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Activation of B Cells by a Dendritic Cell-Targeted Oral Vaccine

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Abstract

Production of long-lived, high affinity humoral immunity is an essential characteristic of successful vaccination and requires cognate interactions between T and B cells in germinal centers. Within germinal centers, specialized T follicular helper cells assist B cells and regulate the antibody response by mediating the differentiation of B cells into memory or plasma cells after exposure to T cell-dependent antigens. It is now appreciated that local immune responses are also essential for protection against infectious diseases that gain entry to the host by the mucosal route; therefore, targeting the mucosal compartments is the optimum strategy to induce protective immunity. However, because the gastrointestinal mucosae are exposed to large amounts of environmental and dietary antigens on a daily basis, immune regulatory mechanisms exist to favor tolerance and discourage autoimmunity at these sites. Thus, mucosal vaccination strategies must ensure that the immunogen is efficiently taken up by the antigen presenting cells, and that the vaccine is capable of activating humoral and cellular immunity, while avoiding the induction of tolerance. Despite significant progress in mucosal vaccination, this potent platform for immunotherapy and disease prevention must be further explored and refined. Here we discuss recent progress in the understanding of the role of different phenotypes of B cells in the development of an efficacious mucosal vaccine against infectious disease.

Keywords

B-1 cells; *Bacillus anthracis*; germinal centers; humoral immune response; *Lactobacillus*; mucosal vaccine; oral vaccine

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CONFLICT OF INTEREST STATEMENT

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INTRODUCTION

The surfaces of the mucosae are the most vulnerable locations for microbial challenge. However, parenteral vaccines often fail to confer protection at mucosal surfaces, and local immune responses are essential for protection against diseases that occur mainly by the mucosal route [1]. Vaccine delivery at one mucosal surface elicits the immune protection at all mucosal surfaces, as well as potentially inducing systemic immunity. Additionally, mucosal vaccines offer many advantages; in particular, oral administration is a fairly simple and inexpensive approach to deliver a vaccine *en mass*. However, antigens delivered by this route could be subjected to proteolysis within the stomach. To confer protection from degradation, certain bacteria that can thrive within the harsh conditions of the orogastric route have been used to express the immunogen of interest. We have successfully utilized beneficial gut bacteria, lactobacilli, which not only deliver antigens to the intestinal mucosa, but also provide several ligands to engage Toll-like receptors (TLRs), which detect conserved microbe-associated molecular patterns (MAMPs). There are several TLR and Nod-like receptor (NLR) ligands that have been used to enhance the immune response against several immunogens; however, bacterial RNA, which is exclusively provided by a live bacterial vector, is found to be the most effective adjuvant to provide long-lasting immunity [2, 3].

The early induction of a sufficient immune response dictates the overall protection provided by a vaccine. We have recently reported that a *Lactobacillus*-based oral vaccine transiently activates both T helper cell (Th)1 and Th17 responses [4]. Considering the propensity for oral tolerance in the gut, a strong Th1/Th17 response is often required to induce an immune reaction in this location. Although a strong Th1 response is induced in the gut with the live *Lactobacillus*-based vaccines, they also allow for a Th2 response to confer strong humoral immunity. Here, we will review recent progress in the understanding of the role of different phenotypes of B cells in the development of mucosal immunity and present data concerning the efficacy of an oral vaccine in the induction of the humoral immune response against oral infection with the Sterne strain of *Bacillus anthracis*.

MUCOSAL VACCINATION

The intestine comprises four concentric layers: the mucosa, submucosa, muscularis propria, and serosa. The intestinal epithelium of the mucosa is endodermal in origin and provides a tight barrier between the external environment and the host [5]. Since the mucosal layer forms the external boundary of internal organs, it is a vulnerable entry point for infection by pathogenic microorganisms. Vaccines capable of eliciting mucosal immune responses fortify immunity at mucosal surfaces and protect against infection. However, most licensed vaccines are administered parenterally, and fail to mount a protective local immune response at mucosal surfaces. In fact, only seven licensed vaccines are routinely administered mucosally (all delivered via the oral route except for intranasal Flumist®); these vaccines target poliomyelitis, cholera, typhoid, rotavirus, and influenza infections [1]. Effective mucosal immunization recruits effector molecules at the mucosal surfaces to neutralize pathogenic insults before considerable damage to the host can occur. Although mucosal

surfaces are present at very distinct and functionally diverse locations, vaccination at one mucosal site can confer protection at all of the mucosal surfaces.

However, while different mucosal locations induce comparable immune responses, not all mucosal surfaces are acceptable for mass vaccination. For example, vaccinations via the genital and rectal mucosae have shown a lot of promise, but are not practical approaches toward mass vaccination. In addition, immunologic features of the female reproductive tract change with the menstruation cycle [6–8]. In small animals, rectal inoculation has been attempted, but it was ineffective in larger animals and humans [9–11]. Consequently, in order to develop a mucosal vaccine for future human use, the route of vaccination is practically limited to nasal and oral administration. Vaccines delivered by the intranasal route are easy to administer and result in both mucosal and systemic immune responses [12, 13]. However, nasal administration of antigens or pharmaceuticals often results in their presence in the cerebral spinal fluid via absorption by rich capillary beds surrounding olfactory neurons, especially when adjuvant is present [14–16]. Two recent human clinical trials, in which human immunodeficiency virus (HIV)-1-derived antigen was delivered by the intranasal route, resulted in side effects related to the nervous system, such as Bell's palsy and damage to the olfactory nerves [17–20]. The possibility of these neurologic side effects and the ease of oral delivery have led many vaccinologists to pursue the oral route of vaccination.

ORAL VACCINES

The epithelial surface of the intestines not only composes a physical barrier, but also harbors critical molecular and cellular components of immune activation. The interplay of various cells at the intestinal surface affects antigen uptake and processing and the generation of an immune response, ultimately influencing the immunologic outcome—tolerance versus immunity. Oral administration of antigen(s) appears to be an attractive choice to vaccinate *en mass* because needle-free administration of vaccines does not require skilled individuals and is cost-effective. However, oral vaccination poses problems regarding degradation of the immunogen due to the low pH and proteases present within the gastrointestinal (GI) tract. Additionally, the GI tract is constantly exposed to dietary antigens and commensal bacteria, which induce oral tolerance. Nevertheless, there are a few successful oral vaccines available against human pathogens, such as poliovirus, *Salmonella typhi*, *Vibrio cholera*, and rotavirus. All of these vaccines are live attenuated pathogens that are efficient in antigen protection and their delivery to the target cells [1]. Learning from these strategies, we have used bacteria from the *Lactobacillus* genus to deliver antigens to intestinal immune cells without degradation from gastric acid and digestive enzymes. Consumed for centuries, lactobacilli are considered safe for human consumption, and certain species of *Lactobacillus* are important components of the commensal gut microbiota [21]. Additionally, researchers have developed both inducible and constitutive expression vectors that are effective in lactobacilli [22].

TARGETED DELIVERY OF ANTIGEN TO DENDRITIC CELLS

Antigen delivery to professional antigen presenting cells (APCs) reduces the requirement for a higher dose of immunogen to generate an immune reaction [23]. Traditionally, an antibody-antigen complex has been used to deliver antigen directly to APCs [24–29]. Although the use of antibody-antigen complexes was effective, this strategy cannot be used for the delivery of antigen at the gut mucosa because of possible degradation in stomach. Additionally, designing an expression vector to express a secretory antibody-antigen complex is technically much more complicated. This caveat can be overcome by the use of a twelve amino acid-long peptide that was discovered by phage library screening to bind directly to dendritic cells (DCs) [30]. This DC-targeting peptide has been shown to deliver the protective antigen (PA) of *B. anthracis* to intestinal DCs [31]. Thus, we genetically modified lactic acid bacteria to secrete PA tagged with the DC-targeting peptide (DCpep), which protected mice from lethal challenge with the Sterne strain of *B. anthracis* after vaccination by oral gavage [31].

ANTIGEN CAPTURE BY DENDRITIC CELLS

DCs have specialized functions in the gut and are essential mediators of intestinal homeostasis by inducing tolerance to gut commensal microbes while eliciting protective immune responses against pathogens [32]. The type of immune response induced by DCs is dependent upon the microbe encountered, the type of PRRs expressed and activated on the DCs, and the local cytokine/chemokine levels in the microenvironment; these parameters determine the specific response induced, including Th1 (producing IFN γ); Th2 (producing interleukin (IL)-4, IL-5, and IL-13); Th17 (producing IL-17); and regulatory T cells (Tregs) [33]. In order to interact with both commensal and potentially pathogenic microorganisms, the intestinal tract harbors specialized immune cells within the lamina propria (LP) and other immune follicles (Peyer's patches, colonic patches). There are several distinct populations of DCs present in the murine LP; however, two unique subsets that are limited to the gut are CD11b⁺CD103⁺ DCs and CD11b⁺CX₃CR1⁺ DCs [33]. In steady state, CX₃CR1⁺ DCs outnumber CD103⁺ DCs by 3 to 5-fold. Additionally, CD103⁺ DC are a non-dividing population and under continuous flux; whereas, the turnover of CX₃CR1⁺ DCs is slow and these cells are considered true residents of the GI tract [34]. Interdigitating CX₃CR1⁺ DCs sample intestinal luminal bacteria and their antigens directly across the epithelial cell layer and commence immune activation [34]. Additionally, gut-specific CD103⁺ DCs capture soluble antigens such as those secreted from lactobacilli and present them to T cell subsets in the mesenteric lymph nodes (MLNs) to initiate immune activation and/or regulation. Both invasive and non-invasive bacteria are sampled by LP DCs via transepithelial dendrites; however, commensal bacteria and pathogenic bacteria are transported to different locations by the LP DCs. DCs carrying commensal bacteria often end up in the Peyer's patches, whereas DCs carrying pathogenic bacteria migrate to the MLNs, for reasons that are unclear. CD103⁺ DCs capture soluble antigens and maintain a constant flux toward MLNs in a CCR7-dependent manner, where they prime naïve T cells [35]. In the steady state, both of these resident DC subsets maintain oral tolerance to commensal pathogens and food antigens by expressing retinoic acid (RA) and supporting the expansion of Tregs [36]. A sustained immune-regulatory environment in the gut is not amenable for the generation of a

protective immune response against pathogenic bacteria. Thus, the differential expression of ligands for pattern recognition receptors (PRRs) by vaccine vectors like lactic acid bacteria enhances adjuvanticity and helps to counteract the tolerogenic environment of the gut [37–39]. Although different antigens have been delivered to the gut mucosa with the use of different strains and species of lactobacilli [37, 40–48], in our experience, *L. gasseri* has better adjuvanticity when compared to other common *Lactobacillus* species [49–51]. Using *L. gasseri*, we have delivered PA of *B. anthracis* to successfully vaccinate mice against lethal challenge [31]. Another group has also utilized this bacterium for the successful delivery of *Salmonella* antigens [50].

INDUCTION OF HUMORAL IMMUNE RESPONSE TO AN ORAL VACCINE

The humoral immune response is mediated by antibodies, which are produced by activated B lymphocytes. The physiological function of antibodies is to neutralize and eliminate the antigens that elicited their secretion. Oral vaccination should induce a strong humoral immune response against the delivered antigen, and should not be limited to the common mucosal isotype, IgA. B cells also capture antigens and present them in the context of MHC II; however, the uptake of antigen is very specific and depends on the B cell receptor (BCR) expressed. Interactions between an antigen-sensitized B cell and an activated T cell that recognizes the MHC II-loaded peptide, initiate certain irreversible changes in the B cell. These changes including class switching, somatic hypermutation (SHM), and maturation and development into memory and plasma cells, depend upon a complex interaction between B cells, DCs, and T cells to form a germinal center (GC) [52]. First recognized over 125 years ago, GCs are sites within secondary lymphoid organs where clonal expansion of B cells, SHM, and affinity-based selection occur, resulting in the production of high-affinity antibodies [52].

We have found that the introduction of a live oral *Lactobacillus*-based vaccine induces the production of antibody in two phases, (i) immediate [4] and (ii) late production [31, 53]. The immediate enhancement in antibody production suggests a T-independent (TI) process, which is an orchestrated event involving a variety of innate immune cells that confers early protection against common microbial pathogens. In addition, the T cell-dependent pathway is often associated with an inflammatory reaction that could disrupt the mucosal epithelial barrier. To compensate for these limitations, the intestinal mucosa has developed a faster TI pathway that generates IgA in response to highly conserved microbial signatures recognized by TLRs [54]. In mice, TI IgA production involves B-1 cells from the peritoneal cavity and the intestinal LP, as well as conventional B-2 cells from isolated lymphoid follicles [54]. These B cells release low-affinity IgA (and IgM) in the absence of help from CD4⁺ T cells via CD40L ligation [55].

ROLE OF B-1 CELLS IN ORAL IMMUNIZATION

The role of B cells in adaptive immunity has been extensively studied; however, the discovery of B-1 cells that express CD5 on their surface and perform innate immune functions has raised questions about their involvement in innate immunity [56]. B-1 cells, which includes CD5⁺ B1-a and CD5[−] B1-b subsets, differ from conventional follicular B

cells (B-2 cells) in that they develop from fetal liver progenitors and are maintained by self-replacement, as opposed to B-2 cells that develop from bone marrow precursors [57]. They represent the majority B cell subpopulation in pleural and peritoneal cavities [58], are found in low numbers in the spleen and intestine (<5%), and are very rare in lymph nodes and bone marrow [59]. They continuously traffic to and from the pleural and peritoneal cavities through the omentum in a process that requires CXC-chemokine ligand 13 (CXCL13), which is likely produced by resident macrophages [60]. In steady state, B-1 cells produce natural IgM to provide a crucial barrier against common pathogens [61, 62]. In fact, B-1 cells account for approximately 80% of IgM present in serum [63, 64]. These B-1 cells also undergo the process of class switching; however, whether they undergo the process of SHM is not clear. They switch to a variety of different isotypes *in vitro*, but *in vivo*, they preferentially class-switch to become IgA-secreting cells. IgA-secreting B-1 cells in the intestinal LP develop from precursors in the peritoneal cavity in a process that depends on IL-5 production [65, 66]. The majority of IgA secretion in the gut lumen is produced by B cells present in the Peyer's patches [67] in response to commensal microbes; however, B-1 cells also contribute to the common pool of IgA with the capability to neutralize common pathogens [61, 68]. B-1 cells also work as phagocytic cells in a pro-inflammatory environment and present antigens to CD4⁺ T cells [69, 70]. B1-cells respond to a variety of stimuli, including TLR ligands like glycolipids and polysaccharides and other non-proteinaceous antigens [71–74].

Recently, it has been suggested that B-1 and B-2 memory responses have differentially evolved to complement each other to protect the host from pathogen invasion [75]. B-2 memory induction and the T cell-dependent maturation in GCs leads to a slow, but high-affinity primary response that enhances the quality and degree of recall responses, while B-1 memory is more rapidly induced for immediate protection against infection that lacks affinity maturation and may be confined to recall responses against an antigen in the context of inflammation [75]. Activation of B-1 cells has been shown to provide long-term protection to *Streptococcus pneumonia* [62], *Borrelia hermsii* [76], and *Francisella tularensis* [74]. B-1 cells also secrete certain cytokines such as IL-6 and IL-10, and provide a conducive environment for immune activation and regulation [77]; however, we have only seen an increase in IL-6 production from this cell population in response to the oral vaccine (Fig. 1). The presence of B-1 cells in the gut is well established, but their role in oral vaccination has not been demonstrated. Given their capacity to mount long-term immunity and contribute to the cytokine pool, we have evaluated their numbers and functions post-vaccination. Oral vaccination increased the cellular pool in the LP of the colon, and they class switched to produce IgA (Fig. 1). Currently we are investigating additional functions of these cells during oral immunization.

TLRs facilitate TI IgA responses either by activating B cells directly or by inducing the release of the B cell-activating factor of the TNF family (BAFF, also known as BLyS or TNFSF13b) and its homolog, a proliferation inducing ligand (APRIL, also known as TNFSF13) from innate immune cells. B cells express TLRs on their surfaces to better respond to sudden microbial insults. Activation of B cells by engagement of TLRs leads to the expression of activation-induced cytidine deaminase (AID), an enzyme required for

class switching and SHM. Mice with attenuated production of IgA due to AID deficiency demonstrate an expansion of anaerobic commensal bacteria in the gut, hyperplasia of isolated lymphoid follicles, and increased susceptibility to intestinal pathogens [78]. Expression of AID can also occur because of the interaction between transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI, also known as TNFRSF13b) and its ligands, BAFF and APRIL. These TACI-ligands are released by intestinal epithelial cells, DCs, and other innate immune cells in the gut in response to recognition of bacterial TLR ligands by TLRs [79, 80].

Lactobacilli express a variety of TLR ligands in the form of lipoteichoic acid (LTA, a TLR2 ligand), muramyl dipeptide (MDP, a TLR2 and NOD2 ligand), bacterial RNA (an NLR ligand), and CpG DNA (a TLR9 ligand) that can induce the expression of AID either directly by B cells or indirectly through interactions with TACI and its ligands on B cells. In our quantitative real time PCR analysis, expression of BAFF and APRIL were elevated along with a significant increase in the expression of the transcription factor, B lymphocyte-induced maturation protein 1 (Blimp-1, also known as *Prdm1*), and B-cell maturation antigen (BCMA, also known as *Tnfrsf17*) in colonic tissues. BCMA is expressed by plasma cells and is a receptor for both BAFF and APRIL; BCMA-dependent signaling enhances the survival of B cells by activating NF- κ B and MAPK pathways (Fig. 2) [81]. Blimp-1 results in plasma cell survival by increasing the expression of the transcription factor, X-box binding protein 1 (XBP1), which induces the unfolded protein response, a protective mechanism in plasma cells, and helps to control the endoplasmic reticulum stress that is caused by the immense production of antibodies [82]. Interestingly, Bcl-6, a transcription factor required for follicular helper T cell (T_{FH} cells) differentiation, and the percentage of GC B cells were also elevated in the colon of mice vaccinated with the *L. gasseri*-based vaccine (Fig. 2). This increase in Bcl-6 in the gut suggests early development of gut-associated lymphoid follicles, as Bcl-6 expression is upregulated in B cells that recognize T cell-dependent antigens and is required for GC formation. The proposed function of Bcl-6 is to enable accelerated proliferation as well as the tolerance to genomic damage that occurs during clonal expansion and SHM in the GCs by repressing genes encoding molecules involved in DNA damage sensing and cell cycle checkpoint blocks [83]. Bcl-6 serves to generate GC B cells by (i) silencing the anti-apoptotic molecule, Bcl-2, in GC B cells, which is vital during affinity-based selection and to prevent autoimmunity secondary to somatic hypermutation; (ii) repressing the expression of p53 and ATR, which allows GC B cells to tolerate DNA damage induced by rapid proliferation and AID-induced somatic hypermutation; (iii) preserving GC B cells' identity through the silencing of the plasma cell master transcription factor, Blimp-1, and thereby decreasing GC exit to become a plasma cell; and (iv) downregulating the expression of critical mediators of BCR and CD40 signaling, and thus possibly fine-tuning the responsiveness of GC B cells to selective signals [52, 84, 85]. For example, it has recently been demonstrated that weak, antigen-independent constitutive BCR signaling facilitated spontaneous plasma cell differentiation *in vitro* and *in vivo* in response to TLR agonists or CD40 and/or IL-4 ligation [86].

We also detected *Cxcl13* transcripts in the colon, a chemokine secreted by stromal cells for the recruitment of leukocytes at GCs. An increase in *Cxcl10* was also seen, which is secreted

by resident macrophages in response to IL-6. CXCL10/IP-10 has been shown to amplify the production of IL-6 by B cells, sustaining the STAT3 signals that lead to plasma cell differentiation [87]. Additionally, the presence of *Cxcl12* suggests a CXCR4-dependent control over B cell compartmentalization, which only allows mature B cells to migrate to form GC centers (Fig. 2).

Generation of long-lasting humoral immunity depends upon the development of GCs, the formation of which begins around six days after primary immunization [52]. GC B cell development requires cognate T cell/B cell interactions and provides memory B cells and long-lived plasma cells [88]. Until now, there have been several reports demonstrating the efficacy of oral vaccines [89]; however, the location of GC B cell development or the optimized schedule that would provide a protective response have not been determined. An additional consideration of oral vaccine administration is the likelihood of multiple dosing causing tolerance to the antigen at the cost of immunity. Therefore, minimal and maximum doses should be determined to achieve the optimal response with such a vaccine. We have evaluated GC B cell development post-oral vaccination. We utilized two different vaccination schedules, one that provided protection earlier [31, 53] and one that we consider to be the minimal requirement for protection (Fig. 3A & 4A). We defined GC B cells by the decreased expression IgD, increased binding to peanut agglutinin (PNA) and high levels of Fas on B220⁺ B cells [52]. Upon activation, B cells decrease the expression of IgD on their surfaces. Only a small percentage of these activated cells acquire the phenotype of GC B cells, as defined by the concomitant expression of Fas and binding to PNA. We have examined these expression patterns in various organs, such as the colon, Peyer's patches, MLNs, spleen, and bone marrow for development of GC B cells; however, an increase in GC B cells was found only in the MLNs and spleens of these vaccinated mice (Fig. 3 & 4). These data demonstrate the sites of immune reaction as a result of our oral vaccination strategy. With our minimalistic vaccination approach, in which we orally administered the vaccine one time and repeated the oral dose one month later, we discovered that immunity was boosted. There was a five-fold higher percentage GC B cells in the MLNs, but at the cost of the percentage of GC B cells in the spleen. A decrease in GC B cells in MLNs with increasing numbers of gavages suggests a possible induction of tolerance due to persistent presence of antigen; MLNs are a site known for the induction of oral tolerance [90].

MUCOSAL TOLERANCE

Oral tolerance is the state of local and systemic immune unresponsiveness that is induced by oral administration of an antigen. The generation of tolerance against an immunogen has been considered to be a roadblock in the development of oral vaccination strategies. The CD103⁺ DC population, which specifically resides in the LP of the gut, captures soluble antigens and migrates to MLNs to interact with a diverse population of T cells [91]. CD103⁺ DCs have the unique ability to generate gut-homing FoxP3⁺ Tregs [92, 93]. These DCs metabolize retinoids from food into RA, which in turn, induces the expression of the gut homing markers, CCR9 and $\alpha 4\beta 7$, on T cells [94–97]. RA produced by these cells also helps in the generation of Tregs by altering histones at the *foxp3* locus [98]. At present, RA is considered a master regulator of immune responses in the gut due to its ability to override the cytokine signaling, which may otherwise drive a Th17 response [99–101]. The

transcriptome of stromal cells from MLNs is different from that of any of the other lymph nodes [102] and along with CD103⁺ DCs, these stromal cells also support the generation of Tregs [103]. The generated Tregs are not only capable of controlling general inflammation in the gut and MLNs, but also inhibit the process of GC formation and B cell maturation [100–103]. Therefore, multiple feeding of antigen would potentially result in an antigen-specific tolerance and could inhibit the immune process instead of enhancing it. When a group of mice fed with an oral vaccine once and then boosted once after one month were compared with a group of mice fed the oral vaccine for four consecutive weeks and then boosted, the second group who were fed over consecutive weeks had a lower percentage of GC B cells (Fig. 3 & 4), which supports the idea of inhibition of GC B cells upon multiple oral exposures to antigens. Therefore, one should be cautious regarding the vaccination schedule of an oral vaccine, where overexposure could undermine protection from the pathogen.

CONCLUDING REMARKS

The mammalian immune system has evolved into innate and adaptive branches that produce integrated immune responses against pathogens. However, the conventional notion that the innate immune system is nonspecific and lacks memory, while the adaptive immune system is defined by specific antigen recognition and memory is no longer a correct or useful categorization of host immunity [108]. In our model of oral vaccination, we found upregulation of certain signature genes important for GC formation and other processes of B cell development and maturation (Fig. 5). Within the colons of mice vaccinated with the targeted oral vaccine, we found an accumulation of B-1 cells that express IgA, and IL-6. The roles played by B-1 cells in innate and acquired immunity are still being resolved and is a subject of investigation in our laboratory. GC formation is a hallmark of a Th2-type adaptive immune response and was found more frequently in the vaccinated mice, primarily in their MLNs. We are now investigating the potential generation of Tregs in mice that receive multiple vaccinations and the impact of vaccination on the microbial composition of the gut. Until now, the site of immune activation during oral vaccination was unknown; herein, we provide some evidence that mesenteric lymph nodes and the colon are sites of B cell development with oral targeted vaccination.

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Fig. (1). Accumulation and activation of B-1 cells in the colon

(A) Schematic diagram of vaccination schedule. (B) Groups of C57BL/6 mice ($n = 3$) were orally gavaged with 10^9 CFU of *L. gasseri* expressing PA-DCpep [31]. B-1 cells were evaluated by surface expression of CD5 on CD19⁺ B cells in MLNs, Peyer's patches, and colons. Mice treated once with the vaccine are represented as "Vac" and the mice treated with both the first vaccine and then boosted one more time as shown in vaccination schedule (A) are represented as "Vac-boost". (C) Percentage of CD5⁺ B cells in the lamina propria of different groups of mice. (D&E) Isolated lamina propria cells were also stained with Abs against IL-6 and IgA and analyzed by flow cytometry. Data represent the percentage CD5⁺ B cells expressing IL-6 (D) and IgA (E). Antibodies used were purchased from eBioscience (San Diego, CA) or BioLegend (San Diego, CA). Animal protocols were approved by IACUC at the University of Florida. Data represent mean SEM $\pm$ from two independent experiments. * $P < 0.05$ compared with PBS.

Fig. (2). Gene expression profile of colonic tissues post-vaccination

(A) Groups of C57BL/6 mice ($n = 3$) were orally gavaged with 10^9 CFU of *L. gasseri* expressing PA-DCpep [31] and sacrificed on days 1, 3, and 7 post-vaccination and total RNA isolated from distal portions of the colons. Quantitative real-time PCR (Bio-Rad, Hercules, CA) was performed to evaluate genes important in B cell development. The results represent the ratio of fold change in 18s-rRNA-normalized gene activity in bacteria-treated mice compared to the PBS-treated mice. (B) Representative heat-map corresponding to (A). Data represent mean SEM $\pm$ from two independent experiments. * $P < 0.05$ ** $P < 0.01$ compared with PBS.

Fig. (3). A conventional schedule of oral vaccination leads to a limited increase in germinal center B cells at mesenteric lymph nodes

(A) Schematic diagram of vaccination schedule. (B&C) Groups of C57BL/6 mice ($n = 3$) were orally gavaged with 10^9 CFU of *L. gasseri* expressing PA-DCpep [31]. The development of germinal center B cells was evaluated by surface binding of peanut agglutinin (PNA) and Fas on IgD⁺B220⁺ B cells in MLNs (B) and spleen (C). Left panel shows representative dot plots and compiled data from two independent experiments (total $n=6$). (D) Groups of C57BL/6 mice ($n = 3$) were orally gavaged with 10^9 CFU of *L. gasseri* expressing PA-DCpep. The activation of B cells in the MLNs was evaluated by downregulation of IgD from cell surfaces. Data show the percentage of B cells that exhibited decreased IgD on their surfaces. Data represent mean SEM $\pm$ from two independent experiments. * $P < 0.05$ and ** $P < 0.01$ compared with PBS.

Fig. (4). A minimalistic schedule of oral vaccination leads to a strong increase in germinal center B cells at mesenteric lymph nodes

(A) Schematic diagram of the vaccination schedule. (B&C) Groups of C57BL/6 mice ($n = 3$) were orally gavaged with 10^9 CFU of *L. gasseri* expressing PA-DCpep [31]. The development of germinal center B cells was evaluated by surface binding of peanut agglutinin (PNA) and Fas on IgD^{low}B220⁺ B cells in the MLNs (B) and spleen (C). Left panel show the representative dot plots and the compiled data from two independent experiments (total $n=6$). Data represent mean SEM $\pm$ from two independent experiments. * $P<0.05$ and ** $P<0.01$ compared with PBS.

Fig. (5). Schematic diagram illustrating the events in the colon and mesenteric lymph nodes post-oral vaccination

Orally fed bacteria, which are recognized by epithelial cells and interdigitating DCs, serve as a complex adjuvant and the source of secreted bacterial antigens, and are taken up by DCs. Activated epithelial cells and DCs secrete B cell activating factors (e.g., APRIL, BAFF) that provide a niche for B-1 cells that contribute IL-6 and IL-10 to the cytokine pool, and, at the same time, class-switch to the secretory IgA. Simultaneously, DCs capture the secreted targeted vaccine, and migrate to the mesenteric lymph nodes to activate CD4⁺ T cells. The subsequent complex interactions between DCs, follicular T cells (T_{FH} cells), and B cells result in the development of germinal center (GC) B cells. After some GC B cells mature into plasma cells, they produce high affinity antibodies in the circulation. Memory B cells developed in the process are ready for subsequent pathogenic challenges.