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Impaired Mucosal Immunity in L-Selectin-Deficient Mice Orally Immunized with a *Salmonella* Vaccine Vector

David W. Pascual, 2 Michelle D. White, Trina Larson, and Nancy Walters

Lymphocyte trafficking in the gastrointestinal tract is primarily mediated by interactions with the mucosal addressin cell adhesion molecule 1 and its lymphocyte ligand, \( \alpha_4 \beta_7 \), and partly by L-selectin (L-Sel) interactions with peripheral node addressin coexpressed on some mucosal addressin cell adhesion molecule 1. We inquired whether intestinal responses in mice lacking L-Sel would be enhanced. L-Sel-deficient (L-Sel \(^{-/-}\)) mice were orally immunized with either *Salmonella* vaccine vector or *Salmonella* vector-expressing colonization factor Ag I (CFA/I) from enterotoxigenic *Escherichia coli*. In L-Sel \(^{-/-}\) mice, mucosal IgA anti-CFA/I fimbrial responses were greatly reduced, and systemic IgG2a anti-CFA/I fimbrial responses were 26-fold greater compared with C57BL/6 (L-Sel \(^{+/+}\)) mice. L-Sel \(^{-/-}\) Peyer’s patch (PP) CD4\(^{+}\) Th cells revealed IFN-\( \gamma \)-dominated responses and an unprecedented absence of IL-4, whereas the expected mixed Th cell phenotype developed in L-Sel \(^{+/+}\) mice. PP CD4\(^{+}\) Th cell anti-*Salmonella* responses were nearly nonexistent in L-Sel \(^{-/-}\) mice immunized with either *Salmonella* vaccine. Spleenic CD4\(^{+}\) Th cell anti-*Salmonella* responses were reduced but did show cytokine production in Ag restimulation assays. Increased colonization of PP and spleen was noted only with the *Salmonella* vector in L-Sel \(^{-/-}\) mice, resulting in increased splenomegaly, suggesting that the *Salmonella*-CFA/I vaccine was not as infectious or that the presence of the fimbriae improved clearance, possibly because of reduced neutrophil recruitment. However, sufficient anti-*Salmonella* immunity was induced, because *Salmonella* vector-immunized L-Sel \(^{-/-}\) mice showed complete protection against wild-type *Salmonella* challenge, unlike L-Sel \(^{-/+}\) mice. This evidence shows that L-Sel is important for development of mucosal immunity, and absence of L-Sel is protective against salmonellosis. *The Journal of Immunology*, 2001, 167: 407–415.

The mucosal barrier provides the first line of defense against enteric bacterial pathogens. Further penetration by a pathogen depends largely on whether the host’s adaptive immune system has been armed by previous exposure or vaccination. To prevent infection, oral vaccination offers the means to defend the gut-associated lymphoreticular tissue via Ag uptake by the mucosal inductive tissues, the Peyer’s patches (PP). It is in the PP that naive immune B and T cells are appropriately stimulated to initiate their differentiation and allow migration to gut effector tissues such as the intestinal lamina propria. Once recognition of the pathogen has occurred in the mucosal inductive tissue, the preferential return of protective lymphocytes to mucosal effector tissues is believed to occur via specific molecules referred to as cell adhesion molecules on lymphocyte cell surfaces. The conventional belief is that the mucosal and peripheral immune systems are separate entities with little crossover between them. This theory is largely supported by earlier observations that peripheral immunizations failed to adequately stimulate mucosal immune responses (1, 2). It was only mucosal immunization that would permit protective immunity. This observed segregation between the mucosal and peripheral immune systems is more appropriately defined by the expression of particular integrins (3–5). These integrins specifically interact with addressin molecules expressed on the high endothelial venules (HEV) to allow retention of specific lymphocyte subsets in either peripheral or mucosal tissues. For example, the HEV of peripheral lymph nodes express the peripheral node addressin (PNAd) that allows selective retention of lymphocytes expressing L-selectin (L-Sel) (6, 7). In contrast, the lymphocyte trafficking to the PP is dominated by \( \alpha_4 \beta_7 \) interactions with mucosal addressin cell adhesion molecule 1 (MAdCAM-1) expressed on PP HEV (3, 4), and in vitro assays show that lymphocyte attachment to PP HEV is blocked by mAbs to \( \alpha_4 \) or \( \beta_7 \) integrins (4, 8). However, PNAd also is expressed in the PP, but only associated with MAdCAM-1 (4, 9). The presence of PNAd on MAdCAM-1 suggests that L-Sel does contribute to lymphocyte migration in the PP. In fact, lymphocyte migration in PP was shown to be partially blocked by a mAb specific for L-Sel (10) and diminished in PP from L-Sel-deficient (L-Sel \(^{-/-}\)) mice (11–13). Thus, PNAd can contribute to trafficking in the PP via association to its cellular ligand L-Sel. More recently, it was shown that perhaps the nasal-associated lymphoid tissues exhibited physical traits resembling more of a peripheral rather than a mucosal lymphoid tissue (14). Such evidence of crossover in addressin use suggests that a distinction of action between the peripheral and mucosal immune systems may require additional consideration.

Recent work has focused on understanding mucosal immunity resulting from oral immunization with mucosal adjuvants to promote either enhanced Th2 cell-dependent (15–17) or Th1 cell-dependent (18–23) immunity. The recent development of live vector delivery systems (24–30) that take advantage of the invasive properties or intracellular requirement of the organism tends to bias host immunity along Th1 cell-dependent pathways. Only recently...
has it been shown that live vector systems can convert from Th1-type to Th2-type biases by mode of passenger Ag expression (24, 25). In fact, we have recently shown that the extracellular secretion of enterotoxigenic Escherichia coli fimbrial adhesin, colonization factor Ag I (CFA/I), by an attenuated Salmonella vaccine vector results in a biphasic Th cell response that supports elevated levels of specific secretory IgA (S-IgA) Abs (25). This response is characterized by an early, rapid induction of IL-4– and IL-5-dependent responses followed by an incremental induction of Th1 cell (IFN-γ)-dependent responses. The level of Th2-type immunity mimics what can be obtained by mucosally adjuvanted, soluble protein immunizations. Importantly, both the mucosal and systemic immune compartments are immunized by Salmonella vaccine vectors because Salmonella vectors are particularly adept at targeting mucosal inductive sites (31–33). Because of the nature of its pathogenicity, the Salmonella can ultimately reach the systemic immune compartment via the mesenteric lymph nodes, resulting in a resolvable bacteremia (31, 32).

With the recent generation of the L-Sel−/− mouse (11, 34–36), a number of studies have confirmed the important role L-Sel plays in peripheral lymphoid cell trafficking. Furthermore, a number of immune parameters have been evaluated to assess the role of L-Sel. L-Sel−/− mice show diminished delayed-type hypersensitivity responses (34) and primary Ag-specific T cell proliferative responses (34), but not T cell proliferative responses as a result of mitogen stimulation (37). This failure in Ag-specific T cell proliferative responses was not attributed to defective Ag presentation because this aspect remained functionally intact and capable of presenting Ag to wild-type T cells (35). The lack of L-Sel also had an impact on humoral responses and was dependent on the type of Ag and route of administration. No significant changes in Ab responses to keyhole limpet hemocyanin were evidenced in L-Sel−/− mice when compared with similarly immunized C57BL/6 (L-Sel+/+) mice (34). More pronounced augmentations in serum Ab to T cell-independent type 2 Ag was observed when Ag was given i.p. as opposed to s.c. (37).

Collectively, studies to date have focused on immune deficits derived subsequent to peripheral immunization in L-Sel−/− mice. With the noted L-Sel dependency observed in the nasal-associated lymphoid tissues (14), coupled with the observations that L-Sel does contribute to lymphocyte migration in the PP (10–13), we questioned the relevance of L-Sel on mucosal immunity. Although past studies have shown limited changes in Ag-specific responses subsequent to peripheral immunization, the importance of L-Sel in providing mucosal immunity to an infectious agent remained undetermined. This was of particular interest because it was recently shown that L-Sel is preferentially expressed by CD4+ Th1 cells regulated by IL-12 (38). Here we pose the question of whether a deficiency in L-Sel impacts mucosal immunity. To address this question, studies were focused on our Salmonella-CFA/I vaccine showing a biphasic CD4+ Th cell response with concomitant stimulation of elevated S-IgA Abs as a means to probe mucosal responses. Our results from oral immunization of L-Sel−/− mice with Salmonella-CFA/I vaccine show diminished mucosal, but not systemic, IgA responses to CFA/I fimbriae. Such reduction in S-IgA was linked to the remarkable absence of IL-4-producing CD4+ Th2 cells. Instead, elevated numbers of IFN-γ-producing CD4+ Th1 cells were induced. Furthermore, vaccinated mice orally challenged with wild-type Salmonella were completely protected in contrast to similarly vaccinated L-Sel+/+ mice. Hence, our data suggest that Th1 cell development is not impaired in the gut-associated lymphoreticular tissue, but rather Th2 cell development is substantially attenuated in L-Sel−/− mice.

Materials and Methods

Mice

Breeder pairs of homozygous L-Sel−/− mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME) and were bred and maintained at the Montana State University Animal Resource Center (Bozeman, MT). C57BL/6 L-Sel−/− mice also were obtained from The Jackson Laboratory. All mice were maintained in horizontal laminar flow cabinets, and sterile food and water were provided ad libitum. All animal care and procedures were in accordance with institutional policies for animal health and well-being.

Oral immunization with Salmonella typhimurium

The S. typhimurium-CFA/I vaccine vector, strain H696, and CFA/I fimbrial expression are maintained by plasmid with a functional asd gene to complement the lethal chromosomal Δasd mutation and stabilize CFA/I expression in the absence of antibiotic selection (20). As a result, CFA/I fimbriae are expressed on the S. typhimurium vector cell surface as functional fimbriae (39). L-Sel+/+ and L-Sel−/− mice (10/group) pretreated with an oral 50%-saturated sodium bicarbonate solution received a single oral dose of 5 × 10^8 CFU of the S. typhimurium-CFA/I construct or the plasmid control strain H647, which lacks the CFA/I operon. Amount of vaccine administered was confirmed by plating serial dilutions of the inoculum on Luria-Bertani agar plates.

Ab ELISA

CFA/I-specific end point titers from dilution of immune sera or fecal extracts (25) were determined by an ELISA as described previously with purified CFA/I fimbriae Ag (40). End point titers were expressed as the reciprocal dilution of the last sample dilution giving an absorbance ≥ 0.1 OD units above the OD_{415} of negative controls after a 1-h incubation.

Lymphoid cell isolation

Groups of mice were euthanized 4 wk subsequent to oral immunization to collect lymphoid tissues. Splenic lymphocytes were isolated by conventional methods (19, 20, 25). PP lymphocytes were isolated as described previously (19, 25). Both procedures yielded >95% viability with trypan blue exclusion. Enriched CD4+ T cell fractions were isolated by a negative selection procedure (25).

Cytokine ELISPOT assays

Cytokine secretion by stimulated lymphocytes was detected by the cytokine-specific ELISPOT assays (19, 25). Splenic and PP CD4+ T cells were cultured at 5 × 10^6 cells/ml with equal numbers of feeder cells treated with mitomycin C (50 µg/ml; Sigma, St. Louis, MO) in either medium only or medium with 10 µg/ml CFA/I fimbriae or 10 µg/ml of alkaline-treated Salmonella non-LPS extracts (41) for 2–3 days at 37°C. Subsequently, the cells were analyzed by cytokine-specific ELISPOT assays.

S. typhimurium colonization

L-Sel−/− and L-Sel+/+ mice were orally immunized with the Salmonella-CFA/I construct or the isogenic Salmonella vector. Two weeks subsequent to infection, spleens and PP were removed aseptically and weighed. Tissues were homogenized and plated onto MacConkey’s agar (Difco, Detroit, MI) and incubated overnight at 37°C, and colonies were subsequently counted.

Flow cytometry

To assess splenic neutrophil levels, spleens were homogenized as described previously, and single-cell suspensions of individual whole spleens were immunomostained with mAbs to mouse neutrophils, SK208 (kindly provided by Dr. M. A. Jutila, Veterinary Molecular Biology, Montana State University), a rat IgG2a mAb with similar specificity to SK105 (42), or RB6–8C5 mAbs (43). A cell-culture supernatant for SK208 mAb was reacted with the cells for 30 min on ice, and binding was detected with a 1/500 dilution of PE-conjugated goat anti-rat IgG Ab (Southern Biotechnology Associates, Birmingham, AL). After washing, cells were incubated with 1% rat serum for 20 min to bind free arms of the goat anti-rat IgG Ab. Then cells were incubated with a 1/200 dilution of biotinylated CD11b (Mac-1 chain) mAb (clone M1/70; BD Pharmingen, San Diego, CA) for 30 min on ice, and binding was detected with a 1/1000 dilution of streptavidin-Alexa 488 (Molecular Probes, Eugene, OR) for 30 min on ice. Neutrophils were identified as SK208+ and Mac-1+.
To discern differences in B and T lymphocyte levels subsequent to *S. typhimurium* vaccine immunization, splenic mononuclear cell preparations were obtained subsequent to Ficoll-Hypaque (Lymphocyte M; Accurate Chemical, Westbury, NY) gradient centrifugation (25). PP lymphocytes were obtained as described above. Splenic and PP lymphocytes were immobilostained with either a 1/200 dilution of FITC-conjugated B220 mAb (clone RA3-6B2; BD Pharamingen), FITC-conjugated anti-CD3 mAb, PE-conjugated anti-CD4 mAb (clone RM4-5; BD Pharamingen), PE-conjugated anti-CD8 mAb (clone 53-6.7; BD Pharamingen), or FITC-conjugated pan NK cell mAb (BD Pharamingen) for 30 min on ice. FL1 and FL2 gains and compensations were set by the analysis of single-color FITC or PE. Two-color analyses were performed with a FACSCalibur (BD Biosciences, Mountain View, CA), and up to 50,000 events/sample were collected.

Salmonella challenge studies

Wild-type *S. typhimurium* strain H71 was kindly provided by Dr. D. M. Hone (Institute of Human Virology, Medical Biotechnology Center, University of Maryland, Baltimore, MD). The wild-type *S. typhimurium* strain was cultured as described previously (44). Groups of L-Sel+/− and L-Sel+/+ mice were given sterile PBS (vehicle) orally or immunized orally with *Salmonella* vector only. Four weeks subsequent to immunization, mice were pretreated with 50%–saturated sodium bicarbonate solution and then infected with 5 × 10^7 CFU/0.2 ml H71 strain. Amount of bacteria given was confirmed by plating serial dilutions of bacterial suspensions on LB agar plates. Mice were observed twice daily and extent of survival was recorded for 2 wk.

Statistical analysis

The Student t test was used to evaluate differences between variations in Ab titers, cytokine production levels, tissue weights, and extent of colonization. A paired t test was performed to discern differences in neutrophil recruitment. The Kaplan-Meier method (GraphPad Prism; GraphPad, San Diego, CA) was applied to obtain the survival fractions after infection with a lethal dose of wild-type *S. typhimurium*. With the Mantel-Haenszel log rank test, the p value for statistical differences between vehicle and immunized mice was discerned at the 95% confidence interval.

Results

Oral Immunization of L-Sel+/− mice with Salmonella-CFA/I results in diminished Ag-specific mucosal IgA responses

L-Sel mediates naive lymphocyte homing to peripheral lymphoid tissues via its interaction with the HEV-expressing PNAδ (6, 7), which results in lymphocyte accumulation in peripheral lymph node. Although lymphocyte homing to PP is largely dependent on αβ,β−MADCAM-1 interactions, PNAδ also colocalizes with MADCAM-1, implicating the involvement of PNAδ-L-Sel to lymphocyte homing in the PP (4, 9, 10). Thus, L-Sel does contribute to lymphocyte trafficking in the mucosal compartment. To test the role of L-Sel on intestinal immunity, L-Sel−/− and L-Sel+/+ mice were orally immunized with either an attenuated *Salmonella* construct expressing CFA/I (strain H696) or the Salmonella vector only (strain H647). Four weeks subsequent to immunization, coproantibody and serum IgG titers were measured with a CFA/I-specific ELISA. Surprisingly, it was observed that the copro-IgA response was substantially diminished when compared with that obtained for L-Sel−/− mice (p = 0.003), whereas no significant differences were observed for serum IgA anti-CFA/I titers (Fig. 1A). The serum IgG and IgG subclass responses also varied. Serum IgG anti-CFA/I titers were 4-fold greater for the L-Sel−/− mice (p = 0.003) than those obtained for L-Sel+/+ mice (Fig. 1B). This enhancement in serum IgG titers was attributed primarily to the 26-fold greater IgG2a anti-CFA/I response (p < 0.001) by the L-Sel−/− vs L-Sel+/+ mice (Fig. 1B). Both the IgG1 and IgG2b anti-CFA/I responses were similar for L-Sel−/− and L-Sel+/+ mice (Fig. 1B). Thus, these results suggest that there are diminished mucosal IgA responses associated with L-Sel deficiency and an augmentation in systemic IgG immunity. Mice immunized with vector only resulted in no mucosal or serum anti-CFA/I Ab responses (data not shown), consistent with what has been observed previously (25).

Oral Immunization of L-Sel−/− mice with Salmonella-CFA/I construct induces attenuated mucosal and normal systemic CFA/I fimbiae-specific CD4+ Th cell responses

To inquire why the mucosal IgA responses were reduced, CFA/I-specific CD4+ T cell responses were evaluated from L-Sel+/− and L-Sel+/+ mice orally immunized with the Salmonella-CFA/I construct. To determine whether immune CD4+ T cells were induced in the PP, enriched CD4+ T cells from the PP and spleen were obtained. These cells were cultured by conventional means (25) and restimulated in vitro with purified CFA/I fimbiae. After 2–3 days of in vitro Ag restimulation, CD4+ T cells were analyzed by cytokine ELISPOT assays to quantitate CFA/I-specific cytokine responses (Fig. 2). Immune L-Sel−/− PP CD4+ T cells showed elevated numbers of IFN-γ spot-forming cells (SFC), representing a 5-fold increase when compared with those induced by CFA/I restimulated L-Sel+/− PP CD4+ T cells (p = 0.02; Fig. 2A). There was an absence of IL-4 (p = 0.02) but a substantial increase in the number of IL-10 SFC (p < 0.001) in L-Sel−/− mice when compared with PP L-Sel+/− CD4+ T cells. This increase in IL-10 SFC was most likely stimulated by the increased presence of IFN-γ. Such a combination of elevated IFN-γ and IL-10, along with the absence of IL-4, may account for the diminished mucosal IgA responses. In contrast, the systemic compartment showed no significant difference in the CD4+ Th cell cytokine responses when restimulated with CFA/I fimbiae (Fig. 2B). Because no significant differences were shown by the CFA/I-restimulated splenic CD4+ T cell cultures, this evidence suggests that the absence of L-Sel did not impact the induction of these CD4+ Th cell responses. Although there are differences in serum IgG2a anti-CFA/I fimbral Ab levels, the observed elevation by the immunized L-Sel−/− mice may be attributed to the derivation of

![](http://www.jimmunol.org/doi/fig/1)
immune B cells from the mucosal compartment because it is here that substantial increases in IFN-γ-producing cells were noted.

**Oral immunization of L-Sel−/− mice with Salmonella-CFA/I or Salmonella vector showed loss of Salmonella-specific CD4+ Th cell responses in PP**

To assess CD4+ T cell immunity to Salmonella Ags, immune PP CD4+ T cells from L-Sel−/− and L-Sel+/+ mice were evaluated by cytokine ELISPOT assays 4 wk after immunization. CD4+ T cells were restimulated in vitro with intracellular Salmonella Ags extracted with NaOH (41) from Salmonella H647. L-Sel−/+ PP CD4+ Th cells from mice immunized with Salmonella vector (strain H647) only showed elevations from three experiments (n = SEM) in IFN-γ (621 ± 61) and IL-6 (1057 ± 316) but also showed some coinduction of IL-4 (142 ± 43) and IL-10 (94 ± 15) SFC per 1 x 10^6 CD4+ T cells. These responses were statistically elevated for each cytokine SFC response when compared with those obtained in L-Sel−/− mice (Fig. 3, A–D). Salmonella Ag-restimulated L-Sel−/− PP CD4+ T cells showed greatly reduced IFN-γ (12.5 ± 4.2), IL-6 (10.5 ± 0.71), and no IL-4 or IL-10 SFC responses (Fig. 3, A–D). This evidence suggests that the Salmonella vector is not being recognized in the mucosal compartment of L-Sel−/− mice. To inquire whether the addition of the immunogenic CFA/I fimbriae to the Salmonella would improve recognition of Salmonella Ags, PP CD4+ Th cell responses from L-Sel−/+ mice were analyzed. They showed reduced cytokine SFC responses in comparison to the same type of mice immunized with Salmonella vector only (Fig. 3, A–D). Nonetheless, PP CD4+ Th cell responses from Salmonella-CFA/I-vaccinated L-Sel−/− mice on Salmonella Ag-restimulation showed no IFN-γ, IL-6, or IL-10, and minimal IL-4 (2 ± 0.71) SFC per 1 x 10^6 CD4+ T cells. Thus, the weak mucosal IgA responses may partly be attributed to the failure by the Salmonella-CFA/I vector to stimulate PP CD4+ Th cells in L-Sel−/− mice.

**FIGURE 2.** Oral immunization with the Salmonella-CFA/I vaccine shows enhanced numbers of IFN-γ-producing CD4+ Th cells in the mucosal compartment compared with the systemic immune compartment. PP (A) and splenic CD4+ T cell (B) populations were harvested 4 wk after oral immunization and restimulated with CFA/I fimbriae in the presence of feeder cells for 2 days and then examined for cytokine secretion by the cytokine ELISPOT method. L-Sel−/− mice showed both Th1- and Th2-type responses to CFA/I fimbriae. In contrast, the L-Sel−/− mice showed elevated numbers of IFN-γ-producing and IL-10-producing, but not IL-4-producing, CD4+ Th cells, whereas no significant changes in splenic CD4+ Th cell responses were observed between L-Sel−/− and L-Sel+/+ mice. Values are expressed as the mean ± SEM of two experiments of SFC per 1 x 10^6 CD4+ T cells. Values were corrected for cells cultured in the absence of CFA/I fimbriae. *, p < 0.001; **, p = 0.02.

**Oral immunization of L-Sel−/− mice with Salmonella-CFA/I or Salmonella vector showed reduced Salmonella-specific CD4+ Th cell responses in spleen**

To ascertain whether similar attenuations in mucosal Salmonella-specific CD4+ Th cells derived from L-Sel−/− mice would hold true for their splenic responses, CD4+ Th cell anti-Salmonella responses from mice orally immunized with either Salmonella-CFA/I or Salmonella vector were assessed. Splenes were harvested from the same mice described previously. As depicted in Fig. 3, E–H, splenic CD4+ Th cells from L-Sel−/− mice immunized with Salmonella vector showed, subsequent to in vitro restimulation with Salmonella Ag, elevations in IFN-γ (569 ± 130) and IL-6 (1054 ± 24) SFC per 1 x 10^6 CD4+ T cells, with detectable increases in IL-4 (8.3 ± 1.1) and IL-10 (14.6 ± 2.1). L-Sel−/− mice orally immunized with the Salmonella vector did show ~4-fold reductions in IFN-γ (162 ± 102) and IL-6 (248 ± 129) SFC per 1 x 10^6 CD4+ T cells (Fig. 3, E–H). Minimal changes were observed in IL-10 SFC, and no significant difference was noted in IL-4 SFC. This evidence suggests that at least in the...
systemic compartment, the Salmonella vector is being recognized by L-Sel^{-/-} mice.

Examination of the differences in splenic cytokine SFC responses against Salmonella Ags by mice orally immunized with Salmonella-CFA/I showed similar reduction in the magnitude of cytokine responses in L-Sel^{-/-} mice. The L-Sel^{+/+} mice displayed the expected Th responses dominated by IFN-γ and co-induction of IL-4, IL-6, and IL-10 (Fig. 3, E–H). Although a mixed Th cell response was observed for mice orally immunized with Salmonella-CFA/I, the IFN-γ SFC responses were reduced by 3.5-fold, and a significant increase by 66% in the number of IL-4 SFC responses was observed, whereas IL-6 and IL-10 SFC responses were significantly reduced (Fig. 3, E–H). Unlike the mucosal compartment, the systemic compartment retained the ability to recognize the Salmonella vectors, although there was still an impairment in this recognition when compared with L-Sel^{+/+} mice.

The reduced CD4^{+} Th cell responses to the Salmonella Ags were not attributed to reduced levels of CD4^{+} Th cells in the spleen (Table I) or PP (Table II). Mononuclear splenic and PP cell fractions were obtained from L-Sel^{-/-} and L-Sel^{+/+} mice orally immunized 2 wk earlier and immunostained to analyze differences in stimulation of CD4 and CD8 T cell fractions. L-Sel^{-/-} mice orally immunized with the Salmonella vector showed a 50% increase of splenic CD4^{+} T cells when compared with naive control, and more so than that observed for immunized L-Sel^{+/+} mice (Table I). Similar increases also were obtained for CD8^{+} T cells. No significant increases in CD4^{+} T cells were observed for either L-Sel^{-/-} and L-Sel^{+/+} mice orally immunized with the Salmonella-CFA/I vaccine (Table I). No significant reductions in PP CD4^{+} T cells for either Salmonella vector- or Salmonella CFA/I-immunized L-Sel^{-/-} or L-Sel^{+/+} mice (Table II). A slight reduction in the percentage of CD8^{+} T cells in Salmonella vector-immunized L-Sel^{-/-} mice was observed. Thus, the noted reduction in responsiveness by CD4^{+} Th cells was not attributable to a lack of CD4^{+} T cells.

Expression of the CFA/I fimbriae by Salmonella reduces the extent of splenomegaly caused by Salmonella infection

Tissue weights of PP and spleens were measured from Salmonella vector-infected and Salmonella-CFA/I-infected L-Sel^{+/+} and L-Sel^{-/-} mice. These evaluations were conducted to discern whether either Salmonella strain showed increased infectivity in L-Sel^{-/-} mice. In uninfected L-Sel^{-/-} mice, the spleens were enlarged by ~58% compared with L-Sel^{+/+} mice (p < 0.001) when PP were not significantly different in weights (Fig. 4). Evaluation of weights throughout the infection (21 days) with Salmonella vector (Fig. 4A) showed significant increases in the splenic tissues for both L-Sel^{-/-} and L-Sel^{+/+} mice and only moderate changes in the L-Sel^{-/-} PP. Although increases were observed in the spleen, the L-Sel^{-/-} mice did show the most dramatic increases by 21 days, with nearly 400% in some instances, whereas the L-Sel^{+/+} mice showed, at most, a 69% increase. The increases of splenic weights for L-Sel^{-/-} and L-Sel^{+/+} mice were not as dramatic when orally immunized with the Salmonella-CFA/I vaccine (Fig. 4B). The L-Sel^{+/+} mice showed only a slight increase on 14 and 21 days postinfection of not >27%. The splenic weights for L-Sel^{-/-} mice only significantly increased 14 days postinfection, showing a moderate increase of 40% (p = 0.012). PP from neither the L-Sel^{-/-} nor L-Sel^{+/+} mice immunized with Salmonella-CFA/I vaccine showed any increases in weight (Fig. 4B). In fact, the L-Sel^{+/+} showed a significant reduction by 22% (p = 0.003) at 14 days postinfection. Thus, these data suggest that an inflammatory response is greater in the spleens of the Salmonella vector-infected L-Sel^{-/-} mice than in the spleens of the L-Sel^{+/+} mice. Moreover, the presence of the CFA/I fimbriae on the Salmonella also reduces these inflammatory responses. This is particularly striking by the reduced splenic weights of L-Sel^{-/-} mice when orally immunized with the Salmonella-CFA/I vaccine and compared with the same mice immunized with Salmonella vector. Although not as dramatic, similar comparison was evident in L-Sel^{+/+} mice.

The absence of L-Sel delays clearance of Salmonella vector from the PP

To assess whether the increased splenomegaly caused by vaccination with the Salmonella vaccines is attributable to increased colonization, both L-Sel^{+/+} and L-Sel^{-/-} mice were orally immunized with Salmonella vector only. Two weeks after immunization, PP and spleens were procured and serial dilutions of homogenates were assessed for extent of colonization on MacConkey’s agar. L-Sel^{-/-} mice orally immunized with the Salmonella vector did show a significant increase in extent of colonization by greater than 5-fold (p = 0.008) when compared with similarly immunized L-Sel^{+/+} mice (Fig. 5A). Likewise, the spleens of the L-Sel^{-/-} mice did show a nearly 3-fold increase (p = 0.026) in the extent of colonization. Thus, the increased colonization by the Salmonella vector suggests that L-Sel^{-/-} mice may not be able to clear the Salmonella infection as rapidly as L-Sel^{+/+} mice. Degree of colonization also was assessed in Salmonella-CFA/I vaccinated mice. There were no significant differences in the extent of colonization of PP or spleen by L-Sel^{-/-} mice (Fig. 5B). This evidence suggests that the expression of the CFA/I fimbriae reduces the degree of colonization by both L-Sel^{+/+} and L-Sel^{-/-} mice, thereby limiting the degree of inflammation.

Table I. FACS analysis of splenic lymphocytes from L-Sel^{+/+} and L-Sel^{-/-} mice orally immunized with Salmonella vector (H647) or Salmonella-CFA/I (H696) control

<table>
<thead>
<tr>
<th>% B220⁺</th>
<th>% CD3⁺</th>
<th>% CD4⁺</th>
<th>% CD8⁺</th>
<th>% NK⁺</th>
<th>% CD4⁺ NK1.1⁺</th>
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</thead>
<tbody>
<tr>
<td>L-Sel^{+/+}</td>
<td></td>
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</tr>
<tr>
<td>Normal</td>
<td>73.4 ± 1.2</td>
<td>19.9 ± 1.4</td>
<td>12.6 ± 1.1</td>
<td>8.5 ± 0.35</td>
<td>4.1 ± 0.26</td>
</tr>
<tr>
<td>H647-infected</td>
<td>68.7 ± 1.4 (0.012)</td>
<td>18.9 ± 4.3 (NS)</td>
<td>13.9 ± 0.6 (NS)</td>
<td>5.3 ± 0.75 (0.003)</td>
<td>3.2 ± 0.81 (NS)</td>
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<tr>
<td>H696-infected</td>
<td>67.2 ± 3.0 (0.029)</td>
<td>22.4 ± 5.1 (NS)</td>
<td>14.6 ± 2.1 (NS)</td>
<td>7.9 ± 3.70 (NS)</td>
<td>5.3 ± 0.66 (0.043)</td>
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<tr>
<td>L-Sel^{-/-}</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Normal</td>
<td>68.5 ± 5.7</td>
<td>23.0 ± 5.0</td>
<td>18.1 ± 3.0</td>
<td>7.2 ± 0.85</td>
<td>3.8 ± 0.25</td>
</tr>
<tr>
<td>H647-infected</td>
<td>52.7 ± 2.9 (0.002)</td>
<td>41.8 ± 5.2 (0.002)</td>
<td>27.1 ± 2.6 (0.004)</td>
<td>14.0 ± 2.30 (0.003)</td>
<td>4.0 ± 0.61 (NS)</td>
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<tr>
<td>H696-infected</td>
<td>61.5 ± 3.3 (NS)</td>
<td>32.8 ± 4.2 (0.024)</td>
<td>24.1 ± 3.8 (NS)</td>
<td>10.2 ± 1.60 (0.026)</td>
<td>5.1 ± 0.79 (0.036)</td>
</tr>
</tbody>
</table>

* Splenic mononuclear cells from normal or orally immunized L-Sel^{+/+} or L-Sel^{-/-} mice were immunostained to determine levels of the various subsets 2 wk after immunization. Values represent the mean ± SD of five mice per group.
Table II. FACS analysis of PP lymphocytes from L-Sel+/+ and L-Sel−/− mice orally immunized with Salmonella vector (H647) or Salmonella-CFA/I (H696) construct

<table>
<thead>
<tr>
<th></th>
<th>% B220+</th>
<th>% CD3+</th>
<th>% CD4+</th>
<th>% CD8+</th>
<th>% NK+</th>
<th>% CD4+ NK.1.1+</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Sel+/+</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Normal</td>
<td>80.6 ± 0.8</td>
<td>16.4 ± 2.2</td>
<td>12.7 ± 0.5</td>
<td>4.2 ± 0.23</td>
<td>1.9 ± 0.51</td>
<td>0.11 ± 0.09</td>
</tr>
<tr>
<td>H647-infected</td>
<td>68.1 ± 3.2 (&lt;0.001)</td>
<td>23.8 ± 2.5 (0.004)</td>
<td>11.8 ± 1.1 (NS)</td>
<td>4.8 ± 0.99 (NS)</td>
<td>3.4 ± 0.39 (0.003)</td>
<td>0.19 ± 0.03 (NS)</td>
</tr>
<tr>
<td>H696-infected</td>
<td>76.7 ± 2.2 (0.016)</td>
<td>17.2 ± 1.9 (NS)</td>
<td>12.2 ± 1.0 (NS)</td>
<td>4.0 ± 1.0 (NS)</td>
<td>3.7 ± 0.45 (0.002)</td>
<td>0.16 ± 0.05 (NS)</td>
</tr>
<tr>
<td>L-Sel−/−</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>68.2 ± 2.9</td>
<td>26.9 ± 2.8</td>
<td>17.3 ± 1.2</td>
<td>8.9 ± 1.41</td>
<td>5.0 ± 0.72</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>H647-infected</td>
<td>60.1 ± 13.2 (NS)</td>
<td>29.6 ± 6.9 (NS)</td>
<td>15.6 ± 2.2 (NS)</td>
<td>6.4 ± 0.92 (0.025)</td>
<td>9.4 ± 3.74 (0.032)</td>
<td>0.39 ± 0.14 (NS)</td>
</tr>
<tr>
<td>H696-infected</td>
<td>64.6 ± 9.2 (NS)</td>
<td>26.1 ± 7.1 (NS)</td>
<td>17.7 ± 5.4 (NS)</td>
<td>8.6 ± 1.80 (NS)</td>
<td>3.4 ± 0.91 (0.033)</td>
<td>0.23 ± 0.14 (NS)</td>
</tr>
</tbody>
</table>

a) PP mononuclear cells from normal or orally immunized L-Sel+/+ or L-Sel−/− mice were immunostained to determine levels of the various subsets 2 wk after immunization. Values represent the mean ± SD of five mice per group.

The observed splenomegaly may be attributed in part to reduced ability to recruit neutrophils

From the previous studies, it is clear that the adaptive immune system in the L-Sel−/− mice remained functional in the systemic compartment as evidenced by the B and CD4+ T cell responses to the CFA/I fimbriae. Because increased colonization of the spleen was observed with L-Sel−/− mice orally immunized with the Salmonella vector, we questioned whether neutrophil recruitment may be affected by the absence of L-Sel (45, 46). Whole splenic cell preparations were obtained from unimmunized, Salmonella vector-immunized, and Salmonella-CFA/I-immunized L-Sel+/+ and L-Sel−/− mice, and neutrophil population was defined by immunostaining with Mac-1 and SK208 mAbs. Normal splenocytes from L-Sel−/− mice showed increased colonization of the spleen. Both L-Sel−/− and L-Sel+/+ mice were orally immunized with either Salmonella vector (H647) or Salmonella-CFA/I vector (H696) to measure changes in weight. L-Sel−/− mice showed a 278% increase by 3 wk. For mice immunized with the CFA/I fimbriae diminishes the inflammatory responses. Data are representative of two experiments. *, p ≤ 0.002; **, p ≤ 0.012; ***, p ≤ 0.04.

FIGURE 4. Oral immunization with the Salmonella vector (strain H647), not Salmonella-CFA/I (strain H696) vaccine results in increased splenomegaly. L-Sel−/− and L-Sel+/+ mice were orally immunized with either Salmonella vector (A) or Salmonella-CFA/I vaccine (B). Tissue weights were performed at weekly intervals on individual spleens and PP to measure changes in weight. L-Sel−/− mice immunized with the Salmonella vector showed increases in PP for 3 wk, whereas L-Sel+/+ mice showed a significant increase only at 7 days. Both strains showed significant increases in spleens, but more so for the L-Sel+/+ mice, which showed a 278% increase by 3 wk. For Salmonella CFA/I-immunized L-Sel−/− mice, no significant changes in PP weight were observed, whereas the L-Sel+/+ mice showed a significant reduction (~22%) at 14 days after immunization. Both L-Sel−/− and L-Sel+/+ mice immunized with the Salmonella-CFA/I vaccine showed increased spleens by 14 days, but the increases were greater in the L-Sel+/+ mice than in the L-Sel−/− mice, 40 vs 20%, respectively. Yet these increases were less than those in mice orally immunized with Salmonella vector, suggesting the expression of the CFA/I fimbriae diminishes the inflammatory responses. Data are representative of two experiments. *, p ≤ 0.002; **, p ≤ 0.012; ***, p ≤ 0.04.

FIGURE 5. The increased splenomegaly observed in L-Sel−/− mice orally immunized with Salmonella vector is partly attributed to the increased colonization of the spleen. Both L-Sel−/− and L-Sel+/+ mice were orally immunized with Salmonella vector (A) or Salmonella-CFA/I vaccine (B). Two weeks after immunization, PP and spleens were homogenized and evaluated for the extent of Salmonella colonization. For mice immunized with Salmonella vector, the L-Sel−/− mice (n = 9) showed 5- and 3-fold greater colonization in PP and spleens, respectively, than L-Sel+/+ mice (n = 8). For mice immunized with Salmonella-CFA/I vaccine, no significant differences in colonization of PP or spleens were observed between L-Sel−/− (n = 6) and L-Sel+/+ mice (n = 7), *, p = 0.008; **, p = 0.026.
spleens were immunostained with Mac-1 and SK208 mAbs. The Mac-1+ (25), despite repeated oral doses as others have shown (47–49).

The Mac-1+ SK208 cells represent neutrophils. Unimmunized L-Sel−/− (B) and L-Sel+/+ mice (A) showed similar levels of Mac-1+ SK208+ cells. However, subsequent to immunization with Salmonella vector (C and D) or Salmonella-CFA/I vaccine (E and F), only the L-Sel+/+ (C and E, p < 0.025), not the L-Sel−/− (D and F), mice showed increased numbers of Mac-1+ SK208+ cells. Data are representative of four to five mice per group.

when compared with vehicle controls, with a median survival time of 9 days (p < 0.026). Vaccinated L-Sel−/− mice (n = 9) showed complete protection (p < 0.009), and in fact, these mice survived well beyond 2 mo after completion of the experiment, whereas the median survival time for vehicle controls was 13 days. For the vaccinated mice, the absence of L-Sel clearly enhanced the survival (p < 0.01). Thus, the combination of adaptive immunity and the attenuated neutrophil response possibly mediated protection to wild-type Salmonella challenge.

Discussion

To test the impact of L-Sel on gastrointestinal immunity, we applied our Salmonella vaccines based on work from previous studies showing that the expression of enterotoxigenic E. coli fimbrial Ags provokes Th2 cell development (24, 25). Consistent with our previous findings in BALB/c mice (25), a single oral dose of S. typhimurium-CFA/I vaccine was sufficient to stimulate elevated mucosal IgA Ab responses in L-Sel+/+ mice. This was largely influenced by the costimulation of CFA/I-specific Th2 cells. The effectiveness of this vaccine also is apparent when compared with past unsuccessful attempts to elicit mucosal IgA Abs with an attenuated E. coli-CFA/I construct in conjunction with choleragen (25), despite repeated oral doses as others have shown (47–49). However, the unexpected finding from this study was the impaired S-IgA anti-CFA/I responses by L-Sel−/− mice while systemic IgA responses remained unabated. Although past studies have not evaluated mucosal responses in L-Sel−/− mice, focusing instead on responses subsequent to peripheral immunization, investigators could demonstrate a slight increase in total serum IgA and increases in Ag-specific serum IgA responses (37). In our studies, typically 4 wk subsequent to oral immunization with Salmonella fimbrial vaccines (24, 25), elevated fimbrial-specific S-IgA responses were obtained in normal mice. This apparent failure to elicit elevated S-IgA responses in L-Sel−/− mice does appear to be related to two notable observations. First, these mice showed 5-fold increases in the number of CFA/I-specific IFN-γ-producing CD4+ T cells in the PP, but no significant differences in the number of the same cells in the spleen. The second observation is the absence of IgA-promoting, IL-4-producing CD4+ Th2 cells in L-Sel−/− PP. Although our study does show enhanced CD4+ Th1 cell presence, it seems unlikely that such elevation in IFN-γ-producing cells can solely diminish IgA-promoting CD4+ Th2 cells. Clearly, the selective absence of IL-4 does suggest that this cytokine is required for productive mucosal IgA generation against CFA/I fimbriae.

Our data show that mucosal immunity is interrupted in L-Sel−/− mice, which is attributed to a failure to stimulate CD4+ Th2 cells and surrendering to CD4+ Th1 cell development. Such a discrepancy does not appear to be due to the lack of CD4+ T cells, and the reason for the reversal in Th2 cell bias of the PP (1, 2) remains unclear. Perhaps L-Sel−/− mice have become more predisposed to the proinflammatory pathway. In a recent study, it was shown that in vitro polarized CD4+ Th2 cells lack L-Sel expression, whereas polarized CD4+ Th1 cells show L-Sel expression (38). This L-Sel expression was dependent on IL-12. Whether IL-12 influences expression of L-Sel in vivo and what its importance to CD4+ T cell development is remains to be determined, especially in view of what we observed in L-Sel−/− mice. Future studies will examine whether L-Sel−/− mice are biased in their abilities to produce Th1 cell-promoting cytokines.

Nonetheless, such selective reduction in mucosal IgA did not impact the ability to generate serum IgG responses. One of the hallmarks of the Salmonella-CFA/I vaccine, in addition to its ability to stimulate elevations in mucosal IgA Abs, is the stimulation of equivalent levels of serum IgG1 and IgG2a anti-CFA/I Ab titers (25). Although the serum IgG1 and IgG2a anti-CFA/I titers were similar in L-Sel−/− mice, there was a shift in the bias toward IgG2a in L-Sel−/− mice. Serum IgG2a anti-CFA/I titers were significantly elevated by 26-fold in L-Sel−/− mice, suggesting that a
portion of these Abs may have been mucosally derived. We previously have found increased numbers of fimbrial-specific IgG-producing cells in the PP subsequent to oral immunization with *Salmonella* vaccines (20, 24). The increased numbers of IFN-γ-producing CD4+ T cells in the PP suggest that these cells could induce the observed IgG2a generation. Alternatively, these IgG2a Abs could be peripherally derived subsequent to systemic exposure to the *Salmonella*-CFA/I vaccine. Previous studies examining peripheral immunization of L-Sele−/− mice showed increases in all IgG subclass responses (37), whereas, in our study, only IgG2a Abs were increased. Such evidence suggests that in the absence of L-Sel, the route of immunization plays a role in determining the type of Ab responses generated. Indeed, L-Sel does have an important role in subsequent ability to provoke mucosal IgA Ab responses, more so perhaps than previously expected. In contrast, the spleen did not show a significant difference in the development of CD4+ Th cell immunity to CFA/I fimbriae.

A number of factors support the notion that the *Salmonella*-CFA/I vaccine does not necessarily follow conventional pathways for its clearance. As such, the ability of this vaccine to colonize the PP and spleen is reduced when compared with the extent of colonization evident with the *Salmonella* vector. No significant differences in CFU levels in PP or spleen were observed for Salmo-

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References


coli and Salmonella typhimurium vectors that express colonization factor antigen of enterotoxigenic E. coli (ETEC) in the absence of the CFA/I positive regulator cdtA. Infect. Immun. 65:4933.


