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Villous B Cells of the Small Intestine Are Specialized for Invariant NK T Cell Dependence[1]

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Abstract

B cells are important in mucosal microbial homeostasis through their well-known role in secretory IgA production and their emerging role in mucosal immunoregulation. Several specialized intraintestinal B cell compartments have been characterized, but the nature of conventional B cells in the lamina propria is poorly understood. In this study, we identify a B cell population predominantly composed of surface IgM^+IgD^+cells residing in villi of the small intestine and superficial lamina propria of the large intestine, but distinct from the intraepithelial compartment or organized intestinal lymphoid structures. Small intestinal (villous) B cells are diminished in genotypes that alter the strength of BCR signaling (Bruton tyrosine kinase^{xid}, Gαi2$^{-/-}$), and in mice lacking cognate BCR specificity. They are not dependent on enteric microbial sensing, because they are abundant in mice that are germfree or genetically deficient in TLR signaling. However, villous B cells are reduced in the absence of invariant NK T cells (Jα18$^{-/-}$ or CD1d$^{-/-}$ mice). These findings define a distinct population of conventional B cells in small intestinal villi, and suggest an immunologic link between CD1-restricted invariant NK T cells and this B cell population.

Homeostasis and protection from pathogens in the intestine are the result of a complex orchestration of function among multiple host cell types (epithelial cells, T cells, NK cells, dendritic cells, and B cells), resident commensal microbiota, and itinerant alien

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microorganisms. The intestine, particularly in the colon, bears a large microbial commensal population that has critical roles in food catabolism, production of fermentative metabolites required by epithelial cells, interbacterial control of alien microbial species, and induction of inflammatory quiescence (1–4). On the host side, B cells play one important role in this bacterial commensalism, particularly through the production of secretory IgA or IgG that provides low inflammatory mechanism for barrier and neutralization functions (3,5,6). Functionally, somatic mutation of B cells is important for the quality of the mucosal secretory Ig response and control of intestinal microbiota (7,8). B cells may play a nonreplaceable role in mucosal Ag presentation, as evidenced by impaired mucosal T cell immunity in $CD19^+$-deficient mice and the predominance of B cell lymphotoxin (LT)[3]-α production in mucosal lymphoid tissue formation (9,10). Moreover, recent studies have pointed to a unique immunoregulatory role played by B cells in the control of autoaggressive intestinal inflammation (11, 12).

Developmentally, a key locus for B cells in the intestine is at the host-microbial interface, at lymphoid structures within and immediately subjacent to the intestinal epithelium. Peyer's patches (PP) are well-known germinal center-like structures, which together with isolated lymphoid follicles (ILF) are typically critical for IgA formation in response to enteric Ags (5,13–15). ILFs are a second class of structures, comprised of hundreds of small subepithelial follicles, comprised almost entirely of conventional IgM^+ B cells, with small numbers of mature T cells, dendritic cells, and lymphoid progenitors (7,16–19). A perhaps distinct structure is the lymphocyte-filled small intestinal villus, which bears conventional B cells as a minor population, and is comprised mainly of T lymphocytes (20).

Whole-mount and immunohistochemical analyses indicate that the ILF compartment includes structures of varying size, morphologic coherence, and level of maturation (germinal center formation). However, in terms of B cell numbers, it is a large compartment exceeding the PP compartment, and encompassing the majority of nonplasma B cells in the intestine. Studies have included their structural and genetic characterization and their participation in the immune response to enteric microbial challenge. Like other mucosal lymphoid sites, B cell expression of LTα is an inductive requirement of LTβR stromal cells in ILF organogenesis (7,16–19). Cryptopatches are a third class of structures identified for their role in early lymphoid progenitor development (21–24). Recent genetic and microbial experimentation further suggests that cryptopatches and ILFs may comprise solitary lymphoid follicles representing a continuum of lymphocyte development, recruitment, and activation (25).

Finally, B cells have been identified outside these organized mucosal lymphoid structures in the lamina propria. Morphologic and immunophenotypic marker assessment in situ indicate that these B cells are mainly comprised of plasma cells and their immediate progenitors, in part including cells programmed by a primitive T cell-independent process (26–29). Although conventional B cells have been identified in the lamina propria, it is uncertain whether they represent contaminants from organized mucosal lymphoid structures, or authentic lamina propria residents.

In this study, we report that conventional intraepithelial preparations harbor B cells derived from intestinal superficial lamina propria (SLP), including the small intestinal villi, and the SLP of the large intestine. We find that there is an uncommon, but readily detectable mucosal B cell population with distinctive surface markers, ontogeny, and segmental intestinal distribution. SLP B cell formation was strictly dependent on LTα sufficiency, and diminished

[3]Abbreviations used in this paper: LT, lymphotoxin; Btk, Bruton tyrosine kinase; ILF, isolated lymphoid follicle; IRF-3, IFN regulatory factor-3; LPL, lamina propria lymphocyte; PP, Peyer's patch; SLP, superficial lamina propria; SPF, specific pathogen free; TRIF, Toll/IL-1 receptor domain-containing adaptor inducing IFN-β.

in mice genetically modulated for BCR signal strength or BCR cognate Ag recognition. However, SLP B cells were not reduced by the absence of enteric microbiota or TLR sensing. Instead, they were dependent on genetic traits required for invariant NK T interaction, indicating a link between these B cells and invariant CD1-restricted Ag presentation in the intestinal mucosa.

Materials and Methods

Mice

C57BL/6, BALB/c, CBA/J, CBA/N (Bruton tyrosine kinase (Btk)^{xid}), C3H/HeJ (TLR4^{P712H} missense mutation) (30), C3H/FeJ, muMT (31), MD4 (C57BL/6 background) (32), muMT (C57BL/6 background), and CD1d$^{-/-}$ (BALB/c background) (33) mice were obtained from The Jackson Laboratory. LT$\alpha^{-/-}$ mice (C57BL/6 background) were bred at the Washington University animal facility. TLR2$^{-/-}$ (34), MyD88$^{-/-}$ (35), IFNAR$^{-/-}$ (36,37), and IFN regulatory factor-3 (IRF-3) $^{-/-}$ (38) animals (gift from T. Taniguchi, University of Tokyo, Tokyo, Japan) (all on the C57BL/6 background) were bred and maintained in the University of California Department of Laboratory and Animal Medicine. Some MyD88$^{-/-}$ mice were also bred at the vivarium of Cedars Sinai Medical Center; the phenotypes of MyD88$^{-/-}$ mice, including mucosal B cell properties, were indistinguishable from these two colonies. Toll/IL-1 receptor domain-containing adaptor inducing IFN-β TRIF$^{-/-}$ (*lps2*) (39) animals were a gift from B. Beutler, Scripps Research Institute (La Jolla, Ca). Jα18$^{-/-}$ (C57BL/6 background) mice and heterozygote controls were obtained from La Jolla Institute for Allergy and Immunology.

Germfree mice (129/SvEv) were obtained from the Center for Gastrointestinal Biology and Disease Gnotobiotic Core Facility and National Gnotobiotic Rodent Resource Center, North Carolina State University. Some experiments were also performed with germfree Swiss Webster mice purchased from Taconic Farms. Most mice were analyzed at the North Carolina Gnotobiotic Center. Some mice were received at University of California by shipment in sterile isolation containers, and sacrificed for tissue harvest immediately after arrival. Sterility of germfree mice was documented on a monthly basis by fecal Gram stain and aerobic and anaerobic cultures of the feces and bedding. For selected mice, sterility of cecal contents was documented Gram stain and cultures at the time of necropsy.

Gαi2$^{-/-}$ mice (40) (C57BL/6 and 129Sv mixed background) were bred at the University of California Department of Laboratory and Animal Medicine. MD4 mice (32) bear trangenes for anti-hen egg lysozyme Ig. To suppress leakage of B cells bearing Abs for divergent specificities, we prepared double-mutant mice for MD4 and muMT (which blocks development of B cells). Homozygous muMT mice were crossed with mice hemizygous for the MD4 transgene; F_1 heterozygous mice were screened for MD4 via PCR and intercrossed. F_2 mice were screened for MD4 and muMT by PCR, and muMT homozygosity was determined via antilysozyme reactivity of splenic B cells via flow cytometry.

Mice were housed with sterilized cages, bedding, rodent chow (egg free), and water. Each room is HEPA filtered, and all mice are maintained in microisolator cages that are opened only in flow hoods using sterile procedure. Unless otherwise specified, animals examined in this study were all male between 8 and 10 wk of age and were age matched within each experiment. All procedures were performed under approved University of California Animal Research Committee protocols.

Cell isolation

Small intestine (including jejunum and ileum) and large intestine (including cecum and colon) were harvested. To isolate superficial and deep lamina propria lymphocytes (LPL), small and large intestine were harvested, PP were removed, and tissue was cut open longitudinally. The washed tissue was cut into 1-cm segments. Epithelium associated (intraepithelial lymphocytes and superficial LPL; see below) were released with two consecutive washes with 1 mM DTT (Sigma-Aldrich), shaking at 220 rpm for 20 min at 37°C. Deep LPL were released from the remaining tissue with collagenase (Sigma-Aldrich) treatment at 1 mg/ml, shaking at 220 rpm for 40 min at 37°C. Epithelium-associated lymphocytes and deep LPL were further isolated via a discontinuous 40:70% Percoll gradient (Amersham Biosciences). The yield of viable cell numbers for each specimen was enumerated by hemocytometer counts and trypan blue exclusion.

Whole-mount analysis

Visualization of intestinal B cells in situ was performed, as previously described (18). Small intestines were removed intact, flushed with PBS, opened along the mesenteric border, and mounted lumen facing up. Intestines were incubated three times in HBSS (BioWhittaker) containing either 5 mM EDTA or 1 mM DTT at 37°C, with shaking for 10 min to remove epithelial cells. Intestines were then fixed in 10% phosphate-buffered formyl saline and treated with 1% H_2O_2 for 15 min at room temperature, as above. Intestines were incubated in a blocking solution of 50 mM Tris (pH 7.2), 150 mM NaCl, 0.6% Triton X-100, and 0.1% BSA for 1 h at 4°C to block nonspecific Ab binding, and then incubated with rat anti-mouse B220 Ab (BD Pharmingen) diluted in the above solution overnight at 4°C. Intestines were washed three times in blocking solution and incubated with a HRP-conjugated goat anti-rat IgG Ab (Jackson ImmunoResearch Laboratories) diluted in blocking solution at room temperature for 1 h. Intestines were washed three times and incubated in diaminobenzidine metal peroxide substrate, as above. Intestine whole mounts were examined under a dissecting microscope at an original magnification of ×25– 65. Photo documentation at low power was obtained using a Zeiss Stemi 2000-c stereomicroscope and a Nikon 5000 digital camera. Video and photo documentation at higher power was obtained using a Zeiss Axioskop 2 plus upright microscope and a Nikon 5000 camera. To count the numbers of $B220^+$cells within individual ILFs, video documentation of individual ILFs was obtained while focusing up and down through the ILFs. The numbers of $B220^+$cells within each ILF were counted on each video by an individual unaware of the treatment group.

Immunofluorescence

Rolled distal small intestine from wild-type C57BL/6 mice was embedded in OCT, frozen, sectioned, and fixed in −20°C acetone for 20 min, blocked with 10% normal goat serum and anti-CD16, and then stained with mouse anti-E-cadherin FITC (Molecular Probes), goat anti-mouse IgM Alexa 546 (Molecular Probes), and rat anti-mouse B220 Cy5 (Molecular Probes) for 30 min. Images were acquired using Zeiss LSM 510 with Plan-Apochromat ×20 0.75N/A air objective.

Flow cytometry

Cells were stained using standard 96-well microtiter plate-staining technique. Cells were plated at 2.5×10^5/well and stained with 0.125 μg Ab/well (0.5 μg Ab/10^6 cells). Abs were obtained from BD Pharmingen, including monoclonal anti-mouse IgM, IgD, α_4 integrin, CD19, CD1d, CD23, CD21/CD35, CD45, CD45RA (B220), and CD103 (α_E integrin). Isotype and species-matched control Abs were mouse or rat IgG2a or IgG2b. Staining was conducted for 30 min on ice, followed by washing and fixation in 2% paraformaldehyde. Data acquisition was performed at the University of California Jonsson Cancer Center Flow Cytometry Core Facility

using BD FACSCalibur (BD Biosciences), and analysis was conducted using BD CellQuest software. In some cases, absolute numbers of lymphocyte subsets in intestinal compartments were calculated as the product of the number of viable cells from the specimen (see above), and the frequency of the lymphocyte subset determined by flow cytometry.

Statistics

Statistical analysis was conducted using GraphPad Prism software (graphpad.com). To examine age and strain variability, each data set was examined for Gaussian distribution via a normality test, and log transformed when necessary to allow for further analysis. Linear regressions were conducted to examine the contribution of age on ILF variability. Regressions were plotted with a 95% confidence (dashed lines) and contribution of age to variability defined by r^2. For determination of significance of differences seen in between two groups, two-sided Student's *t* test with a 95% confidence interval was conducted. For all statistical tests, significance was defined as $p < 0.05$.

Results

Isolation of SLP B cells of small intestinal villi

We began this study by evaluating the distribution of conventional B cells present in the intestine. To exclude PP-associated cells, we excised PP before intestinal processing. Epithelium-associated (DTT-released) and lamina propria (collagenase-released) lymphocytes were separately isolated from small (jejunum and ileum) and large (cecum and colon) intestine. Flow cytometry was then performed, gating on the lymphocyte scatter interval, and assessing cells stained for CD19 (or B220) and IgM expression.

A representative example of flow analysis is shown in Fig. 1. In both the small and large intestine, 10–15% of the lymphocyte scatter-gated population from epithelium-associated, DTT-released fraction was $CD19^+IgM^{moderate}$ in C57BL/6 mice (Fig. 1*a*). Similar results were obtained using either CD19 or B220 for B cell discrimination, and >90% of epithelium-associated $CD19^+$ cells were $B220^+$ (data not shown). By four-color staining, DTT-released small intestine B cells ($CD19^+IgM^+$) were also $IgD^{high}CD23^+CD21^{dim}$ (Fig. 1*b*) and $CD5^-CD11b^-$ (data not shown). A similar phenotype was observed in LPL B cells, except that they were recovered at ~25% of the level of DTT B cells, and they were negative for CD21 and CD23 expression (possibly a result of CD23 sensitivity to collagenase treatment) (Fig. 1*b*). The small numbers of isolated LPL B cells are consistent with the low abundance of LPL vs ILF B cells reported in a recent microdissection study (15).

A population of epithelium-associated IgM^-IgD^+ B cells was also detected in the small intestine (Fig. 1*c*), which was notable for a $CD23^-$ subpopulation that was ~5% membrane or cytoplasmic IgA^+ by flow cytometry (Fig. 1*c* and data not shown). This is in agreement with the understanding that IgD single-positive B cells are enriched for cells undergoing class switching (e.g., IgA) and plasma cell differentiation (41), and in IgA precursor B cells of the ILF compartment (15). Other IgM^-IgD^+ B cells might represent cells in a state of peripheral antigenic tolerance (see *Discussion*) (42,43).

Initially, we surmised that the epithelium-associated, DTT-released B cells were ILF B cells. To test this idea, we used intestinal whole mounts stained with B220 to visualize and enumerate in situ the abundance of ILFs and ILF-associated B cells using EDTA or DTT (Fig. 2). EDTA treatment is required to permeabilize the tissue for whole-mount anti-B220 staining (18). As is well known, EDTA treatment releases intraepithelial T cells. However, we observed that EDTA alone does not release intestinal B cells (Fig. 2*a*). We predicted that if the DTT-released B cells were derived from ILFs, then the number of ILFs would be reduced in DTT-treated

tissue, compared with EDTA-treated tissue. Accordingly, we compared the entire intestine of C57BL/6 mice using EDTA-treated or DTT-treated whole mounts, as described in *Materials and Methods*. To our surprise, we observed no difference between the treatment groups in the numbers of ILFs as defined by clusters of $B220^+$ cells (data not shown).

It was also possible that DTT treatment released only some of the B cells from each ILF, so that the number of ILFs would not be changed, but the number of B cells per ILF would be reduced. To evaluate this, we examined the numbers of $B220^+$ cells within ILFs from the EDTA- and DTT-treated intestinal whole mounts. To minimize variability of $B220^+$ cells within ILFs from different mice and different regions of the intestine, we examined adjacent sections of C57BL/6 intestine treated with either EDTA or DTT. As shown in Fig. 2*b*, counts of $B220^+$ cells from these adjacent sections confirmed that there was no difference in the numbers of $B220^+$ cells within ILFs between the two treatment groups. These findings indicated that B cells released by DTT treatment did not derive from the ILF compartment.

Upon further immunohistochemical examination of the whole mounts at high power, we noted that an additional population of $B220^+$ cells could be identified outside of ILFs, distributed within villi (outlined by lines at low power, and by arrows at high power, in Fig. 2, *c* and *d*, respectively). These B cells were scattered throughout the villi, but were absent at the periphery of the villus (where the epithelial cells reside). Thus, they were localized in the villous lamina propria, but not in the intraepithelial compartment of the villi.

The villous population of $B220^+$ B cells was readily detected in the intestinal villi of EDTA-treated whole mounts (arrows), but depleted in the DTT-treated whole mounts (Fig. 2*d*). These findings are further demonstrated in scanning video microscopy (see supplemental video 1).[4] Because DTT, but not EDTA released B cells from the intestinal villi, we expected to find increased B cells in the DTT vs EDTA wash fluid. As expected, flow cytometric analysis of the released cells from whole mounts treated with EDTA or DTT confirmed that B cells were much increased in the DTT vs EDTA wash fluids (Fig. 2*a*).

A schematic representation of mucosal B cell subsets in the small intestine (Fig. 3*a*) summarizes the foregoing results and findings with cryostat confocal immunofluorescent examination (Fig. 3, *b*–*e*). As a positive control of immunofluorescence staining of B cells, tissue was examined in which PP was not excised. This was the only case in this study in which PP was left intact before analysis. PP and ILF with $B220^+$ and IgM^+ B cells are demonstrated in Fig. 3, *b* and *e*, respectively. Intestinal B cells are present in the SLP (Fig. 3, *c* and *d*), as well as small subglandular lymphoid aggregates (Fig. 3*e*). Like in the whole mounts, extensive viewing of 10-μm cryostat sections for $B220^+$ cells confirmed that B cells were localized exclusively in the lamina propria; no intraepithelial B cells were observed (data not shown).

Taken together, these findings indicate that the epithelium-associated, DTT-released B cells were not derived from ILFs or the intraepithelial compartment, but instead from the SLP. In the small intestine, these DTT-released B cells were localized, at least in part, to intestinal villi. The localization of this SLP compartment in the large intestine was less certain, but presumably in the region lamina propria between colonic glands. Accordingly, we will refer to the small intestine population as villous B cells, and to the small and large intestine populations together as SLP B cells.

Homing and retention markers of SLP B cells

Recent B cell immigrants to the intestinal mucosal lymphoid structures express $\alpha_4\beta_7$, due to its interaction with mucosal addressin cell adhesion molecule-1 expressed on the mucosal

[4]The online version of this article contains supplemental material.

lymphoid endothelium (44). Therefore, we predicted that SLP B cells would express $\alpha_4\beta_7$ integrin to facilitate their homing to the gastrointestinal mucosa; we assessed their expression of the homing molecule$\alpha_4\beta_7$ integrin and the retention molecule $\alpha_E\beta_7$ integrin (44,45). As expected, α_4 integrin was expressed on SLP IgM^+ B cells in both the small and large intestine (Fig. 4*a*). Lymphocytes that intercalate within the intestinal epithelial layer express$\alpha_E\beta_7$ integrin (CD103), which promotes heterotypic $\alpha_E\beta_7$:E-cadherin interaction of intraepithelial T cells with intestinal epithelial cells (45,46). Because our immunolocalization data indicated that SLP B cells were excluded from the intraepithelial compartment, we predicted that they would not express $\alpha_E\beta_7$. As shown in Fig. 4*a*, they indeed were CD103 (α_E integrin) negative in both small and large intestine, and thus lacked this molecular trait required for proficient penetration of the intraepithelial compartment.

The LTα system is required for formation of specialized intestinal lymphoid compartments, such as PP and ILFs (16,17). To test whether LTα is required for formation of SLP B cells, we compared the abundance of $CD45^+$ cells in LT$\alpha^{+/+}$and LT$\alpha^{-/-}$ on the C57BL/6 background (Fig. 4*b*). LTα sufficient and deficient mice had similar numbers of total epithelium-associated $CD45^+$ lymphocytes, in agreement with the minimal effect of LTα deficiency on the levels of epithelium-associated T cells. However, LT$\alpha^{-/-}$ mice were entirely deficient in SLP B cells. As previously reported, these mice were also deficient in LPL B cells (data not shown) (19). Accordingly, SLP and deep LPL B cells share a developmental requirement for LTα, characteristic of B cells in organized mucosal lymphoid compartments.

Numerical heterogeneity of SLP B cells: segmental localization and age

SLP B cells reside at the host-flora interface, and the composition of the enteric flora differs along the intestine. We therefore examined the distribution of villous B cells along the length of the intestine. Small intestine was divided into three equal-length segments (11–12 cm each). In the large intestine, the cecal appendix was excised, and the remaining large bowel was divided into two equal segments (7–8 cm each). DTT-released lymphocytes from each segment were immunostained for flow cytometry, and the SLP B cells ($CD19^+IgM^+IgD^+$) were tabulated as percentage of total lymphocytes. As shown in Fig. 4*c*, SLP B cells were present at low levels in the jejunum, but reached high levels in the ileum and cecum. However, they dropped again to lower levels in the subsequent segments of large intestine.

We considered that segmental variability may have been due to technical issues affecting precision/accuracy. To further address this, the same segmental quantitation was performed on four different mice. This yielded similar relative differences between segments in each mouse, but the levels of recovered B cells in each mouse varied by a factor of 2 (data not shown). On this basis, normalizing and pooling data for detailed segmental analysis (Fig. 4*c*) was not feasible. However, these findings confirm the segmental difference of SLP B cells, and suggest that local factors play a role in their homing or persistence, which may be particularly favorable in the ileo-cecal region. It also indicates that SLP B cells present in our small and large intestine preparations are mainly due to ileal and cecal populations, respectively. The cellular details of this variation is uncertain, reflecting a combination of changes in abundance of mucosal B cells, IgA^+ $CD19^+$ B cells, and mucosal T cells, and in these fractions.

We next asked whether age or gender might affect total numbers of SLP B cells (Fig. 4, *d* and *e*, for small and large intestine, respectively). A nonsignificant difference was observed in SLP B cell distribution of C57BL/6 mice between 9 and 13 wk of age: ~6% variability in SLP B cell distribution can be seen in the small (r^2 =0.06) and large ($r^2 < 0.01$) intestines. Finally, we compared the effect of gender on SLP B cell formation. However, no intra-or intergroup differences were observed when male and female mice were stratified (data not shown). These

findings indicate that SLP B cell levels reach a stable, adult level not long after weaning, and are unaffected by gender.

BCR signaling requirement of SLP B cells

B cell subsets have differential requirements for signaling strength mediated via BCR for their presence and formation. To better understand the biochemical and potential biologic properties of SLP B cells, we examined mice with known alterations in BCR signal strength, to assess their effect on SLP and LPL B cell formation.

Btk is a positive intermediary in BCR signaling, and is required for development of splenic follicular and peritoneal B-1 B cells (47,48). We therefore compared intestinal B cell levels in age-matched wild-type CBA/J mice and CBA/N mice, a substrain bearing the nonfunctional *xid* missense mutation of Btk (Btk^{xid}) (Fig. 5*a*). SLP B cells in Btk^{xid} mice were deficient in both the small ($p = 0.02$) and large ($p = 0.04$) intestine. As in the SLP compartment, the absolute numbers of LPL $Btk^{-/-}$ B cells also were quite low in the small intestine (35% of wild type), but were not reduced in the large intestine (data not shown). Therefore, signaling dependent on Btk is necessary for the normal formation of SLP B cells of the small intestine.

The heterotrimeric G protein Gαi2 is a negative regulator of BCR signaling (49), and a positive signaling intermediate for certain chemokine receptors (50), each differentially required by certain B cell subsets. Pharmacologic and genetic analysis revealed that tonic Gαi2 signaling mediates a substantial physiologic attenuation of BCR signaling. In G$\alpha i2^{-/-}$ mice, splenic B cell development was selectively perturbed by a deficiency of marginal zone B cells and their splenic progenitors, transitional type-2 B cells; peritoneal B-1a B cell formation was also profoundly deficient (49). We therefore compared wild-type and G$\alpha i2^{-/-}$ mice for levels of SLP B cells in the small and large intestine (Fig. 5*b*). In the small intestine, SLP B cells were deficient ($p = 0.01$). However, G$\alpha i2^{-/-}$ mice had no significant abnormality in large intestinal SLP B cells ($p = 0.65$). As in the SLP compartment, the absolute numbers of LPL G$\alpha i2^{-/-}$ B cells also were selectively reduced in SI, but not LI (30 and 100% of wild type, respectively; data not shown). These findings suggest that among the intestinal B cell populations, SLP and LPL B cells of the small intestine are particularly susceptible to inappropriate BCR signaling.

Role of BCR specificity in SLP B cell formation

The selective deficiency of SLP B cells in Btk^{xid} and G$\alpha i2^{-/-}$ mice suggests a requirement for proper tuning of BCR signaling for efficient formation of this intestinal B cell population. However, we wanted to further ascertain whether this requirement involved Ag specificity of the BCR. To address this issue, we compared three groups of age-matched C57BL/6 bred in our colony, as follows: MD4 (bearing the hen-egg lysozyme Ig transgene); MD4: $muMT^{-/-}$ mice, whose B cells strictly bear the lysozyme transgene (due to a critical null mutation in the endogenous Ig locus); and wild-type mice bred in the same room. As shown in Fig. 5*c*, SLP B cells in MD4 and MD4:$muMT^{-/-}$ mice were equally reduced compared with wild-type mice. Numerical reductions (3-and 2-fold) were observed in the small and large intestine, respectively; this reached statistical significance in the small intestine. Therefore, efficient SLP B cell formation required their capacity for cognate adaptive Ag recognition.

Role of microbial sensing on SLP B cell formation

We next tested whether this B cell population might instead require enteric microbiota and innate microbial sensing. First, we compared the levels of age-matched 129/SvEv mice reared in specific pathogen-free (SPF) or germfree conditions. Surprisingly, germfree mice preserved the SLP B cell population (their mean numbers were actually increased, and this reached statistical significance in the small intestine) (Fig. 6*a*). Evaluation of SPF and germfree mice from age-matched outbred Swiss Webster strain showed comparable results (data not shown).

It was possible that SLP B cell formation might be induced, directly or indirectly, by innate sensing of microbial products present in the sterile animal chow or other environmental exposure. To test this idea, we first evaluated mice bearing missense or null mutations of TLR4 (Fig. 6*b*) and TLR2 (Fig. 7*a*). However, SLP B cells were preserved in both these mutant mice compared with mice matched for strain background, age, and vivarium conditions. We realized that this negative result may have reflected redundancy in TLR signaling through the different TLR family members. Therefore, we next analyzed mice bearing null mutations in key TLR signaling adaptor proteins. These included MyD88 (used by most TLR isoforms) and TRIF, a MyD88-independent signaling pathway used by TLR3 and TLR4, which through IRF-3 induction elicits IFNβ expression and IFNβ-receptor signaling (39,51–54).

The results of this extended analysis are shown in Figs. 6 and 7. SLP B cells were not reduced in either MyD88$^{-/-}$ or TRIF$^{-/-}$ mice (Fig. 6, *c* and *d*, respectively). SLP B cells were also preserved in mice deficient in the type 1 IFN receptor IFNAR and IRF-3 (Fig. 7, *b* and *c*, respectively). Like germfree mice, these mutant mice were often numerically increased for SLP B cells, and in some cases reached statistical significance compared with control mice. Taken together, these findings indicate that neither enteric microbial residents, nor any of several TLR-related microbial sensing systems, are necessary or predominant for SLP B cell formation.

We note the issue of variability in SLP B cell numbers. Among >100 wild-type mice analyzed in this study, the total number of recovered SLP B cells per mouse (small plus large intestine) was 2.1 ± 0.1 million (mean ± SEM), and ranged from 1.5 to 4 million (see Figs. 4*b*, 5, 6, and 8, *a* and *b*). It should be noted that in some control groups, the mean levels of SLP B cells differed from our usual C57BL/6 controls in the University of California Center for Health Sciences vivarium. This included elevations for 129/B6 (Gαi2 control) and BALB/c (CD1d control; see below), and a reduction in the C57BL/6 control groups for IFNAR and germ-free (each from different non-Center for Health Sciences vivariums). However, in other cases, a divergent strain (C3H/Fe control for Btk) or vivaria (C57BL/6 controls for TRIF, or Jα18 (see below)) had no effect on mean SLP B cells. This suggests that strain background or vivarium conditions (food, bedding, etc.) in some cases may contribute to SLP B cell levels.

Role of CD1-restricted Ag presentation

The nonclassical MHCI molecule CD1 is specialized for immunologic presentation of lipoglycan Ags derived from microbial, environmental, and autologous metabolic sources (55,56). We therefore wondered whether SLP B cell formation might be dependent on CD1-restricted T cells. To test this idea, we evaluated CD1d$^{-/-}$ mice for levels of SLP B cells. As shown in Fig. 8*a*, these mice displayed a substantial (3-fold) and significant reduction of SLP B cells in the small intestine. There are a variety of CD1-restricted T cell populations, distinguished by their tissue distribution, Ag specificity, and functional markers (57). Among these populations, invariant NK T cells are notable for their clonal homogeneity (including the TCR Jα18 V gene), common structural lipoglycan fine specificity, and predominance in the liver (which is suffused by enteric Ags via portal circulation). When we analyzed mice deficient for invariant NK T cells (Jα18$^{-/-}$ mice), we observed a similar deficiency in SLP B cells (Fig. 8*b*).

It should be further noted that this deficiency was selective for SLP B cells of the small intestine. First, large intestinal SLP B cells were preserved in both CD1$^{-/-}$ and Jα18$^{-/-}$ mice (Fig. 8, *a* and *b*). Second, PP B cells were preserved in Jα18$^{-/-}$ mice, a finding that further distinguishes small intestinal SLP B cells from subepithelial B cells associated with organized lymphoid structures (Fig. 8*c*). Taken together, these findings indicate that SLP B cells are linked, directly or indirectly, to CD1-restricted Ag presentation and invariant NK T cells.

Discussion

This study characterizes the B cell population residing in the SLP of the intestine. They are selectively extracted in the conventional IEL preparation, which does not include B cells from the ILF and PP B cell compartments. Strain backgrounds differing in SLP B cell abundance indicate that genealogically divergent strains of mice all contain SLP B cells, but the relative abundance of SLP B cells depends in part on host genetic factors. Genotypes altering the strength of BCR signaling (Btk^{xid}, $G\alpha i2^{-/-}$) or bypassing cognate BCR recognition (MD4) impair SLP B cell formation, particularly in the small intestine. Resident microbiota and innate microbial sensing are not required for small intestine SLP B cell formation, but SLP B cell levels are substantially reduced in the absence of invariant NK T cells. In this discussion, we consider how these observations refine the understanding of local microenvironment and antigenic selection on the mucosal B cell compartment.

B cells recovered in the bulk epithelium-associated (DTT) preparation were localized to the superficial and SLP lamina propria compartment by direct in situ comparison of this compartment before and after DTT treatment. PP were carefully excised before intestinal analysis, so the isolated B cells were not attributable to this source. They were also distinguished from the ILF compartment, whose structural integrity and abundance were unaffected by DTT treatment. Like other mucosal lymphocytes, SLP B cells express the mucosal homing receptor α_4 integrin. However, they lacked $\alpha_E\beta_7$, an integrin facilitating epithelial interaction of IEL T cells (45). This is consistent with the segregation of SLP B cells from direct epithelial interaction.

Epithelium-associated (DTT-released) conventional B cells ($CD19^+$ and/or $CD45RA/B220^+$, IgD^+ and/or IgM^+, $CD21^+$) have been previously reported at a comparable frequency (5–10% of mucosal lymphocytes) in the mouse, rat, cow, and human (27,28,58–61). As in our study, this compartment was distinguished from deep lamina propria by the paucity of plasma cells, but the microanatomy of subepithelial B cells in those studies was not further specified. In the human small intestine, lymphocyte-filled villi have been noted bearing follicle-associated epithelium, memory (CD45RO-positive) T cells, dendritic cells, and a variable B cell component, with no evidence of immature lymphocytes (20). We have not observed such densely lymphocyte-filled villi in mice, but it is possible that the murine SLP B cells described in the present study may be homologues of B cells in this human compartment. The SLP B cell compartment is notable for its substantial size, because the total numerical recovery of SLP B cells per mouse ($1–2 \times 10^6$) is similar to that of B cells in the entire PP compartment.

Like ILF, PP, and other mucosa-associated follicular lymphoid compartments (16,17), formation of SLP B cells was strictly dependent on LTα sufficiency, and thus suggests that SLP B cells may in part represent progeny from these differentiative sites. Due to the dependence of NF-κB-interacting kinase in LTβR signaling, SLP B cells would seem to be distinguished from the IgM^+ lamina propria population that forms in *aly/aly* mice, a strain naturally deficient in this kinase (26). It is notable that SLP B cells resembled ILF B cells by phenotype ($IgM^{moderate}IgD^{high}CD21^{low}CD23^+CD5^-CD11b^-$) (16), and included minor IgD^+IgM^{low} and IgA^+ populations. IgD^+ IgM^{low} is a phenotype of H chain class switch-committed cells (41), and PP and possibly ILFs provide an important microenvironment for formation of B cells committed to mucosal IgA response. Accordingly, it is possible that SLP B cells may in part represent progeny of B cells from these compartments. Because the IgD^+IgM^{low} phenotype is also a feature of an immunologically tolerant subset (42,43), such SLP B cells may also represent cells with this functional fate.

A novel feature shared by SLP (this study) and ILF (18) B cells is their reduced formation in the absence of cognate Ag receptor function. In this study, we also show that their formation

was substantially reduced in Btk$^{-/-}$and Gαi2$^{-/-}$mice. Btk is a positive regulator in proximal phases of BCR signaling (47,48), and Gαi2 is a negative regulator of BCR signaling, in part through Gi-dependent cross-talk in a common pathway of calcium mobilization (49). This suggests that aberrant BCR signal strength (attenuated and exaggerated in Btk$^{-/-}$and Gαi2$^{-/-}$mice, respectively) impairs SLP B cell formation. Tonic signals mediated via the BCR and coreceptors are critical for the development and function of B cells (49,62,63). Tonic signaling has been implicated in the formation of Ag-independent IgA-producing PP B cells in transgenic EBV LMP2A mice (8), and of IgM^{+}, IgA-producing lamina propria in NF-κB-interacting kinase$^{-/-}$mice (26). Clonal success of such mucosal B cells is also dependent on enteric microbiota and T cell activity, suggesting that signaling from these microbial interactions may compensate for limited antigenic signaling in these B cell populations (see below).

The mechanisms accounting for these threshold effects are still emerging. Follicular competition of B cells may be tuned by BCR signal strength (favoring quiescent vs Ag-activated B cells) through a process complementary to classic CXCR5/CXCL15 follicular homing, resulting in follicular exclusion by high-threshold BCR signaling (64,65). The B cell chemoattractant CXCL13 is distinctively expressed in a variety of mucosal intestinal sites, including PP high endothelial venules (66), and may account for the requirement of its counterreceptor (CXCR5) in PP B cell immigration. CCR7 and CXCR4 are also required for efficient immigration, attributed to the local role of the more ubiquitously expressed CXCL12 (66). In this context, it should be noted that chemokine receptors can be Gi coupled, and CXCR4 and CCR7 couple predominantly to Gαi2 in B lymphocytes (50). Accordingly, the phenotype of Gai2$^{-/-}$mice (a selective deficiency in small intestinal SLP B cells, as well as a selective functional impairment of lamina propria vs PP B cells) (67) may be directly attributable to the selective chemokine receptor for SLP B cells in this microenvironment.

Although SLP B cells reside at an interface with enteric microbiota, their abundance was not dependent on enteric microbiota. First, the SLP B cell distribution was predominant in the small intestine (mainly ileum), where microbial colonization is reduced compared with the large intestine. Second, SLP B cells were not diminished in germfree mice, and actually increased compared with SPF mice. Third, a variety of null mutations in TLR and TLR-coupled signaling molecules preserved SLP B cells, and indeed a modest increase was seen in MyD88$^{-/-}$mice. The reason for elevated SLP B cells in germfree or MyD88$^{-/-}$mice is uncertain, but might suggest a negative role for enteric microbial products and their MyD88-dependent sensing in the formation or fate of SLP B cells. However, these findings certainly indicate that enteric microbial sensing through the TLR system is unlikely to be required for intestinal homing and sustenance of SLP B cells.

Recent studies have delineated different modes by which lumenal stimuli influence intestinal B cell compartments. The abundance of B cells in both ILF and PP compartments is highly dependent on enteric bacteria, as shown by their deficiency in germfree or antibiotic-treated mice, and their expansion in activation-induced cytidine deaminase$^{-/-}$mice with impaired microbial control (8,15,17,18,26,68). However, whereas the formation of PP germinal centers was dependent on the presence of T lymphocytes, the formation of ILFs was not dependent on conventional or NK T cells (18).

In the present study, SLP B cells were selectively reduced in the small (but not large) intestine in mice genetically deficient in invariant NK T cells. CD1 is detectable on nearly all B cells, and cognate CD1-dependent interaction with NK T cells can elicit B cell proliferation and Ab production (69–71), and reciprocally T cell regulatory function (11). Invariant NK T cells are uncommon in the intestinal mucosa itself, and their role in local enteric mucosal regulation is uncertain (57). Although B-NKT interaction might occur at this site, it might instead take place

at a differentiative site where it programs selective B cell trafficking or survival in the SLP intestinal compartment. Because SLP B cells (and invariant NK T cells (72)) are fully preserved in germfree mice, the antigenic drive for this interaction would seem to involve either food Ag or endogenous glycolipids, rather than enteric commensal microbial products (57). Thus, additional studies are required to uncover the basis of the intriguing relationship of villous B cells and invariant NK T cells and this aspect of mucosal biology.

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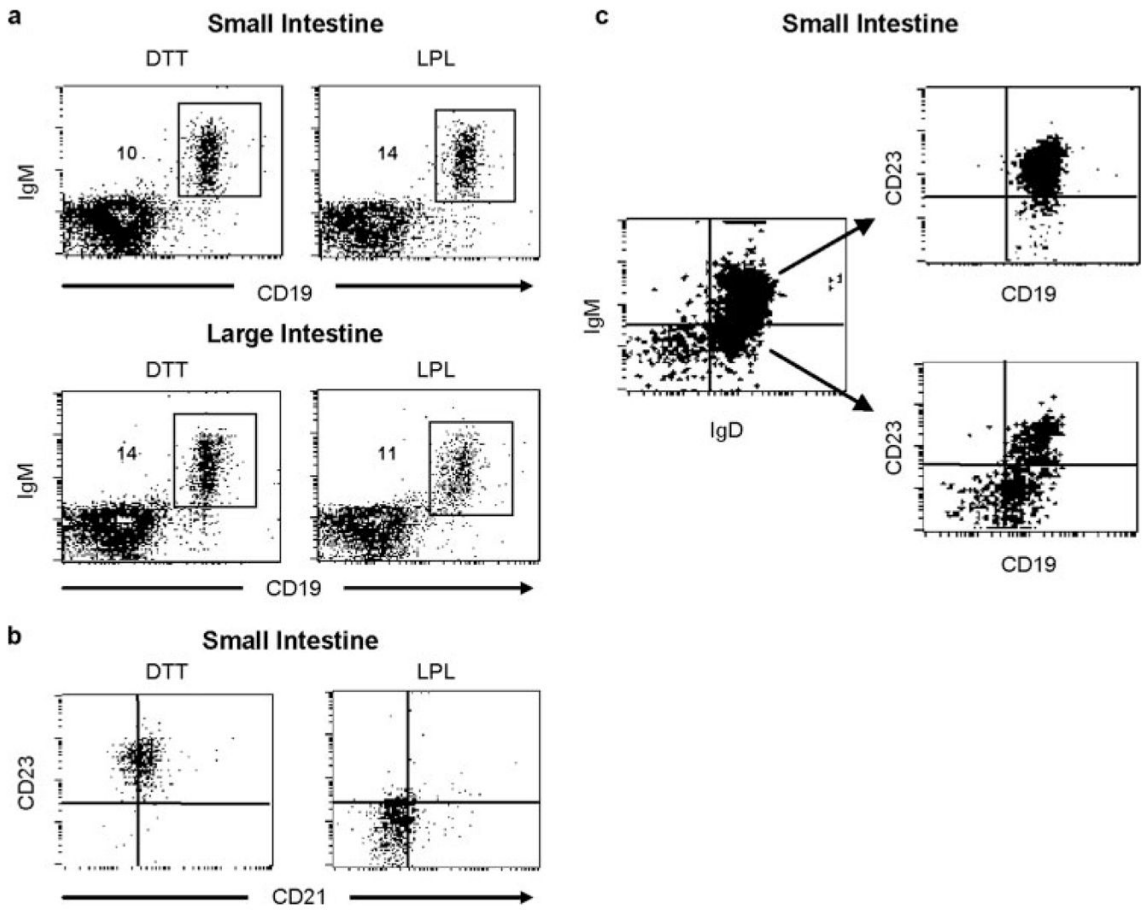

FIGURE 1.

Phenotype of epithelium-associated and LPL B cells. Epithelium-associated (DTT) and lamina propria-associated (LPL) lymphocytes were harvested from small and large intestine of C57BL/6 mice and examined by flow cytometry for expression of surface markers. *a*, Total $IgM^+$and $CD19^+$ cells in the small intestine (jejunum, ileum) and the large intestine (cecum, colon), gated for lymphocyte size and scatter. *b*, CD21 and CD23 phenotype of $CD19^+$ cells in DTT and LPL fractions of small intestine. *c*, Phenotype of IgM- and IgD-expressing small intestine epithelium-associated B cells. *Left panel*, IgM and IgD expression. *Right panel*, CD23 and CD19 expression of IgM^+IgD^+ (*top*) and IgM^-IgD^+(*bottom*) B cells.

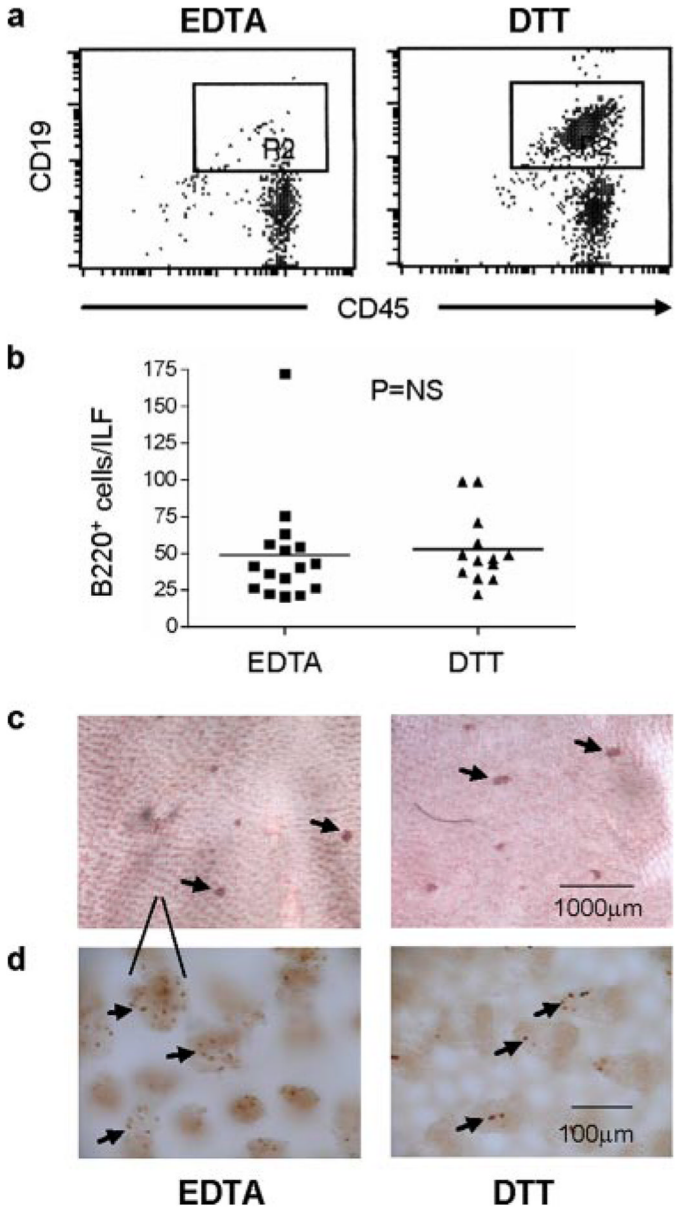

FIGURE 2.

Localization of DTT-released, epithelium-associated B cells. *a*, Flow cytometry of cells released by EDTA (*left panel*) or DTT (*right panel*) treatment, stained for CD45 (leukocyte) and CD19 (B cell), and analyzed using lymphocyte size and scatter gates. *b*– *d*, Whole-mount specimens of C57BL/6 small intestine (2-cm sections, 6 cm from the terminal ileum), treated with EDTA or DTT, were stained for B220 and analyzed by microscopy. *b*, Numbers of $B220^+$ cells within individual ILFs from EDTA- and DTT-treated specimens of adjacent intestine. *c*, Microscopic image of whole-mount intestinal mucosa at low power; original magnification ×20. Examples of ILFs are indicated with arrowheads. Note comparable numbers and size of ILFs in EDTA- and DTT-treated specimens. *d*, Microscopic image of whole-mount small intestinal mucosa at high power, original magnification ×100. Arrows indicate representative villi, and lines show scaling between *c* and *d*. Note that the number of B cells (punctuate brown spots) in each villus is greater in EDTA- vs DTT-treated specimens

(8 and 2 B cells/villus, respectively). Values of *p* were determined by Student's *t* test. Data are representative of four or more independent experiments.

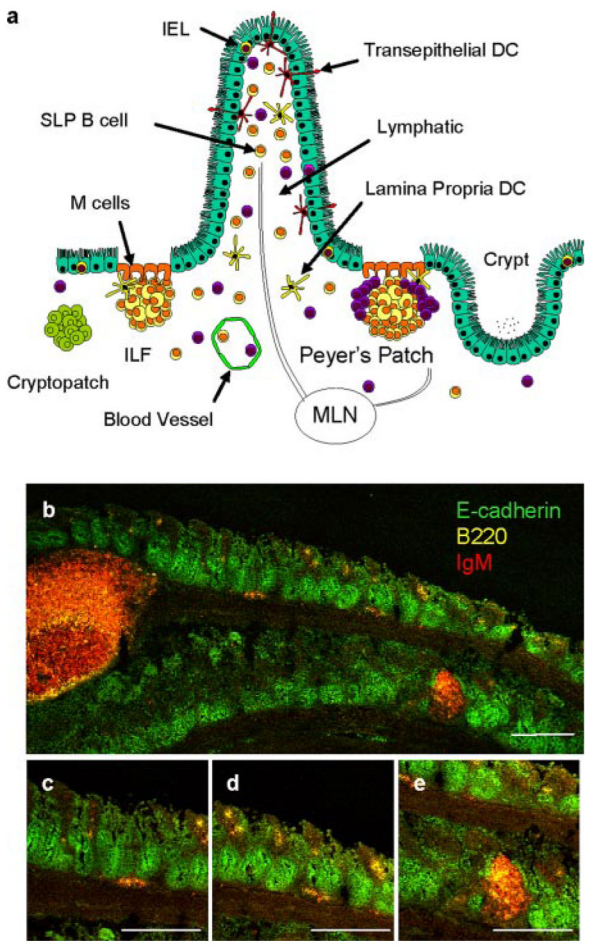

FIGURE 3.

Small intestinal B cell subsets. *a*, Schematic representation of PP, ILF, and SLP B cell subsets in the small intestine. Not to scale. *b*– *e*, B220 (yellow), IgM (red), and E-cadherin (green) immunofluorescent staining of rolled small intestine. *b*, Tilescan of distal small intestine demonstrating intact $B220^{+}IgM^{+}$ PP (*left*) and ILF (*bottom right*). *c*, Villi deficient of B cells, with subglandular $IgM^{+}B220^{+}$ cell aggregate (*d*). Villi bearing $IgM^{+}B220^{+}$ cells and subglandular B cell aggregate (*e*) intact $B220^{+}IgM^{+}$ ILF. Scale bar = 200 μm.

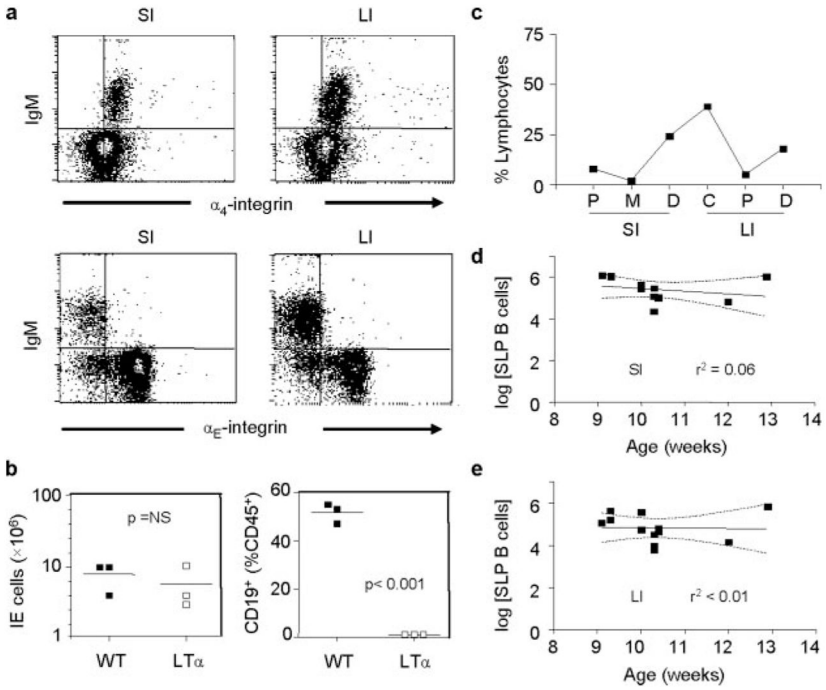

FIGURE 4.

Effects of homing and retention markers, segmental location, and age on superficial LPL B cells. Epithelium-associated cells were isolated by DTT treatment, and the homing molecules on B cells, B cell in LTα knockout mice, and the frequencies of SLP B cells ($CD19^+IgM^+IgD^+$) in various locations of intestine from the mice at different ages were examined. *a*, α_4 and α_E (CD103) integrin expression in epithelium-associated B cells from small and large intestine. *b*, Comparison of wild-type and LT$\alpha^{-/-}$ mice for absolute numbers of total intestinal epithelium-associated $CD45^+$ cells (termed IE) and the frequency of B cells ($CD19^+$ cells) as a percentage of $CD45^+$ cells in the lymphocyte size/scatter gate. Each symbol represents individual mice that were littermates matched for age (9 wk). Values of *p* were determined by Student's *t* test. Data representative of 6 –10 independent experiments for mice aged 8 –12 wk. *c*, Small intestine was divided into three equal-length segments (proximal, P; middle, M; and distal, D; 11–12 cm each). In the large intestine, the cecal appendix (C) was excised, and the remaining large bowel was divided into two equal segments (proximal, P; and distal, D; 7– 8 cm each). Data are representative of results from four mice. *d* and *e*, Effect of mouse age (C57BL/6J) on number of SLP B cells recovered from the small (*d*) and large (*e*) intestine. Symbols are data from individual mice. Data were log transformed and analyzed by regression analysis.

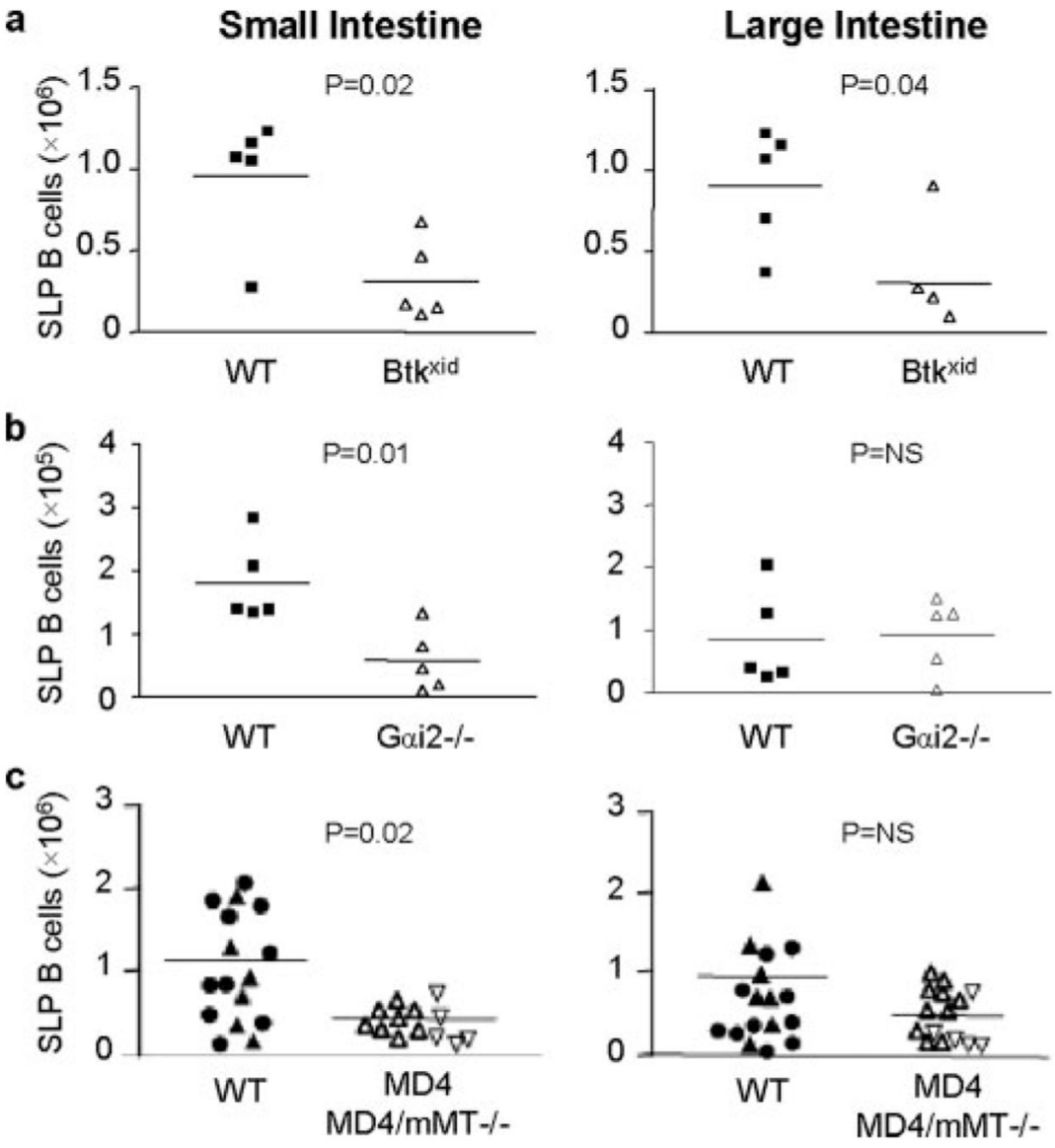

FIGURE 5.

BCR signaling requirements for SLP B cells. The number of SLP B cells ($CD19^+IgM^+$ cells in epithelium-associated lymphocytes) recovered from the small (jejunum and ileum) or large (cecum and colon) intestine was determined by flow cytometry. Symbols are data from individual mice. *a*, Comparison of wild-type Btk (CBA/J) and Btk^{xid} (CBA/N) mice. *b*, Comparison of wild-type G*α*i2 and G*α*$i2^{-/-}$ mice. *c*, Comparison of wild-type (closed symbol), MD4 (open triangle, base down), and MD4: $muMT^{-/-}$ (△, point down) mice. Data were produced from at least five mice, each data point representing a single animal, of each genotype and statistically compared by Student's *t* test.

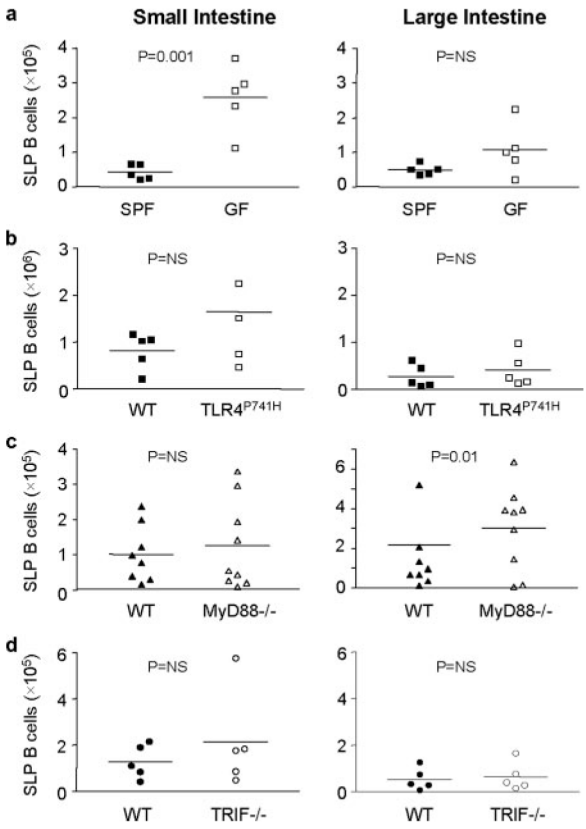

FIGURE 6.

Relationship of SLP B cell numbers to enteric flora and TLR-dependent sensing. DTT-released cells were isolated and counted from the small (*left column*) or large (*right column*) intestine and analyzed by flow cytometry with IgM and CD19 to determine frequency of SLP B cells, and the total number of SLP B cells was calculated. *a*, 129/SvEv mice from the North Carolina National Gnotobiotic Rodent Resource Center and Rodent Core Facility were compared under germfree (□) and SPF conditions (■). *b* and *c*, Comparison of littermates of C57BL/6 mice bearing wild-type (closed symbols) or null mutation (open symbols) of genes involved in TLR sensing or signaling: *b*, $TLR4^{P741H}$; *c*, MyD88; and *d*, TRIF. Values of *p* were determined by Student's *t* test.

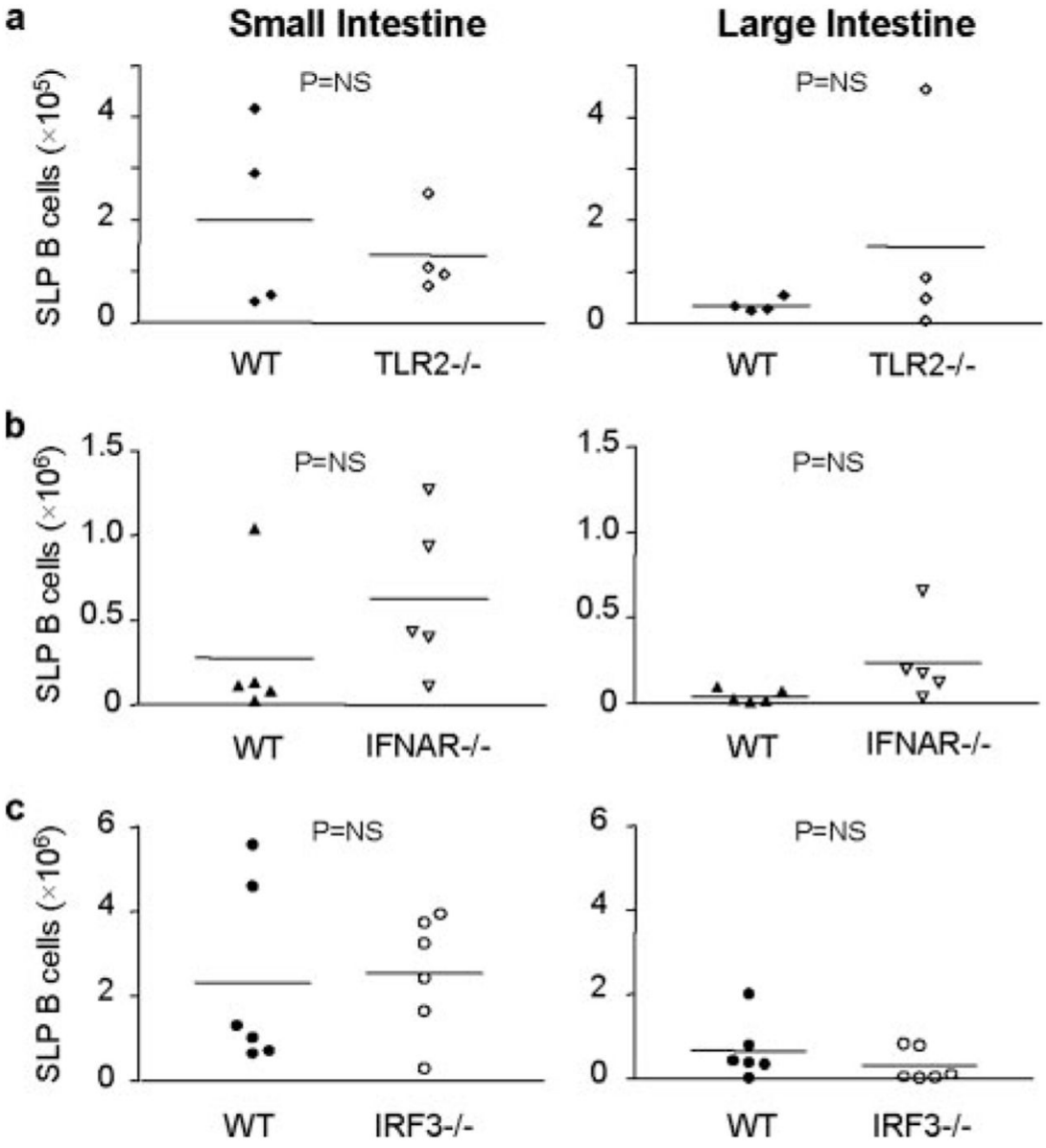

FIGURE 7.

Innate sensing and SLP B cell levels. Animals null for TLR2 (*a*), type 1 IFN receptor (*b*), or the transcription factor IRF3 (*c*) were examined for SLP B cells in small (*left*) and large (*right*) intestine. No significant differences in SLP B cells were seen in animals deficient for any of these innate sensors (open symbols) as compared with wild-type controls (closed symbols).

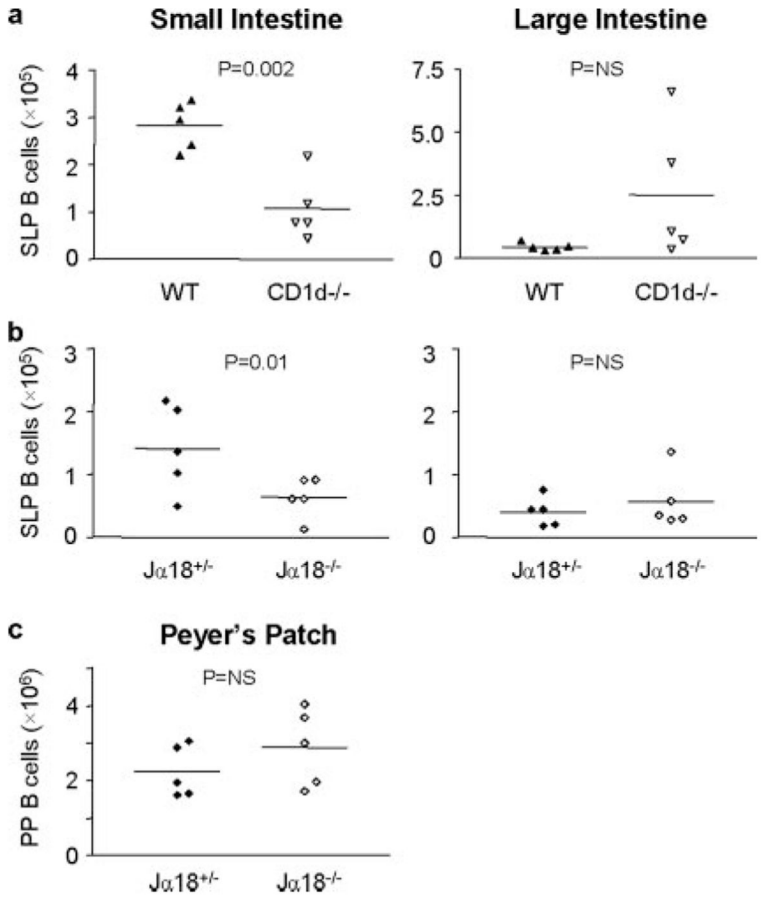

FIGURE 8.

Levels of SLP B cells in CD1$^{-/-}$ and Jα18$^{-/-}$ mice. SLP B cells were isolated from the small (*left column*) and large (*right column*) intestine and quantified as in Fig. 6. *a*, Comparison of wild-type (WT, ▲) and CD1d$^{-/-}$(△) mice. *b*, Comparison of heterozygote (Jα18$^{+/-}$, ◆) and homozygote null (Jα18$^{-/-}$, ◇) mice. *c*, PP were excised from each mouse, enumerated for the number of total cells, and analyzed by flow cytometry by CD19 to determine frequency of B cells, and the total number of PP B cells was calculated; heterozygote (Jα18$^{+/-}$, ◆) and homozygote null (Jα18$^{-/-}$) mice. Values of *p* were determined by Student's *t* test. Data were collected in three or more experiments.