and L-Sel$^{+/+}$ iLP (Figure 5a). No iLP IgG anti-CT-B AFCs were detected (Figure 5b). Examination of NP revealed that no IgA CT-B-specific activity was associated with the $\alpha_E\beta_7^+$ B cells from L-Sel$^{+/+}$ or L-Sel$^{-/-}$ NP (Figure 5c), but instead all the reactivity appeared in the $\beta_7^{low}$ subset. However, some IgG CT-B-specific AFCs were found associated with the NP $\alpha_E\beta_7^+$ subset (Figure 5d).

## Cell-sorted $\alpha_E\beta_7^+$ B cells contain the majority of the IgA CT-B-specific activity in L-Sel$^{-/-}$ HNLN

Fluorescence-activated cell-sorting analyses for L-Sel, $\alpha_4\beta_7$, and $\alpha_E\beta_7$ expression were performed on B220-gated B cells obtained from the NALT, HNLN, and PP (Figure 6). At 6 weeks post-primary immunization, levels of L-Sel were diminished on L-Sel$^{+/+}$ NALT B cells, but both L-Sel$^{high}$ $\alpha_4\beta_7^{low}$ and L-Sel$^{low}$ $\alpha_4\beta_7^{low}$ (double-low) were present in

CLN, SMLN, and PP, whereas L-Sel$^{-/-}$ B cells were all $\alpha_4\beta_7^{low}$ (Figure 6a). Interestingly, on examination of both L-Sel$^{+/+}$ and L-Sel$^{-/-}$ mucosal inductive tissues, each was found to contain $\alpha_E\beta_7^+$ B cells (Figure 6b). Additional studies were then undertaken to discern whether this $\alpha_E\beta_7^+$ B-cell subset was present at earlier time points. Less than 3% of the B cells from naive mice CLN, SMLN, and PP (Figure 6d-f) and ~5% of the B cells from the naive mice NALT were $\alpha_E\beta_7^+$ (Figure 6g). Over the course of the oral immunizations, increases in $\alpha_E\beta_7^+$ B cells were detected mostly in the L-Sel$^{-/-}$ mice, peaking at about 35 days post-primary immunization. Collectively, these results suggest that these increases in $\alpha_E\beta_7^+$ B cells would be CT-B-specific.

To assess whether these $\alpha_E\beta_7^+$ B cells secrete anti-CT-B Abs, B220$^+$ B cells from PP and HNLN were sorted for $\beta_7^{low}$ vs. $\alpha_E\beta_7^+$ and evaluated by CT-B-specific ELISPOT. In L-Sel$^{+/+}$ PP, only the $\alpha_4\beta_7^{low}$ B-cell subset showed CT-B-specific IgA AFCs, unlike the L-Sel$^{-/-}$ PP in which the responses were equally distributed between $\alpha_4\beta_7^{low}$ and $\alpha_E\beta_7^+$ B cells (Figure 7a). The CT-B-specific IgG PP responses also differed between mouse strains. The PP IgG AFCs were equally distributed between both subsets in L-Sel$^{+/+}$ mice, but only $\alpha_4\beta_7^{low}$ subset in the L-Sel$^{-/-}$ mice contained the CT-B-specific B cells (Figure 7b). As shown in Figure 3, minimal to no AFC responses were detected in L-Sel$^{+/+}$ HNLN. Thus, the lack of IgA and IgG AFC responses in the HNLN was not surprising (Figures 3 and 7c,d), as oral immunization is not normally effective in stimulating upper respiratory tract responses. Interestingly, however, increased AFC responses were detected in L-Sel$^{-/-}$ HNLN (Figure 3), and cell-sorting analyses revealed that the majority of these CT-B-specific IgA AFCs were $\alpha_E\beta_7^+$ (Figure 7c). The IgG AFC responses were not significantly different between $\alpha_4\beta_7^{low}$ and $\alpha_E\beta_7^+$ B-cell subsets (Figure 7d).

### L-Sel$^{-/-}$ mice show increased propensity for $\alpha_E$ integrin expression on B cells

As the percentage of L-Sel$^{-/-}$ $\alpha_E\beta_7^+$ B cells increased with time in the HNLN, we questioned whether these B cells show an increased likelihood to express $\alpha_E\beta_7$. Whole HNLN, PP, and splenic lymphocytes from L-Sel$^{-/-}$ or L-Sel$^{+/+}$ mice were cultured for 1–3 days, activated with lipopolysaccharide (LPS) or LPS + CT, or left unactivated. Cells were collected and analyzed for $\alpha_E\beta_7$ expression by flow cytometry. Unstimulated L-Sel$^{-/-}$ HNLN B cells at 48 h showed elevated $\alpha_E\beta_7$ expression when compared to L-Sel$^{+/+}$ HNLN B cells (Figure 8a); no significant differences were seen with PP or splenic B cells. The addition of LPS did not significantly enhance $\alpha_E\beta_7$ expression (Figure 8b), but CT co-stimulation showed significantly greater $\alpha_E\beta_7^+$ B cells for the L-Sel$^{-/-}$ HNLN, PP, and spleens than identical B cells from L-Sel$^{+/+}$ mice (Figure 8c,d). A kinetic analysis clearly showed that the L-Sel$^{-/-}$ HNLN B cells showed the greatest propensity for $\alpha_E\beta_7$ expression (Figure 8d), but L-Sel$^{+/+}$ HNLN B cells did not, suggesting that $\alpha_E\beta_7$ may be driven to expression in the absence of L-Sel.

## DISCUSSION

The results obtained in this study differ from what was observed upon nasal CT immunization[16] in that proximal tissues in L-Sel$^{-/-}$ mice were responsive to oral CT, reaffirming the relevance of $\alpha_4\beta_7$–MAdCAM-1 interactions.[23,25] Of interest, however,

were the distal responses obtained in these mice; in particular, NP CT-B-specific IgA responses became elevated with time in L-Sel$^{-/-}$ mice but not in normal C57BL/6 mice. On investigating mucosal inductive site responses, the NALT and PP responses did not differ between L-Sel$^{+/+}$ and L-Sel$^{-/-}$ mice during the early responses. Clearly, however, by 6 weeks, increases in CT-B-specific AFC responses in the NALT and more prominently in the HNLN of orally immunized L-Sel$^{-/-}$ mice were observed. Remarkably, the absence of L-Sel resulted in long-term elevated NP AFC responses, which may be supported by NALT and/or HNLN, whereas in L-Sel$^{+/+}$ mice, these distal immune responses were significantly less robust and remained unchanged or reduced by 6 weeks post-immunization. These differences between mouse species seemed not to be attributed to selective regurgitation of CT following oral immunization, as this was not observed. Rather, these latter results were consistent with previous reports showing that oral immunization is not as effective for stimulating long-lived B cells in distal mucosal tissues.[26,27] Why then is there selective enhancement in the L-Sel$^{-/-}$ upper respiratory tract?

The most obvious explanation for this result is that responses to immunization in the iLP appear to rely on $\alpha_E\beta_7$ expression by B and T cells.[25,28,29] Therefore, oral immunization possibly stimulated an $\alpha_E\beta_7^+$ subset of B cells that could traffic to the nasal effector sites from the intestinal inductive sites even without expression of L-Sel. Consistent with this explanation, our flow cytometry analyses revealed that the subpopulations of B lymphocytes induced in the effector sites of orally immunized mice were similar to those observed after IN CT immunization.[16,17] L-Sel$^{high}$/$\alpha_E\beta_7^{low}$ and L-Sel$^{low}$/$\alpha_E\beta_7^{low}$ (double-low) B lymphocytes were found in NP and iLP, whereas the $\alpha_E\beta_7^+$ population was more prominent in iLP. Cell-sorting experiments revealed that the $\beta_7^{low}$ population provided the majority of CT-B-specific and total IgA response in all effector sites, excluding the possibility that $\alpha_E\beta_7^+$ B cells compensated in the mucosal effector tissues in the absence of L-Sel. Obviously, oral immunization with CT did not stimulate the $\alpha_4\beta_7^{high}$ subset that migrated to the non-intestinal effector sites, as was expected from the previously described rotavirus studies.[25]

However, the most prominent functional differences observed were with the distal B-cell responses, particularly in the upper respiratory tract. Investigation of the responses occurring in the intestinal and non-intestinal inductive sites may provide more clues as to the mechanism of immune responses following oral CT immunization in L-Sel$^{-/-}$ mice. AFC responses were beginning to increase in L-Sel$^{-/-}$ HNLN by 16 days and continued until at least 42 days. Additionally, both L-Sel$^{+/+}$ and L-Sel$^{-/-}$ NALT AFC responses were not significantly different at 16 days; however, both CT-B-specific and total IgA responses were significantly increased in L-Sel$^{-/-}$ NALT at 42 days post-primary immunization. Although no differences in gut IgA responses were observed, AFC responses in the L-Sel$^{+/+}$ PP were slightly enhanced when compared to L-Sel$^{-/-}$ PP. Therefore, in the absence of L-Sel, there was selective enhancement of immune responses in the nasal inductive and effector sites. This attribute of impacting distal immunity was one of the original tenets upon which the theory of the common mucosal immune system[1-3] was founded but later was shown to be somewhat more variable.[4,5] What is striking about the results presented here is the observation that, indeed, the distal immune responses were augmented and retained for at least 42 days, whereas this was not evident in L-Sel$^{+/+}$ mice.

How the augmentation in the L-Sel$^{-/-}$ upper respiratory tract may come about may be explained in part by the observed results in the HNLN. Although the L-Sel$^{-/-}$ HNLN showed reduced cellularity, overall it had an increased "concentration" of AFCs, suggesting that lymphocyte trafficking to these sites is reduced with a concomitant stimulation of resident B-cell populations, as others have suggested.[30] This was largely based on observations made with L-Sel$^{-/-}$ PLN showing increased numbers of germinal centers[30] following peripheral immunization, again suggesting stimulation of resident B cells. Moreover, the results from the oral CT studies were very similar to those previously observed at 42 days post-IN immunization,[17] suggesting that the head and neck have a larger contribution by L-Sel.[18,19] It has also been shown that activated B lymphocytes can shed L-Sel[31] and that re-expression of L-Sel is important for the trafficking of memory B lymphocytes.[32] Thus, as these B cells seem to be restricted or fixed within these tissues, they perhaps behave as memory B cells. Because of this memory phenotype, they are unable to migrate from the HNLN or NALT as effectively as L-Sel$^{+/+}$ mice, thereby restricting their differentiation to occur locally, which could then account for their increased numbers. Consistent with this idea that these B cells may be more of a memory phenotype is the observation that at 42 days post-primary immunization, the majority of the L-Sel$^{-/-}$ were GL7$^{-}$ (data not shown). Activated B cells have been found to show increased expression of GL7.[33,34]

The most prominent observation of this study was the increased IgA AFC responses in the HNLN in which these B cells were almost entirely $\alpha_E\beta_7^+$. This finding suggests that $\alpha_E\beta_7$ can play an important role in the generation of B-cell immunity. This result was surprising, as $\alpha_E\beta_7$ has not previously been shown to mediate lymphocyte trafficking, but rather appears to play a role in lymphocyte retention in the iLP[35,36] and in the skin.[37,38] However, a novel ligand for $\alpha_E\beta_7$ has been observed,[39] and little is known about the role of $\alpha_E\beta_7$ on B lymphocytes. Although we observed increased presence of $\alpha_E\beta_7^+$ B cells in the HNLN, NALT, and NP at 42 days, no CT-B-specific AFCs were detected in the NALT, and only the L-Sel$^{-/-}$ NP $\alpha_E\beta_7^+$ B cells contained IgG CT-B-specific AFCs. In contrast, HNLN $\alpha_E\beta_7^+$ B cells compensated for the absence of L-Sel mostly during the late-phase responses, as no CT-B-specific $\alpha_E\beta_7^+$ B cells were detectable at 16 days in NALT, HNLN, or NP. These CT-B-specific $\alpha_E\beta_7^+$ B cells appear to be generated locally in the HNLN, as basal levels of $\alpha_E\beta_7^+$ B cells could be detected by day 16, although we cannot exclude their potential derivations from the gut. Indeed, the percentage of $\alpha_E\beta_7$ lymphocytes was increased in L-Sel$^{-/-}$ by day 42 post-immunization, and its contribution to the CT-B-specific response was limited to the gut and the HNLN and, interestingly, not the NP. Further evidence that the $\alpha_E\beta_7$ is induced preferentially by L-Sel$^{-/-}$ mice comes from the *in vitro* studies in which unstimulated or LPS plus CT-stimulated L-Sel$^{-/-}$ HNLN B cells showed increased $\alpha_E\beta_7$ with time when compared to L-Sel$^{+/+}$ mice, which simply did not show modulation. This latter finding supports the cell-sorting experiments for IgA AFC in which no $\alpha_E\beta_7^+$ B cells from the HNLN or PP were present in L-Sel$^{+/+}$ mice, whereas this propensity for $\alpha_E\beta_7$ expression was clearly evident in L-Sel$^{-/-}$ HNLN and PP. Collectively, these studies show that alternative integrins can facilitate mucosal immunity as in the present study, which shows that $\alpha_E\beta_7$ is induced and contributes to sustaining distal IgA responses. Current

work is seeking to understand which adjuvants[38] may enhance $\alpha_E\beta_7$ expression to enable development of vaccine strategies to enhance distal mucosal immunity.

## METHODS

### Mice and immunizations.

Specific pathogen-free C57BL/6N (L-Sel$^{+/+}$) female mice were purchased from the National Cancer Institute (Frederick Cancer Research Facility, Frederick, MD) at 5–6 weeks of age and maintained in the Animal Resources Center at Montana State University (Bozeman, MT). Breeding pairs of L-Sel$^{-/-}$ mice on a B6 background were maintained in the Animal Resources Center, as previously described.[16,17,24] All mice were kept under pathogen-free conditions in individually ventilated cages under HEPA-filtered barrier conditions and 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 and 8 weeks of age. Mice were orally gavaged with 200 μl of 50% saturated $NaHCO_3$ solution and, after 15 min, were immunized with 10 μg CT (List Biological Laboratories, Campbell, CA) in 200 μl sterile phosphate-buffered saline (PBS) and additional boosts on days 7 and 14 with 10 μg CT, similar to that previously described.[40]

### Collection of serum and mucosal samples.

Blood was collected from mice through saphenous vein bleeding. Fresh fecal pellets were collected from individual mice and solubilized in 50 μg ml$^{-1}$ of soybean trypsin inhibitor (Sigma-Aldrich, St Louis, MO) in sterile PBS (10× v/w) by continual vortexing for 30 min at 4 °C and then subjected to microcentrifugation. Nasal washes were performed on euthanized mice by intubating their tracheas to access the nasopharyngeal cavity using a small internal diameter (0.010 inch) tygon tubing (Cole-Parmer, Vernon Hills, IL). The nasal cavity was flushed with 300 μl of sterile PBS, and nasal washes were collected from the nares into microcentrifuge tubes. Sera and supernatants from mucosal samples were frozen until assayed.

### Anti-CT-B ELISA.

The method for anti-CT-B enzyme-linked immunosorbent assay (ELISA) was identical to that previously described.[17] Falcon Micro-test III Flexible assay microtiter plates (BD Biosciences, Oxnard, CA) were coated with 50 μl per well of 5 μg ml$^{-1}$ B subunit of CT (CT-B; List Biological Laboratories) in sterile PBS. Serum or mucosal samples diluted in ELISA buffer were added at 50 μl per well, and plates were incubated at 4 °C overnight. Plates were washed and 50 μl per well of detecting horseradish peroxidase conjugates of goat anti-mouse IgG (γ-chain specific) or goat anti-mouse IgA (α-chain specific) Abs (1 μg ml$^{\alpha}$ Southern Biotechnology Associates, Birmingham, AL) was added, and the plates were then incubated at 37 °C for 1.5 h. Horseradish peroxidase was visualized by the addition of 50 μl per well of ABTS (2,2′-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid]) substrate (Moss, Pasadena, CA). Optical density was determined by reading the plates at 415 nm, and end-point titers were expressed as the reciprocal of the last sample dilution giving an absorbance >0.1 over the value of negative control wells (in the absence of biological fluid)

after a 1-h incubation. Similar dilutions of mucosal samples and serum from non-immune mice showed no Ab titer to CT-B.

### Isolation of inductive tissue lymphocytes.

Parotid gland LN, SMLN (sometimes also referred to as the superficial cervical or mandibular gland LN[41,42]), CLN (sometimes referred to as deep CLN[41]), and PP were isolated from immunized L-Sel$^{+/+}$ and L-Sel$^{-/-}$ mice. Each set of lymphoid tissue was pooled from five mice and washed in RPMI-1640 medium. NALT tissues were collected by removing the soft palates, as previously described,[17,18] followed by collagenase digestion (200 U ml$^{-1}$ collagenase type IV solution; Sigma-Aldrich) in RPMI-1640 media containing 0.08 U ml$^{-1}$ DNase (Promega, Madison, WI). The palates were vigorously agitated on a magnetic stir plate for 45 min at 37 °C; the resulting cell suspensions were removed and filtered through Nitex fabric (Fairview Fabrics, Hercules, CA) and the cells were washed and resuspended in complete medium (RPMI-1640 + 10% fetal bovine serum (Hyclone, Logan, UT) + 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer + 10 mM non-essential amino acids + 10 mM sodium pyruvate + 10 U ml$^{-1}$ penicillin/streptomycin) or fluorescence-activated cell-sorting buffer (Dulbecco's PBS + 2% fetal bovine serum).

For flow cytometry and ELISPOT analysis, CLN, SMLN, and parotid gland LN were removed and subjected to Dounce homogenization.[19] The resulting cell suspensions were filtered through Nitex fabric (Fairview Fabrics), washed with RPMI-1640 medium, and centrifuged at 1,500 r.p.m. for 5 min. Cell pellets were resuspended in fluorescence-activated cell-sorting buffer or complete medium.

### Isolation of effector tissue lymphocytes.

NPs devoid of NALT were removed from the head by scraping the turbinates from the nasal cavity, followed by digestion with 200 U ml$^{-1}$ collagenase type IV solution containing 0.08 U ml$^{-1}$ DNase at 37 °C for 30 min, as previously described.[16,17] For iLP lymphocytes, intestines were extracted from the mouse, the PPs were carefully removed, and fecal material and mucus were flushed from the intestine using RPMI-1640 medium. Intestines devoid of isolated lymphoid follicles[43] were processed, as previously described,[16,17] and NP and iLP lymphocytes were 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 complete medium.

### CT-B-specific and total Ab ELISPOT.

Mixed cellulose ester membrane-bottomed microtiter plates (Multi-Screen-HA; Millipore, Bedford, MA) were coated with 5 μg ml$^{-1}$ CT-B (List Biological Laboratories) or goat anti-mouse IgA or IgG (H-chain-specific) Abs (Southern Biotechnology Associates) in sterile PBS overnight at room temperature. The plates were blocked at 37 °C for 2 h with complete medium. A total of 100 μl of cells from each tissue at varying concentrations ($2\times10^6$–$1.25\times10^5$ lymphocytes per ml) was added to the wells, and the plates were incubated at 37 °C overnight. Cells were removed, and the plates were washed as previously described.[17]

For detection of mouse Ab responses, 100 μl of 1.0 μg $ml^{-1}$ goat anti-mouse IgG and IgA-horseradish peroxidase conjugates (Southern Biotechnology Associates) was added to the wells, and the plates were incubated overnight at 4 °C. After washing, the wells were developed with 100 μl of AEC (3-amino-9-ethylcarbazole) (Moss), and the reaction was allowed to continue until spots developed (~30 min). The reaction was stopped with $H_2O$, the plates were allowed to dry overnight, and spot-forming cells were enumerated by counting under a low-power dissecting microscope (Leica, Buffalo, NY).

### Cell-surface staining for analysis and B-lymphocyte cell sorting.

Abs for staining B lymphocytes were obtained from BD PharMingen (San Diego, CA): FITC-M290 anti-CD103 ($\alpha_E$ integrin), PE-FIB504 rat anti-$\beta_7$, PE-DATK 32 rat anti-mouse $\alpha_E\beta_7$, CyChrome-rat anti-mouse B220 (RA3-6B2), and APC-MEL 14 rat anti-mouse L-selectin. PE-goat anti-mouse IgA (Southern Biotechnology Associates) was used in some of the cell-sorting experiments. FL1, FL2, FL3, and FL4 parameters were set with CaliBrite beads (BD PharMingen), and compensations were set with FACSComp software (CellQuest, Becton Dickinson, San Jose, CA). Four-color analysis was performed using a FACSCalibur (BD Biosciences), and 10,000 events per sample were collected. Cell sorting for $B220^+$ B-cell subsets was performed using a FACSVantage with Turbo-Sort (BD Biosciences), and sorted B-cell subsets were evaluated in CT-B-specific and total IgA or IgG ELISPOT assays.

### *In vitro* activation of B cells with LPS and CT.

To assess whether CT induces $\alpha_E$ expression, whole-cell cultures of HNLN, PP, and spleens were performed from individual L-$Sel^{-/-}$ and C57BL/6N mice. Single-cell suspensions were prepared, as described above, and were either left unactivated or cultured with 4.0 μg $ml^{-1}$ LPS (*Escherichia coli* O55:B5, List Biologicals, Campbell, CA) or 1.0 μg $ml^{-1}$ LPS + 5.0 μg $ml^{-1}$ CT for 24, 48, or 72 h. Cells were harvested and stained for coexpression of $\alpha_E$ and $\beta_7$ by $B220^+$ TCR-$\beta^a$ B cells.

### Statistical analysis.

Results were analyzed using analysis of variance followed by a multigroup comparison test, and *P*-values ⩽0.05 are indicated.

## ACKNOWLEDGMENTS

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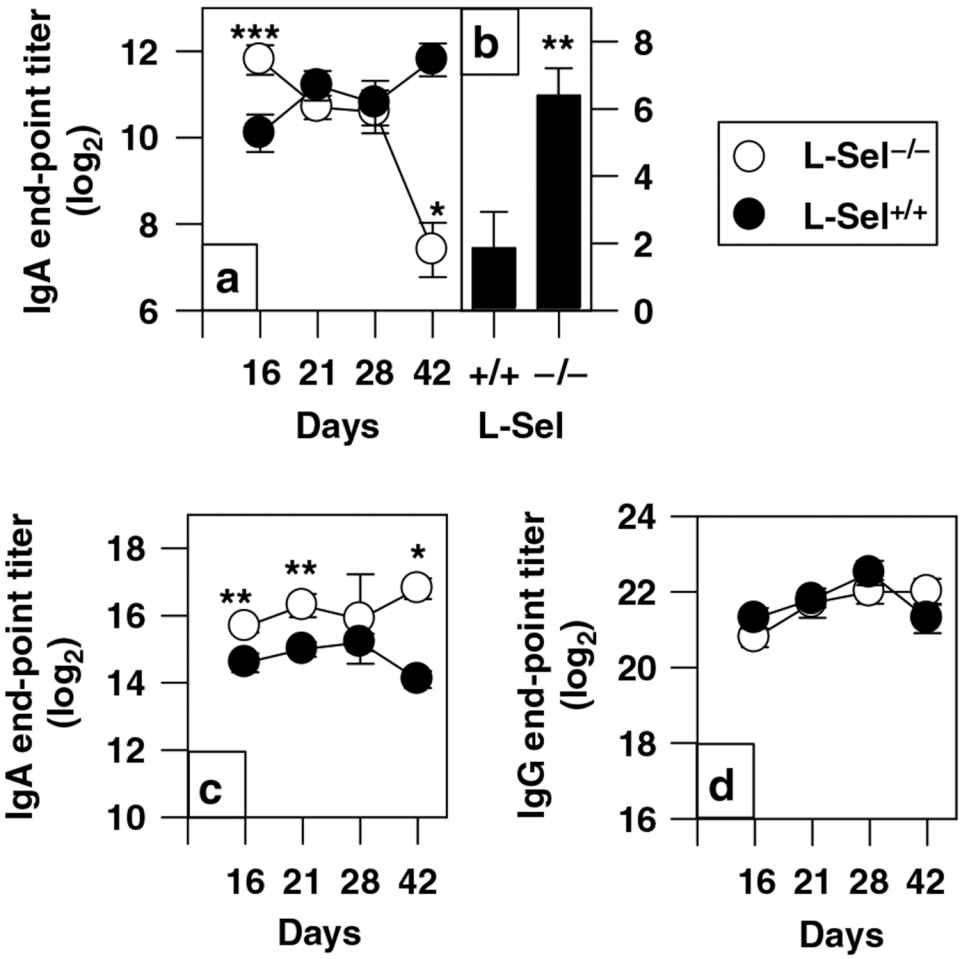


**Figure 1.**

Rapid onset mucosal IgA in L-Sel$^{-/-}$ mice. Depicted are (**a**) fecal IgA and serum (**c**) IgA and (**d**) IgG anti-CT-B titers following oral immunization of L-Sel$^{-/-}$ mice with CT. L-Sel$^{-/-}$ and L-Sel$^{+/+}$ mice were orally immunized on days 0, 7, and 14. Serum and mucosal secretions were collected and assessed for immune Abs by CT-B ELISA. Although L-Sel$^{-/-}$ fecal IgA anti-CT-B Abs decreased at 42 days, (**b**) S-IgA in nasal washes from L-Sel$^{-/-}$ mice remained elevated. Results up to 42 days after primary immunization are depicted as the mean of 10 mice ±s.e.m., and statistical differences between L-Sel$^{-/-}$ and L-Sel$^{+/+}$ mice were determined: *$P < 0.001$; **$P = 0.006$; ***$P = 0.021$. Ab, antibody; CT, cholera toxin; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; L-Sel, L-selectin.

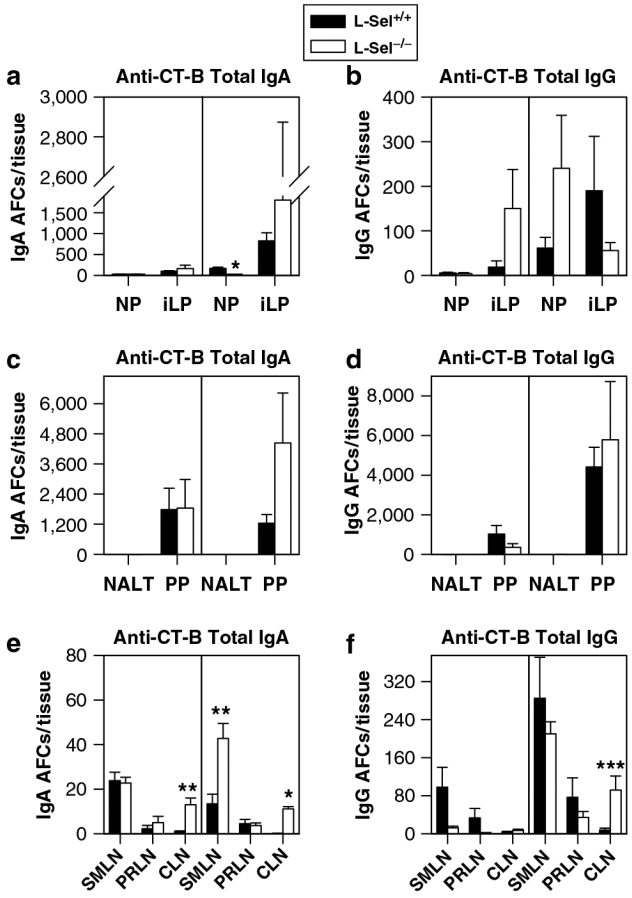


**Figure 2.**

Oral immunization of L-Sel$^{-/-}$ mice with CT results in increased IgA AFC responses in CLN 16 days post-primary immunization. L-Sel$^{-/-}$ and L-Sel$^{+/+}$ mice were orally immunized, as previously described, and on day 16, total lymphocytes from (**a**, **b**) NP and small iLP, (**c**, **d**) NALT and PP, and (**e**, **f**) SMLN, PRLN, and CLN were collected and assayed for anti-CT-B and total (**a**, **c**, **e**) IgA and (**b**, **d**, **f**) IgG AFC responses. CT-B-specific and total IgA AFC responses were slightly enhanced in L-Sel$^{-/-}$ CLN as well as total IgG AFCs. The number of IgA and IgG CT-B-specific AFCs in the other L-Sel$^{-/-}$ HNLN, NALT, PP, NP, and iLP were not significantly different from L-Sel$^{+/+}$ mice. Results depict the mean of three experiments ±s.e.m. per tissue, and statistical differences between L-Sel$^{-/-}$ and L-Sel$^{+/+}$ mice were determined: *$P < 0.001$; **$P \leqslant 0.003$; ***$P = 0.031$. AFC, antibody-forming cell; CLN, cervical lymph node; CT, cholera toxin; HNLN, head and neck lymph node; Ig, immunoglobulin; iLP, intestinal lamina propria; L-Sel, L-selectin; NALT, nasal-associated lymphoid tissue; NP, nasal passage; PP, Peyer's patch; PRLN, parotid gland lymph node; SMLN, submaxillary gland lymph node.

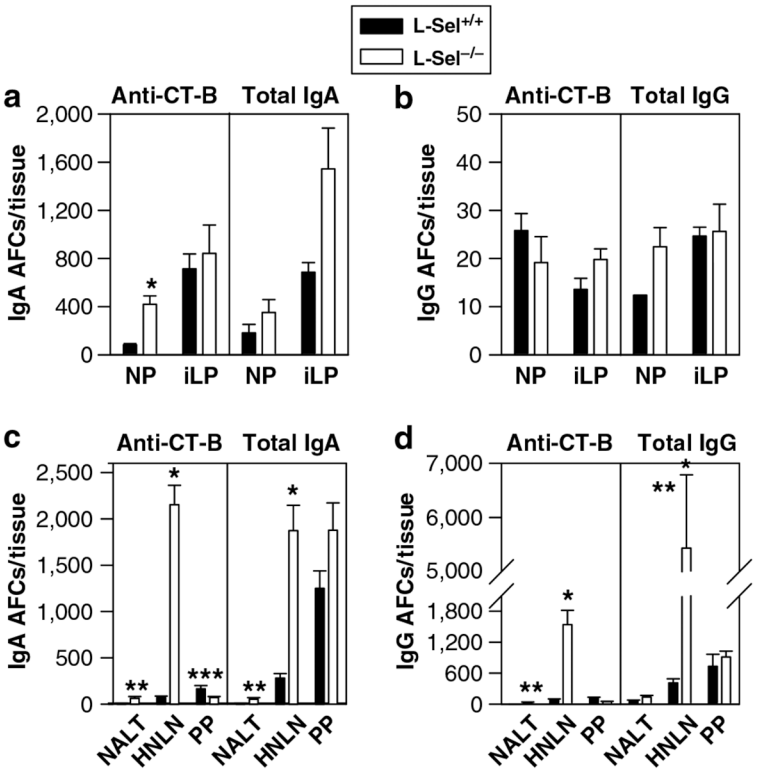


**Figure 3.**

Oral immunization of L-Sel$^{-/-}$ mice with CT results in enhanced IgA AFC responses in NP 42 days post-primary immunization and supported by increases in NALT and HNLN IgA AFCs. L-Sel$^{-/-}$ and L-Sel$^{+/+}$ mice were orally immunized, as previously described, and on day 42, total lymphocytes from (**a**, **b**) NP and iLP and (**c**, **d**) NALT, HNLN, and PP were collected and assayed for anti-CT-B and total (**a**, **c**) IgA and (**b**, **d**) IgG AFC responses. Each L-Sel$^{-/-}$ HNLN was combined and assessed for anti-CT-B activity. CT-B-specific IgA AFC responses were elevated in L-Sel$^{-/-}$ mice when compared to L-Sel$^{+/+}$ mice. IgA and IgG iLP CT-B-specific AFC responses were not significantly different between L-Sel$^{+/+}$ and L-Sel$^{-/-}$ mice; however, dramatic increases in L-Sel$^{-/-}$ NALT and HNLN CT-B-specific IgA and IgG AFC responses were observed when compared to L-Sel$^{+/+}$ mice. Total IgA and IgG AFCs in L-Sel$^{-/-}$ HNLN and total IgG AFCs in L-Sel$^{-/-}$ NALT were also elevated when compared to L-Sel$^{+/+}$ mice. Depicted are the mean AFC responses of three experiments ±s.e.m. per tissue, and statistical differences between L-Sel$^{-/-}$ and L-Sel$^{+/+}$ mice were determined: *$P < 0.001$; **$P \leqslant 0.014$; ***$P = 0.025$. AFC, antibody-forming cell; CT, cholera toxin; HNLN, head and neck lymph node; Ig, immunoglobulin; iLP, intestinal lamina propria; L-Sel, L-selectin; NALT, nasal-associated lymphoid tissue; NP, nasal passage; PP, Peyer's patch; PRLN, parotid gland lymph node; SMLN, submaxillary gland lymph node.

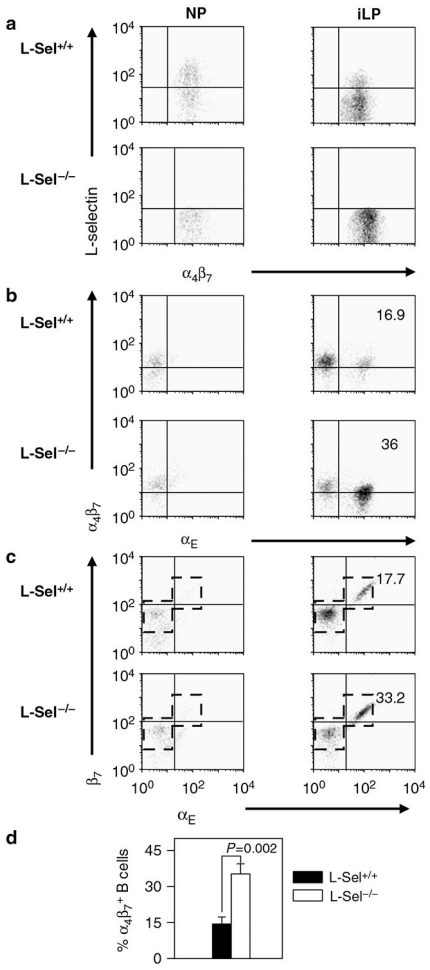


**Figure 4.**

Increased presence of $\alpha_E\beta_7^+$ iLP B cells in the L-Sel$^{-/-}$ mice 6 weeks post-primary oral immunization with CT. L-Sel$^{+/+}$ and L-Sel$^{-/-}$ NP and iLP B cells were analyzed for expression of (**a**) L-Sel and $\alpha_4\beta_7$, (**b**) $\alpha_4\beta_7$ and $\alpha_E$, and (**c**) $\beta_7$ and $\alpha_E$. $\alpha_E\beta_7^+$ B cells remained as a minor subset in the NP, but in the L-Sel$^{-/-}$ iLP they showed (**d**) ~2.4-fold greater percentage than that in L-Sel$^{+/+}$ iLP. (**c**) Boxed histographs represent the populations sorted for B-cell ELISPOT in Figure 5. Data are representative of three experiments except in **d**, which shows the mean±s.e.m. of three experiments. CT, cholera toxin; ELISPOT, enzyme-linked immunosorbent spot; iLP, intestinal lamina propria; L-Sel, L-selectin; NP, nasal passage.

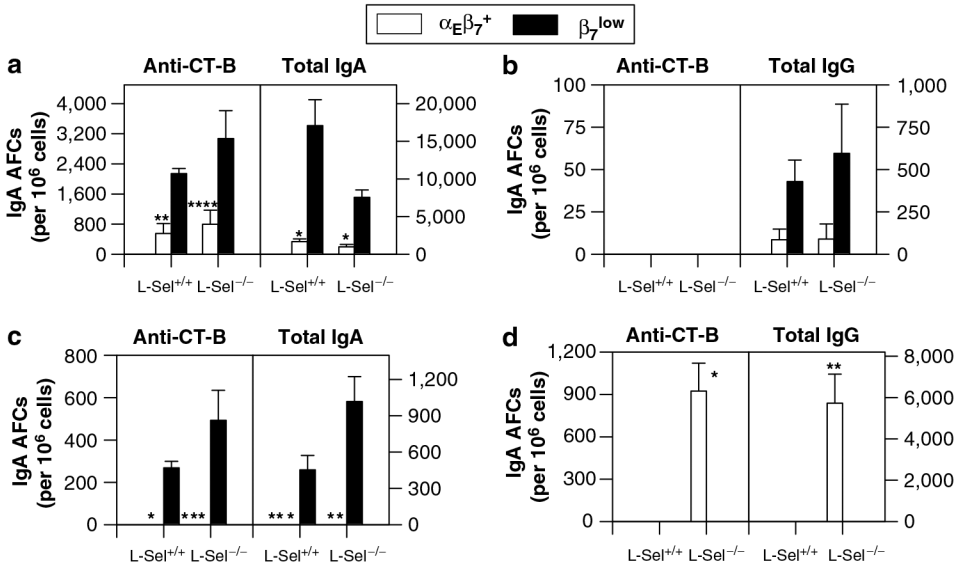


**Figure 5.**

Effector CT-B-specific B cells were mostly L-Sel$^{low}$/$\beta_7^{low}$ in the iLP and NP at 42 days post-primary immunization. Cell-sorting experiments were conducted sorting (**a**, **b**) iLP and (**c**, **d**) NP lymphocytes for $\beta_7$ vs. $\beta_7^{high}$ and $\alpha_E$ expression and assessed by CT-B-specific (left panel) and total (right panel) (**a**, **c**) IgA and (**b**, **d**) IgG ELISPOT. No $\beta_7^{high}$ B cells were found in either L-Sel$^{+/+}$ or L-Sel$^{-/-}$ NP; all the iLP $\beta_7^{high}$ B cells were $\alpha_E\beta_7^+$, not $\alpha_4\beta_7^{high}$, and the $\beta_7^{low}$ B cells were all L-Sel$^{low}$/$\beta_7^{low}$. The majority of the CT-B-specific iLP IgA AFCs were $\beta_7^{low}$. For both L-Sel$^{+/+}$ and L-Sel$^{-/-}$ mice, the L-Sel$^{low}$/$\beta_7^{low}$ B-cell subset contained all of the NP IgA anti-CT-B activity, and for L-Sel$^{-/-}$ mice, $\alpha_E\beta_7^+$ B cells contained the IgG anti-CT-B and the total IgG AFCs. Results depict the mean of three experiments ±s.e.m., and statistical differences between $\beta_7^{low}$ and $\alpha_E\beta_7^+$ B cells were determined: *$P \leqslant 0.002$; **$P \leqslant 0.008$; ***$P < 0.026$; ****$P < 0.05$. AFC, antibody-forming cell; CT, cholera toxin; ELISPOT, enzyme-linked immunosorbent spot; Ig, immunoglobulin; iLP, intestinal lamina propria; L-Sel, L-selectin; NALT, nasal-associated lymphoid tissue; NP, nasal passage.

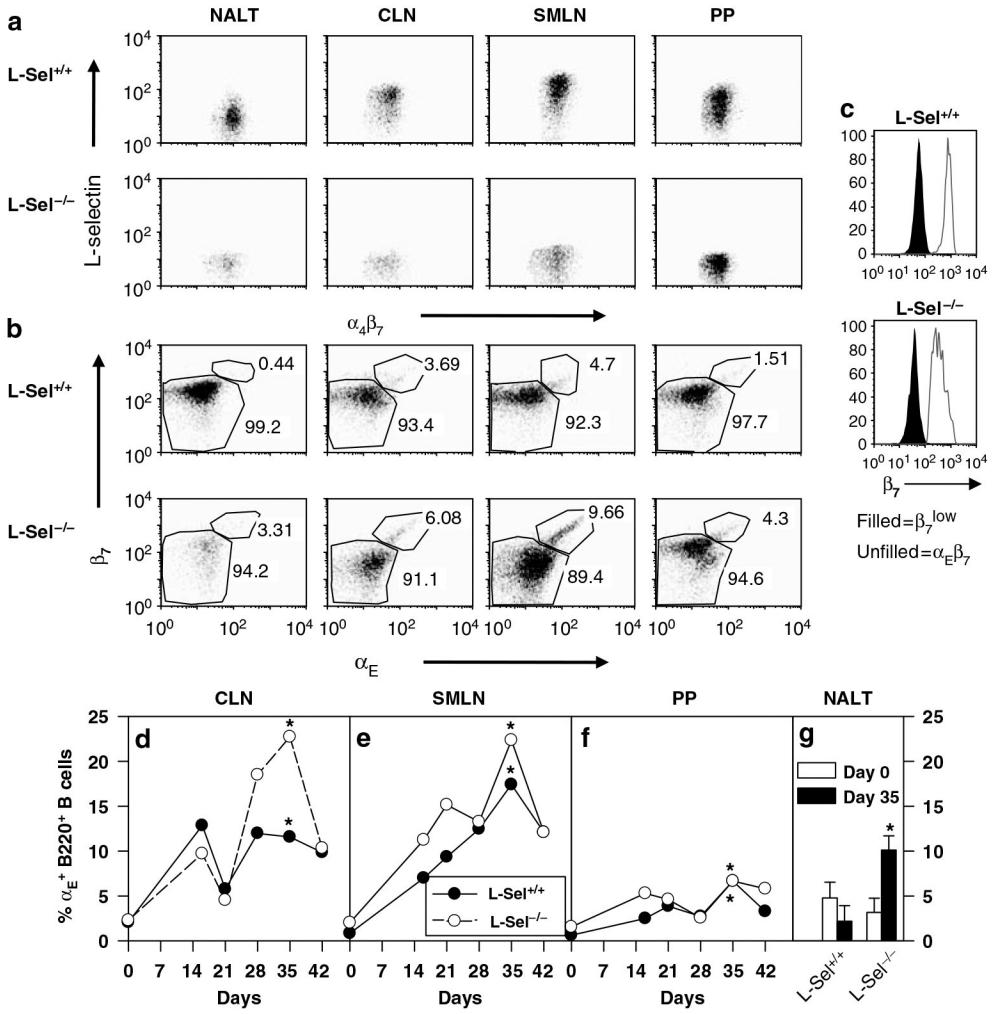


**Figure 6.**

$\alpha_E\beta_7^+$ B cells appear late in mucosal inductive tissues after primary oral immunization with CT in both L-Sel$^{+/+}$ and L-Sel$^{-/-}$ mice. L-Sel$^{+/+}$ and L-Sel$^{-/-}$ NALT, CLN, SMLN, and PP B cells were analyzed for expression of (**a**) L-Sel and $\alpha_4\beta_7$ and (**b**) $\beta_7$ and $\alpha_E$ at 6 weeks post-primary immunization. Expression for $\alpha_E\beta_7$ was evident in each of the mucosal inductive tissues examined. (**c**) Cell-sorting profiles show that the sorted $\beta_7^{low}$ vs. $\alpha_E\beta_7$ B cells were greater than 95% pure. (**d–g**) A kinetic analysis was performed to determine when these $\alpha_E\beta_7^+$ B cells are induced in the (**d**) CLN, (**e**) SMLN, (**f**) PP, and (**g**) NALT. Less than 5% $\alpha_E\beta_7^+$ B cells are present in naive mucosal inductive tissues, but these increase with time, peaking at day 35 post-primary immunization. Each time point represents pooled tissues from five individual mice, and differences between days 0 and 35 were evaluated: $^*P < 0.001$. CLN, cervical lymph node; CT, cholera toxin; ELISPOT, enzyme-linked immunosorbent spot; Ig, immunoglobulin; iLP, intestinal lamina propria; L-Sel, L-selectin; NALT, nasal-associated lymphoid tissue; NP, nasal passage; PP, Peyer's patch; SMLN, submaxillary gland lymph node.

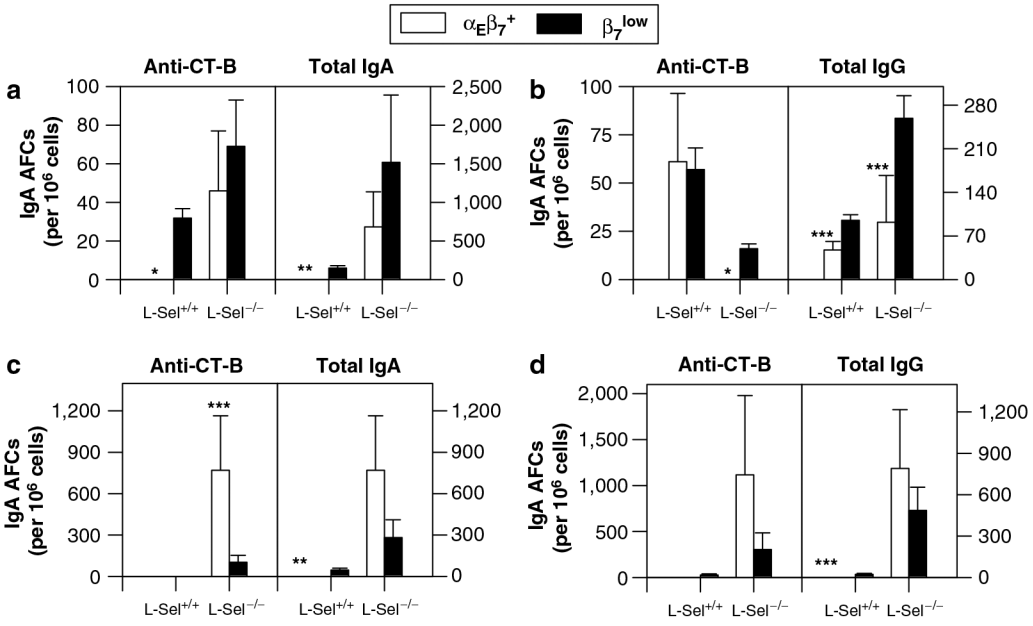


**Figure 7.**

The increased numbers of B cells in the L-Sel$^{-/-}$ HNLN at 42 days post-primary immunization are attributed to increased functional activity by $\alpha_E\beta_7^+$ B cells exhibiting both IgA and IgG anti-CT-B activity. Cell-sorting experiments were conducted on (**a**, **b**) PP and (**c**, **d**) HNLN lymphocytes for $\beta_7$ vs. $\beta_7^{high}$ and $\alpha_E$ expression and assessed by CT-B-specific (left panel) and total (right panel) (**a**, **c**) IgA and (**b**, **d**) IgG ELISPOT. (**a**, **b**) For PP CT-B-specific responses, all the L-Sel$^{+/+}$ IgA AFCs were $\alpha_4\beta_7^{low}$, and IgG AFCs were equally divided between $\alpha_4\beta_7^{low}$ and $\alpha_E\beta_7^+$ subsets. The L-Sel$^{-/-}$ PP IgA AFCs were equally divided between both subsets, and no $\alpha_E\beta_7^+$ IgG anti-CT-B AFCs were detected. (**c**, **d**) For the HNLN CT-B-specific responses, very few to no IgA or IgG AFCs were detected in L-Sel$^{+/+}$ mice. In contrast, the majority of the IgA AFCs were $\alpha_E\beta_7^+$, and the IgG AFC responses were composed of either $\alpha_E\beta_7^+$ or $\alpha_4\beta_7^{low}$ subset. Thus, sustained and elevated IgA anti-CT-B responses are attributed to the $\alpha_E\beta_7^+$ Ag-reactive B cells found late in L-Sel$^{-/-}$ HNLN following oral immunization with CT. The results depict the mean of three experiments ±s.e.m., and statistical differences between $\beta_7^{low}$ and $\alpha_E\beta_7^+$ B cells were determined: *$P \leqslant 0.008$; **$P = 0.02$; ***$P \leqslant 0.048$. AFC, antibody-forming cell; Ag, antigen; CT, cholera toxin; ELISPOT, enzyme-linked immunosorbent spot; HNLN, head and neck lymph node; Ig, immunoglobulin; L-Sel, L-selectin; PP, Peyer's patch.

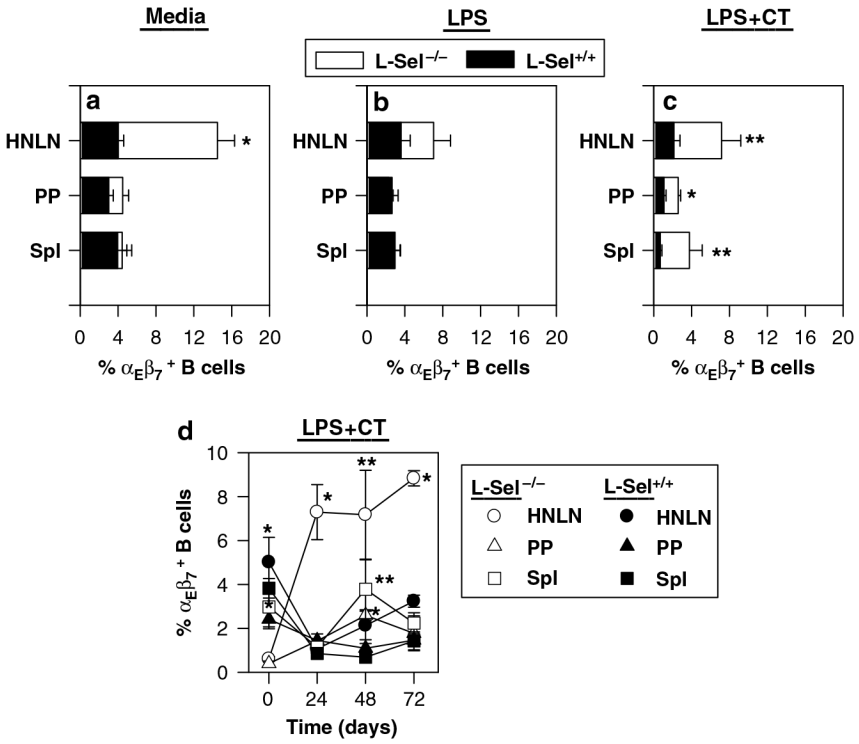


**Figure 8.**

L-Sel$^{-/-}$ HNLN shows increased expression of $\alpha_E\beta_7^+$ B cells. Total HNLN, PP, and Spl lymphocytes were cultured (**a**) without activation or activated with (**b**) LPS or (**c**) LPS plus CT for 48 h. B220$^+$ TCR-β$^-$ lymphocytes were then analyzed for $\alpha_E$ and $\beta_7$ expression. Increased percentages of $\alpha_E\beta_7^+$ B cells were observed with the (**a**) HNLN lymphocytes left unstimulated and (**c**) HNLN, PP, and Spl lymphocytes stimulated with LPS plus CT. (**d**) A kinetic analysis was performed and showed that L-Sel$^{-/-}$ HNLN $\alpha_E\beta_7^+$ B cells showed preferential enhancement at 24, 48, and 72 h with LPS plus CT treatment. Depicted data are the means of six individual mice ±s.e.m., and statistical differences between L-Sel$^{-/-}$ and L-Sel$^{+/+}$ mice were determined: *$P \leqslant 0.001$; **$P \leqslant 0.015$. CT, cholera toxin; HNLN, head and neck lymph node; LPS, lipopolysaccharide; L-Sel, L-selectin; PP, Peyer's patch; Spl, splenic; TCR-β, T-cell receptor-β.