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Lymphoglandular Complexes Are Important Colonic Sites for Immunoglobulin A Induction against *Campylobacter jejuni* in a Swine Disease Model

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Campylobacter jejuni* is a common cause of serious food-borne illness. In an experimental model of human infection, germfree pigs were given *C. jejuni*, the whipworm *Trichuris suis*, dual infections with *C. jejuni* and *T. suis*, or no infection. In dual-infected pigs, a synergistic effect between *C. jejuni* and *T. suis* was observed 27 days after infection, when *T. suis* fourth-stage larvae were found in the proximal colon. In dual-infected pigs, lymphoglandular complexes (LGCs) in the distal colon were substantially enlarged, and *C. jejuni* was detected in cells of the follicle-associated epithelium (FAE) and in cells with macrophage morphology within these follicles. In the study reported here, cell types in colonic tissues from these pigs were analyzed by use of immunohistochemical and morphometric analyses for cell surface markers (IgM, IgG, IgA, CD4, CD8, MHC Class II, and macrophage SWC3a). To our knowledge, we provide the first description of cell types in mammalian LGCs, document that they have all elements necessary for antigen processing, and demonstrate the appearance of IgA germinal centers (GC) in LGCs from *C. jejuni*-infected pigs (single or dual infected). Ileocecal Peyer's patches (ICPP) and mesenteric lymph nodes (MLN) also had IgA GC development if *C. jejuni* was present, but LGCs had the greatest amount of anti-*C. jejuni* staining, and appreciable increase in overall follicle size and size and number of GCs committed to IgA production. LGCs are present in humans and other mammals and are important in other enteric infections.

Disease caused by *Campylobacter* spp. has substantial health and economic impact. *Campylobacter jejuni* is the most commonly reported bacterial cause of food-borne infection in the United States (2, 3, 10, 13, 20), and is a serious emerging problem in the world (1). Also, *C. jejuni* has been linked to Guillain-Barré syndrome, which is a debilitating polyneuritis characterized by fever, pain, and weakness that progresses to paralysis, and often results in long-term disability (9, 35, 36, 43).

The main group at risk for *Campylobacter* enteritis is children. Primary infection is initiated in the gastrointestinal (GI) tract after ingestion of contaminated food or water. *Campylobacter jejuni* may colonize either the small (27) or large (11) intestine, but more often results in colitis or cecitis (11, 27). The presence of abdominal pain, fever, diarrhea, and frank blood and inflammatory cells in stools suggests invasion (7, 11). Histologic examination of colonic biopsy specimens confirms acute colitis with crypt abscesses, depletion of goblet cells, and inflammatory infiltrates of the lamina propria composed of polymorphonuclear neutrophils, lymphocytes, and plasma cells (27, 32). *Campylobacter jejuni* enteritis is initiated in the GI tract, but can become extraintestinal in severe cases (47), particularly in immunocompromised hosts (4). In developing countries, children are infected early in life and have less severe disease than do children in developed countries where infection occurs more commonly later in childhood (45, 46). The disease spectrum includes severe inflammatory illness, mild secretory diarrhea, or an asymptomatic carrier state. In hyperendemic areas, children are exposed earlier to high doses of *C. jejuni*, the duration of disease is shorter, and repeated infection with various *C. jejuni* strains is common, although the role of concurrent infections, including that of parasites, is unknown.

In humans, humoral immunity to *C. jejuni* appears to confer homologous protection. Anti-*C. jejuni*-specific serum IgG, IgM, and IgA concentrations increase during the month after infection (47). Immunoglobulin G confers lasting strain-specific immunity, whereas IgA has an important role in the clearance of *C. jejuni* from the intestine. Additionally, *C. jejuni* are efficiently phagocytosed by polymorphonuclear neutrophils, especially if the bacteria have been previously opsonized (47). Mothers that are constantly exposed often provide passive immunity to their children by secreting IgA and IgG in breast milk (12, 44, 45).

Pigs have been used as an experimental model of *C. jejuni* infection in humans. Germfree pigs given dual infections with the swine whipworm *T. suis* and *C. jejuni* had more frequent and severe diarrhea and more severe pathologic changes than did pigs given no pathogens, *T. suis* alone, or *C. jejuni* alone (33). Dual-infected pigs had significant hemorrhage and inflammatory cell infiltrates in the proximal colon where L4 larvae were found, and intracellular *C. jejuni* associated with abscessed lymphoglandular complexes (LGCs) in the distal colon. Pigs given *C. jejuni* alone developed mild clinical signs of disease and pathologic changes, and bacteria were detected in feces or extracellular sites. Pigs given *T. suis* or no pathogens did not have disease and only minimal pathologic changes. Thus, *C. jejuni* and *T. suis* synergized to

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induce significant disease and site-specific pathologic changes, most notably in LGCs of the distal colon.

LGCs are circumscribed submucosal lymphoid nodules distributed most densely in the rectum and distal portion of the colon of humans (26), swine (33, 37), cattle (28), rats (8), horses (31), and other animals. Grossly they appear as raised nodular domes with a central pore. Histologically, the nodule is a lymphoid follicle with entrapped mucosal crypts opening on the central pore. The M cells with associated leukocytes are found among the cells of the follicle-associated epithelium (FAE). On the basis of histologic and ultrastructural similarities to the Peyer's patches in the small intestine, LGCs have been suggested to recognize and initiate immune responses against foreign agents. The morphologic similarity between porcine and human LGCs suggest potential value of the pig as an animal model for human diseases in which colonic LGCs are altered or in which local colonic immune response (or lack of it) contributes to development or resolution of the disease (37).

In the study reported here, we hypothesized that LGCs are antigen-sampling structures that respond to the presence of *C. jejuni*. To examine this hypothesis, we conducted immunostaining of colonic tissues from germfree pigs experimentally infected with *C. jejuni* alone, *T. suis* alone, *or C. jejuni* and *T. suis*, or not infected (controls). *Campylobacter jejuni* selectively invaded LGCs in either single or dual infections, and led to sensitization and expansion of germinal centers (GCs) within the underlying follicle. These results indicated that the LGC is a major site for induction of immune responses to *C. jejuni*.

Materials and Methods

Pigs. All pigs were obtained by cesarian section at full term of two Landrace x Yorkshire cross sows, as described (34). Gnotobiotic pigs were delivered into germfree incubators and were infected as described (34). Pigs were fed a sterilized complete milk diet (Esbilac, Borden Co., Hampshire, Ill.) for the duration of the study. Pigs were cared for and studied using humane methods that are documented in a protocol approved by the Michigan State University All University Committee on Animal Use and Care. This AUCAUC document (No. 07/02-117-00) follows the guidelines of the Office of Laboratory Animal Welfare, National Institutes of Health entitled the *Guide for the Care and Use of Laboratory Animals* and the Animal Welfare Act Regulations and subsequent amendments.

Experimental design. Tissues from a previous experiment were used for immunohistochemical analysis of cell types in various colonic lymphoid structures (34). Briefly, at three days of age, gnotobiotic pigs were infected with the following pathogens according to group: 1 (n = 5), control group with no infection and sham inoculated; 2 (n = 4), infected with *T. suis* only; 3 (n = 5), infected with *C. jejuni* only; and 4 (n = 6), infected with *T. suis* and *C. jejuni*. Pigs were examined three times daily for adverse signs of infection. Abnormal findings, including description and severity of diarrhea, were recorded. Fecal swab specimens were taken for bacteriologic isolation and identification on days 6 and 27 after infection. At 27 days after infection, pigs were removed from the incubators and humanely euthanatized by intravenous administration of an overdose of barbiturate. Complete postmortem examination was done, and tissues were recovered for various purposes.

Experimentally induced infections. Pigs of groups 2 and 4 were inoculated by gavage with 3,000 embryonated *T. suis* eggs. Embryonated eggs were prepared from adult whipworms pulled free from the colonic mucosa of experimentally infected pigs as described (23). After washing in sterile saline pre-warmed to 37°C, worms were washed in sterile Hanks' balanced salt solution (HBSS) to remove fine debris not visible under the microscope. This was followed by incubation in a 5X-concentrated antibiotic cocktail in RPMI-1640 medium for a 16- to 24-h period. The original 5X cocktail contained 500 U of penicillin (PEN)/ml, 500 µg of streptomycin (STREP)/ml, 1.25 µg of amphotericin B (AMB)/ml, and 350 µg of chloramphenicol (CAP)/ml. A second incubation in a 1X antibiotic cocktail without chloramphenicol was performed for an additional 16- to 24-h period. Worms were then washed repeatedly in sterile HBSS, with at least three changes for a minimum of 2 h each, to remove residual antibiotics. Finally, worms were incubated for 10 days in RPMI-1640 medium containing 1% glucose (4 worms/ml) at 37°C with humidified 5% CO_2 for collection of eggs. Eggs were collected and incubated for 6 weeks to allow embryonation. Eggs were examined microscopically, and embryonated eggs were picked by use of a pipette and placed into individual doses that were delivered by gavage through a feeding needle.

Pigs of groups 3 and 4 were inoculated orally with a low dose (10^6 colony-forming units [CFU]) of *C. jejuni*. The *C. jejuni* strain chosen for infection was second-passage ATCC strain 33292 isolated from a human with enteritis and characterized by testing with various media and conditions to optimize growth and ensure virulence. To test for virulence and to generate low-passage isolates, 3-day-old colostrum-deprived pigs were inoculated orally with a high dose (approx. 5×10^9 CFU) of *C. jejuni* strain 33291, 33292, or 33560. Pigs infected with 33292 developed clinical signs of diarrheal disease within two days after inoculation, and *C. jejuni* were re-isolated from feces and amplified to generate low-passage bacterial stocks. Freezer stocks were maintained in sheep blood at –80°C. Doses for experimentally induced infections were prepared from second-passage frozen stock. Bacteria were streaked onto *Brucella* agar supplemented with 5% sheep blood and were incubated for 48 h at 37°C and 5% CO_2. A single colony was picked and placed into *Brucella* broth, which was incubated at 37°C for 20 h to early logarithmic phase of growth. Confirmation of early logarithmic growth phase was based on growth curve determinations for *C. jejuni* 33292 (data not shown), optical density readings (OD_{560}), and dark field microscopy, in which the majority of the organisms had spiral form and darting motility. Doses were made by resuspending 0.5 ml of the 0.1 OD_{560} inoculum in 49.5 ml of sterile milk warmed to 37°C. To confirm the desired inoculum of approximately 10^6 CFU for each pig, limiting dilution analysis was done on three of the prepared doses of *C. jejuni*.

Tissue preparation. Tissues used for this study included LGCs, mesenteric lymph nodes (MLN), and ileocecal Peyer's patches (ICPP). The LGCs were grossly visualized by their central pore, and were snipped parallel to the mucosa, encompassing the follicle, and extending as deep as the serosa. On the basis of published information (28, 33) and preliminary studies in our laboratory, LGCs were determined to be symmetric around their central axis. Therefore, a cross section through the central pore at the widest spot yields a section where all structural elements can be evaluated (e.g., FAE, GCs, overlying mucosa).

The ICPPs were taken in similar manner from the proximal

Table 1. Specificity of monoclonal antibodies used to identify colonic cell types using immunohistochemical analysis

mAb Designation	Specificity	Subclass	Reference
5C9[a]	IgM-μ	IgG1	(41)
3H5B101R[a]	IgA-±	IgG1	(40)
3H7[a]	IgG-γ	IgG1	(40)
PT90a[b]	CD4	IgG2a	(24)
PT81b[b]	CD8	IgG2b	(24)
MSA3[a]	MHCII-DR	IgG2a	(21)
74-22-15[a]	SWC3a	IgG2b	(42)
Anti-*C. jejuni*[c]	Outer membrane	IgG1	Biogenesis, Sandown, N.H.

[a]Kindly provided by J. K. Lunney, Building 1040, Room 105, BARC-East, Beltsville, Md. 20705.
[b]VMRD, Pullman, Wash.
[c]The anti-*C. jejuni* outer membrane monoclonal antibody (mAb) was used as neat hybridoma supernatants or 1:500 purified ascites fluid.

colon immediately distal to the ileal aperture. The MLN were taken from the antimesenteric border of the colon. Tissue snips were overlayed with O.C.T. compound (Sakura Finetek, Inc. USA, Torrance, Calif.) and snap-frozen in liquid nitrogen. These samples were maintained at –80°C until use. Tissue blocks were sectioned at 4- to 6-μm thickness by use of a cryostat (Reichert-Jung Cryocut, West Germany), and were adhered to either poly-L-lysine (Sigma Chemical Co., St. Louis, Mo.)-coated glass slides or charged glass slides (Fisher Scientific, King of Prussia, Pa.). Sections were fixed in cold (4°C) acetone for 5 min immediately after sectioning. The acetone was allowed to evaporate completely, and the fixed slides were either used immediately or stored at –70°C with added desiccant.

Immunohistochemical staining. Whenever possible, serial sections of a single piece of tissue were subjected to the entire antibody panel (Table 1). The LGCs were sectioned perpendicular to the mucosal surface and through the central pore at their widest spot. The ICPP were also sectioned perpendicular to the mucosa through an area that captured as many follicles as possible. The MLN were cut in cross section.

Tissue sections were rehydrated in 0.05*M* Tris, 0.1% Tween-20, 0.15% NaCl (TTBS) for 10 min. Potentially cross-reactive sites were blocked with 1% bovine serum albumin (BSA)-TTBS (B-TTBS) for 30 min. Primary monoclonal antibodies (mAbs) were diluted 1:10 with B-TTBS (hybridoma supernatants) or 1:500 (purified ascites fluid) (Table 1). Negative controls consisted of: sections treated with B-TTBS only instead of primary antibody, and sections treated with a mixture of anti-rat mAbs of similar mouse isotype. Sections were incubated with primary antibody at room temperature for 3 h, and rinsed with three 5-min washes of TTBS. Primary antibody was detected by use of biotin-labeled sheep anti-mouse antibody [$F(ab')_2$ fragment, cross-absorbed against human serum] diluted 1:100 in B-TTBS. Streptavidin-alkaline phosphatase diluted 1:200 in TBS (no Tween-20) was the tertiary conjugate. Sections were incubated with secondary and tertiary conjugates for 1 h each, followed by three 5-min washes with TTBS. Fast-Red TR/Naphthol AS-MX phosphate was used to develop the alkaline phosphatase, after which sections were counterstained with Gill's I hematoxylin and cover slipped with aqueous mounting medium (Biomeda Corp., Foster City, Calif.).

Morphometric analysis. Briefly, LGCs were sectioned and 25 LGCs from each pig were used to establish the mean diameter (34). Morphometric analysis was conducted on LGCs to provide estimates of cell numbers in stained sections, using the method of Fanuchi and colleagues (17). Five LGCs from each pig were subjected to the panel of mAbs and, on the basis of these results, one representative LGC from each pig was used for statistical comparison of numbers of each cell type within and between groups. This was possible because LGCs taken from the distal colon in a single pig and within groups were highly consistent (34). Cells stained for IgM, IgG, IgA, $CD4^+$, and $CD8^+$ were enumerated at a magnification of 400×, using a Power Macintosh 7100/66 computer with the public domain NIH image program (Wayne Rasband, National Institutes of Health, Bethesda, Md.). Cell numbers were counted in the delimited area of the LGC follicle (e.g., GCs and mantle areas). This area varied in size depending on the infection status of the pig. Similarly, ICPPs and MLNs were cross-sectioned through their widest part and five separate ICPP and MLN sections from each pig were stained with all mAbs. Immunohistochemical staining results for all immunostained cell populations in the LGCs in the colon of experimental pigs 27 days after infection with either T. suis or *C. jejuni*, both pathogens, or neither pathogen were expressed as mean ± SD cell numbers. The range for these morphometric-based cell counts also was determined.

Results

***Campylobacter jejuni* was found mainly in the FAE and GCs of LGCs of experimentally infected pigs.** To summarize the infection results on which this study is based, changes in the GI tract of pigs were characterized as those directly at the site of worm attachment in the cecum and proximal colon and those in the distal colon, and these have been reported (34). The LGCs had significant pathologic lesions, and *C. jejuni* were found in the distal colon (Fig. 1). In all groups, the general anatomic description of LGCs was similar to that of early reports (Fig. 1-3) (6, 18, 37). There was a substantial increase in size of the LGCs in pigs with dual infection of *T. suis* and *C. jejuni* compared with that in pigs of other groups (34). The LGCs appeared enlarged in pigs given only *C. jejuni*, but they were not appreciably different from the LGCs of *Trichuris*-infected pigs or uninfected controls (34). Only colonic tissues from *C. jejuni*-infected pigs reacted positively to anti-*C. jejuni* antibodies. In dual-infected pigs, the main areas that stained were the FAE and GCs of the LGCs (Fig. 1A and 1B). The GCs contained cells that appeared to be macrophages that stayed positively for anti-*C. jejuni* (Fig. 1B). These *C. jejuni*-positive mucosal crypts, that were below the muscularis mucosae and entrapped within the LGCs, were often mucus filled, with extravasated neutrophils (Fig. 1C). In pigs given only *C. jejuni*, the main areas staining were the FAE entrapped within the LGCs.

Germinal center expansion of lymphoglandular complexes occurred only in response to *Campylobacter jejuni*. The most noteworthy finding was increased size of the LGCs due mainly to GC development in all pigs given *C. jejuni*, but not in pigs given *T. suis* or left uninfected (Fig. 2 and 3). In these pigs, LGCs had more GC expansion than did those of other tissues examined (ICPP and MLN) (Fig. 3-5). The cellular composition of lymphoid tissue within the LGCs was mainly the same in all groups, reflecting the structure of a secondary lymphoid tissue, but the number of cells of a particular class was increased depending on infection status (Table 2; Fig. 2). Developing GCs largely accounted for the increases in immuno-

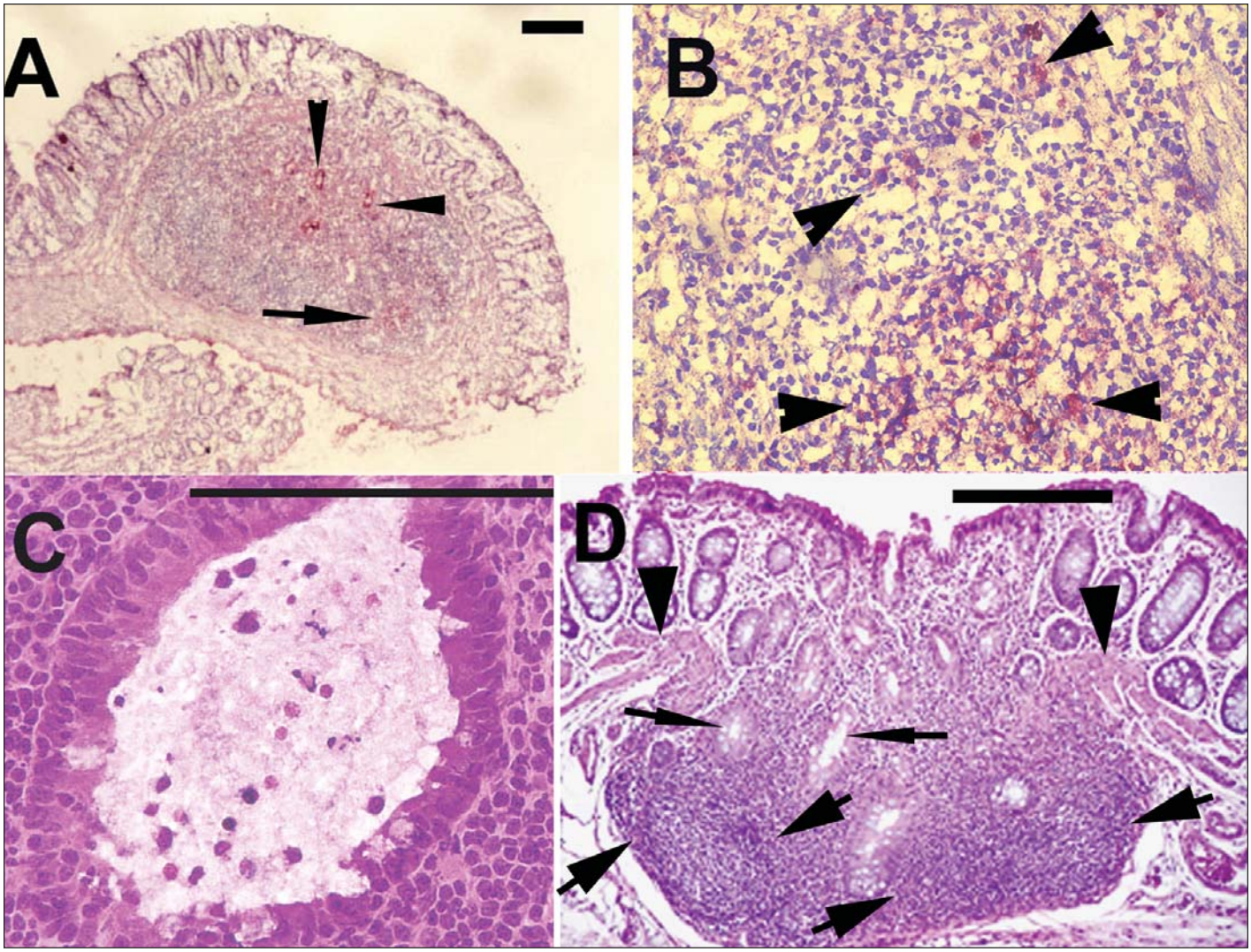

Figure 1. Features of lymphoglandular complexes (LGC) from gnotobiotic pigs infected with *Trichuris suis* and *Campylobacter jejuni*. Immunohistochemical staining (red stain), using a *Campylobacter jejuni* monoclonal antibody (mAb), of LGCs from the distal colon of dual-infected pigs. Bars represent 100 μm. (A) LGC at low power to show the distribution of anti-*Campylobacter* staining in the follicle-associated epithelium of an entrapped crypt (arrowhead) and in the germinal centers (arrow); (B) close-up of cells within the germinal centers that stain for *C. jejuni* (arrowheads); (C) an entrapped, mucus-filled crypt of an LGC with extravasated neutrophils; and (D) LGC stained with H&E to show the general structures including the muscularis mucosae (arrowheads), germinal centers (large arrows), and entrapped crypts (small arrows).

globulin-bearing B-cell (Ig-BC) numbers observed in LGCs of *C. jejuni*-infected pigs. In non-immunostained sections, GCs were located in the basal peripheral regions of the follicle, and could be identified by a cell-dense peripheral mantle that was typically thickest along the mucosal circumference. One or more GCs were present in the cross sections of the LGCs of all *Campylobacter*- and dual-infected pigs, whereas LGCs of *T. suis*-infected and control pigs did not display GCs. Cells expressing different cell surface markers occupied distinct zones of the GC (Fig. 2E-2H). Cells in the basal region of GCs, were positive for major histocompatibility class-II (MHC-II) molecules, but did not react positively for any class of Ig-BC (IgM, IgA, IgG), or other cell surface markers tested (CD4, CD8, or SWC3). Germinal center cores were strongly positive for either IgA or IgM, but not both. The LGCs from 2 pigs of the *Campylobacter* only-infected group had GCs with cores weakly positive for IgG.

IgM-positive B cells accounted for the numerical majority (> 50%) of immunoglobulin-bearing cells in LGC follicles examined from all groups (Table 2; Fig. 2E). In all pigs, the lamina propria overlying and surrounding the follicle was sparsely populated with IgM^+ cells. LGCs from pigs of all infection groups also were examined for IgG^+ cells (Table 2; Fig. 2F). IgG^+ cells were found in the dome areas of all LGCs from pigs that were *Campylobacter*-infected or dual-infected with both pathogens. The pigs with dual infections had more IgG^+ cells visible in these areas. LGC sections from the *T. suis*-only group had one IgG^+ cell each. IgG^+ cells were not found in the lamina propria of any group.

Immunostaining for CD4 and CD8 revealed a significant T-cell compartment within LGC follicles of *C. jejuni*-infected pigs (Table 2; Fig. 2C and 2D). In comparisons of serial sections stained for the various cell surface markers, the majority of the follicle that did not react positively for IgM was positive for a mixture of $CD4^+$ and $CD8^+$ cells. These cells were commonly found in the mantle areas of GCs, as well as scattered throughout the basal regions of the fol-

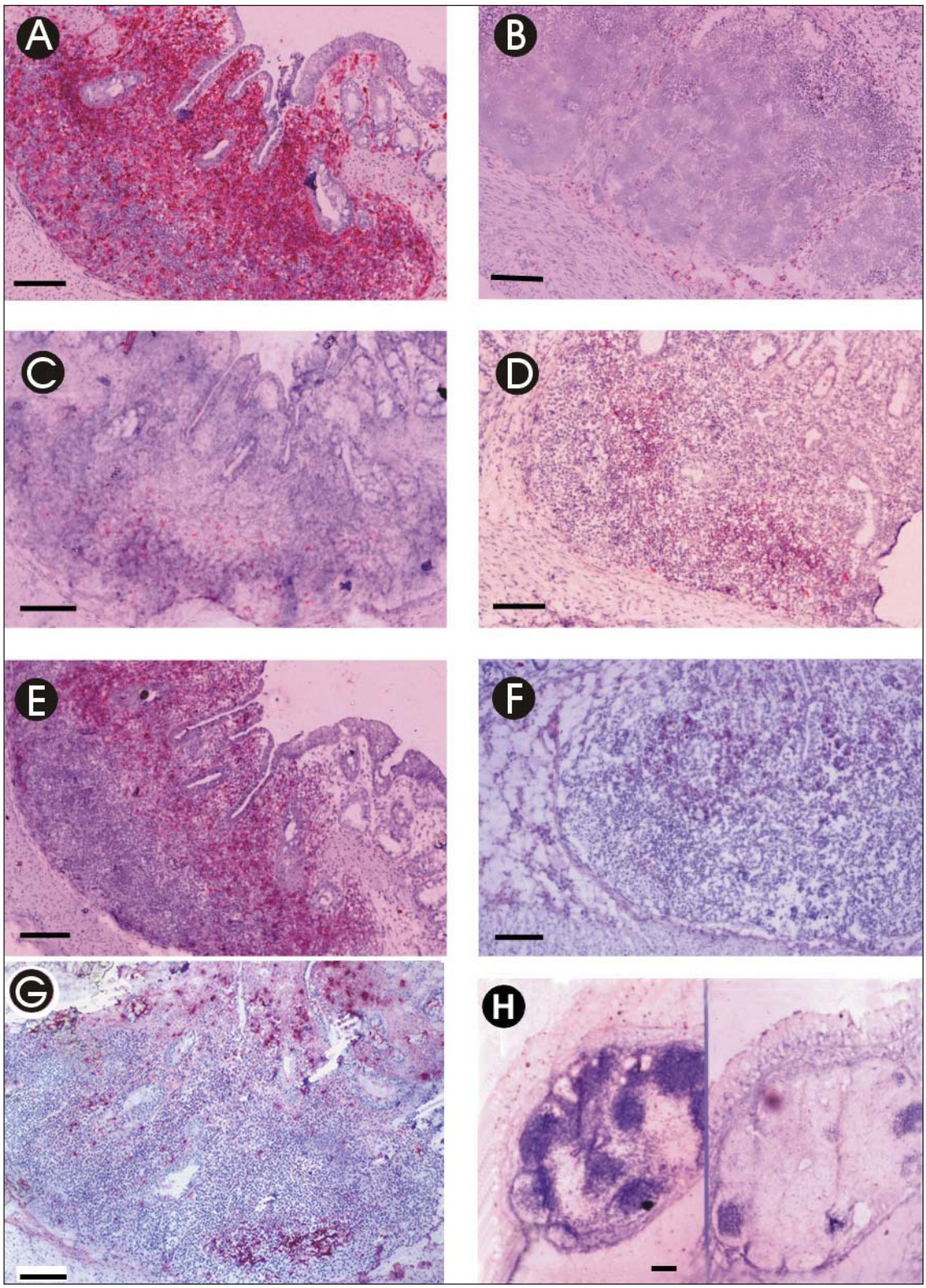

Figure 2. Immunohistochemical staining, using mAbs specific for various cell surface markers, of LGCs from the distal colon of a gnotobiotic pig given *Campylobacter jejuni* and *Trichuris suis* (magnification, 100×). Bars represent 100 μm. Panels A–G are LGCs stained with the mAbs specific for (A) major histocompatibility complex (MHC) class II, (B) macrophage cell surface marker SWC3a, (C) CD4, (D) CD8, (E) IgM, (F) IgG, and (G) IgA. Panel (H) is a lower magnification of an LGC stained with mAbs specific for IgM (left) and IgA (right) (both blue stain with no counter stain) to demonstrate the class switching of individual germinal centers (GCs) in a pig reactive to *Campylobacter jejuni*.

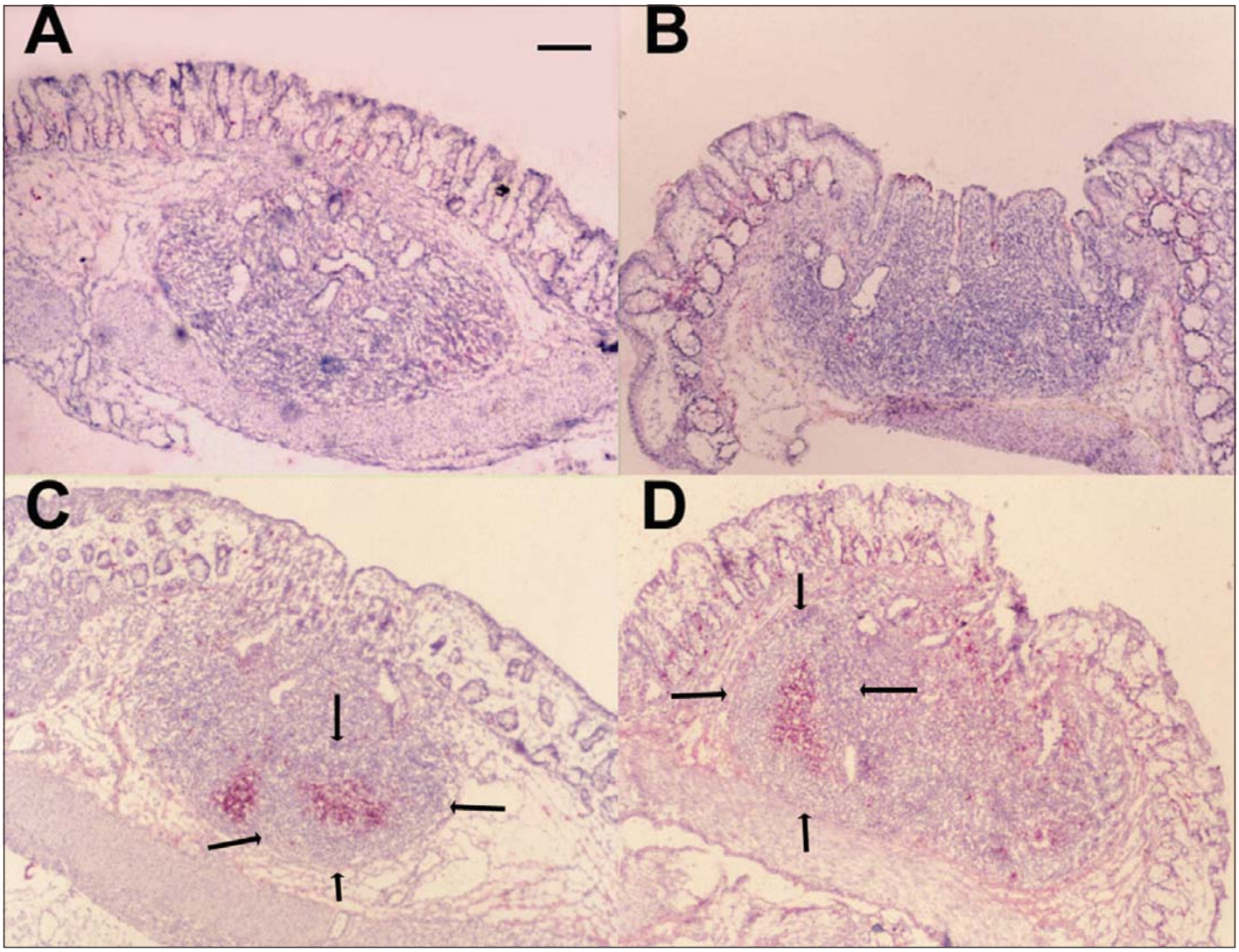

Figure 3. Immunoglobulin A immunohistochemical staining of representative LGCs from the distal colon of gnotobiotic pigs of different infection status. In all panels, the magnification is the same. Bar represents 100 µm. Lymphoglandular complexes are shown from (A) an uninfected pig, (B) a *Trichuris suis*-infected pig, (C) a *Campylobacter jejuni*-infected pig, and (D) a *Campylobacter jejuni* and *Trichuris suis*-infected pig. Note the IgA-positive staining of GC from *C. jejuni*-infected groups (panels C and D) shown in red. Germinal centers from these groups had more cells. Arrows in C and D show the limits of an individual GC. Lymphoglandular complexes from dual-infected pigs (panel D) were significantly larger in diameter than were those from other groups.

licle. Both $CD4^+$ and $CD8^+$ T-cells were found interspersed between the Ig-BC along the leading edge of the GC (Fig. 2C and 2D). In general, the $CD4^+$ staining was weak, with a few CD4 "bright" cells. CD8 staining was stronger than that of the CD4 overall, and more CD8 "bright" cells were seen.

All cells in the B cell-dependent regions of the LGC follicles stained positively for MHC-II (Swine Lymphocyte Antigen, SLA-DR) (Fig. 2A). In LGCs from *C. jejuni*- infected pigs, the majority of the T cell-dependent region was $MHC\text{-}II^+$. In LGCs from the other two groups, a large portion of the cells (approx. 50%) in the T cell-dependent region was MHC-II negative (data not shown). Many cells in the lamina propria were $MHC\text{-}II^+$. Epithelial cells, including those of the FAE of the entrapped crypts, were not reactive with anti-MHC-II antibody.

Macrophages/monocytes (SWC3+ cells) were found primarily in the connective and/or lymphatic tissue surrounding LGC follicles, and in the lamina propria directly overlying the follicles (Fig. 2B). Three of five LGCs from the dual infecting group had 10 or more macrophages in the mucosal dome region of the follicle. The LGC from one pig of each of the *Campylobacter*-only group and the dual group had substantial macrophage infiltration in T-dependent areas (data not shown). In the dual group, anti *C. jejuni* staining within these LGC follicles appeared to coincide with areas of anti-macrophage staining (34) (Fig. 1 and 2).

***Campylobacter jejuni* infection stimulated a switch to IgA production in mucosal lymphoid tissues.** Discrete IgA^+ GCs were observed in LGCs of pigs infected with *C. jejuni*, but not *T. suis* (Table 2; Fig. 2G and 2H and 3C and 3D). This correlated spatially with anti-*C. jejuni*-specific staining in GCs (34). In *Campylobacter*-infected pigs, anti-IgA staining intensely labeled individual cells as well as the interior of mucosal and follicle-associated crypts, suggesting secretion of IgA. Individual IgA-BCs were also common in the lamina propria overlying LGCs from *C. jejuni*-infected pigs. In pigs infected with *T. suis* only, IgA-

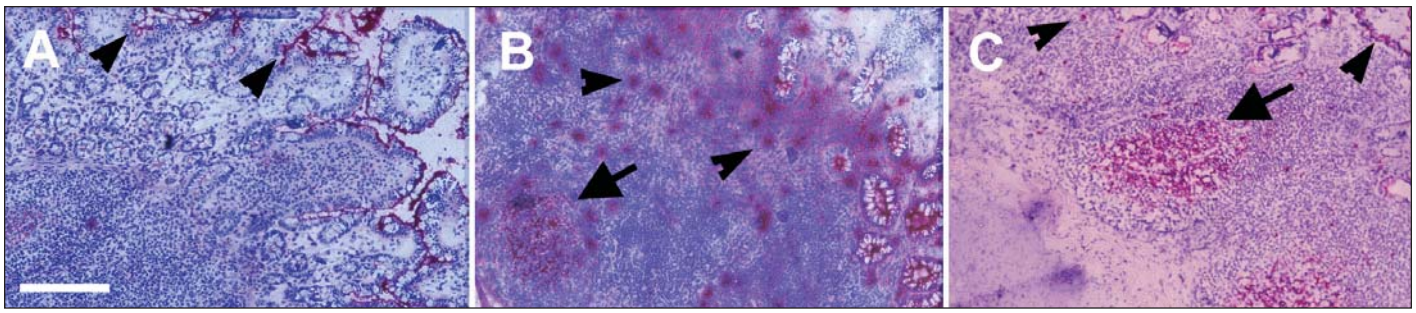

Figure 4. Immunoglobulin A immunohistochemical staining (red staining with a blue counterstain) of representative ileocecal Peyer's patches from the proximal colon of gnotobiotic pigs of different infection status. In all panels, the magnification is the same. Bar represents 120 µm. Ileocecal Peyer's patches are shown from (A) a *Trichuris suis*-infected pig, (B) a *Campylobacter jejuni*-infected pig, and (C) a *Campylobacter jejuni* and *Trichuris suis*-infected pig. Note the IgA-specific staining of GCs in B and C (arrows) and IgA-specific staining of cells in mucosa of A, B, and C (arrowheads).

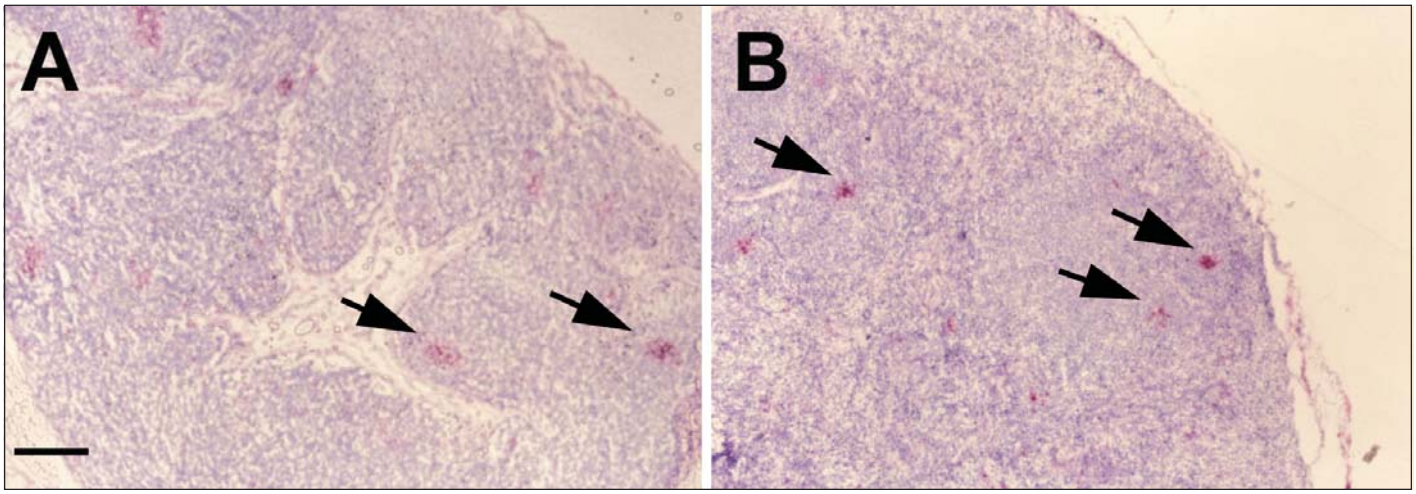

Figure 5. Immunoglobulin A immunohistochemical staining (red staining with a blue counterstain) of representative mesenteric lymph nodes from the antimesenteric border of the proximal colon of gnotobiotic pigs of different infection status. Bar represents 100 µm. Mesenteric lymph nodes are shown from (A) a *Trichuris suis*-infected pig and (B) a *Campylobacter jejuni* and *Trichuris suis*-infected pig. Note the IgA-specific staining of GC in A and B (arrows).

BC were found mainly in tissues from the proximal colon (ICPP and associated lamina propria). Pigs of the control group had few IgA^+ cells in these tissues. A few scattered IgA-BCs were found in the lamina propria of the colonic mucosa, but not in LGCs or their follicular crypts, in *T. suis*-infected and uninfected pigs (Fig. 3).

ICPP and MLN showed IgA production in response to *Trichuris suis* and *Campylobacter jejuni*. The ICPP and MLN follicles varied in size, and GCs were present only in pigs given pathogens. As with the LGCs, cell types within ICPP and MLN were consistent with secondary lymphoid tissue. The ICPP morphology was similar to that described for swine ileal Peyer's patches (14).

The ICPP and MLN from all pig groups given pathogens (*T. suis* only, *C. jejuni* only, and dual infections) were stimulated for IgA production, although the distribution of IgA^+ cells in ICPP of pigs receiving *C. jejuni* differed from that in those receiving only *T. suis* (Fig. 4 and 5). Although IgA-BC were present in the follicles and lamina propria of all three groups, only *C. jejuni*-infected pigs displayed IgA^+ germinal centers.

Staining for other cell surface markers within ICPP GCs was similar to that of LGC GCs. Small follicles were primarily IgM^+, and did not possess visible GC. Larger GCs stained similarly to those of the LGC, with Ig-negative basal regions, IgM^+ and/or IgA^+ cores, and IgM^+ mantles. However, in contrast to LGC GCs of *C. jejuni*-infected pigs, significant numbers of IgA^+ cells were not observed in the predicted downstream track from the core of ICPP GCs in pigs in *T. suis*- and uninfected groups. Also in contrast to the LGC, IgG response was present in some ICPP follicles, with strongly positive cores and significant (approx. 20 to 40) numbers of IgG^+ cells in the dome area; this finding did not appear to be specific to infection status.

The interfollicular lymphoid area of the ICPP was found to be T-cell dependent, with a mixed population of $CD4^+$ and $CD8^+$ cells. The T cells with these markers also were seen in the GC mantles, as in the LGC GCs. No differences in T-cell distribution and abundance were noted in these tissues between infection groups.

Many more $SWC3^+$ cells were associated with the ICPP than the LGC. The lamina propria, follicle domes, and interfollicular areas were heavily infiltrated with these cells. This pattern was seen in all groups. Similar to the LGCs, the majority of cells in the ICPP follicle and surrounding lymphoid tissue stained MHC-II^+. In some instances where GCs were large, mantle areas were negative for MHC-II.

Discussion

The LGCs observed in this study were morphologically similar

Table 2. Summary of immunohistochemical staining results for cell populations in the lymphoglandular complexes (LGCs) in the colon of experimental pigs 27 days after infection with either *Trichuris suis*, *Campylobacter jejuni*, both pathogens, or neither pathogen

	Cell numbers[1]				
Treatment group	IgM-stained cells	IgG-stained cells	IgA-stained cells	CD4-stained cells	CD8-stained cells
Group 1 Uninfected (n = 5)	633 ± 495 (289-1,363)	0	4 ± 1 (3-5)	0	0
Group 2 *Trichuris suis* only (n = 4)	787 ± 524 (416-1,157)	1 ± 0	3 ± 2 (1-4)	17 ± 23 (0-33)	1 ± 1(0-2)
Group 3 *Campylobacter jejuni* only (n = 5)	2017 ± 768 (1,508-3,155)	130 ± 28 (88-146)	548 ± 86 (422-594)	67 ± 51 (29-139)	212 ± 107(122-364)
Group 4 *Trichuris suis* & *Campylobacter jejuni* (n = 6)	1570 ± 459 (1,208-2,330)	230 ± 144 (72-362)	783 ± 240 (539-1,176)	224 ± 290 (0-646)	542 ± 363(7-924)

[1]Values represent cell numbers (mean ± SD). The range is given in parentheses. Cell counts are from one representative LGC from each pig. The LGCs were cut in cross section through the central entrapped crypt at their widest point.

to those identified in the submucosa of the distal colon and rectum in humans (26, 39), swine (33, 37), cattle (29, 30), rats (8), and horses (31). They have also been identified in the stomach of swine (21). To the authors' knowledge, previous studies have not described the distribution of leukocyte subclasses in LGCs of any mammalian species. With the exception of *T. suis* and *C. jejuni*, the pigs of this study were germfree, which allowed examination of the immune cell distribution in unstimulated LGCs, as well as LGCs exposed to defined pathogens. Therefore, both the LGC resting state and its reaction to antigen could be monitored. Additionally, the LGCs were sectioned in a uniform manner, so that comparisons between LGCs from various pigs could be made.

The LGCs were examined as a cross-section through the central pore at the widest spot, which gives a section where all structural elements can be evaluated (e.g. FAE, GCs, overlying mucosa). In the unstimulated LGCs of the *T. suis*-only group, follicles lay well beneath the lamina propria and were composed primarily of IgM-BC, with a distinct T cell-dependent area and no GC. Very few positively staining cells were observed in the lamina propria. As *T. suis* were found only in the proximal colon, antigenic stimulation of these follicles could be assumed to be limited to excretory-secretory products (ESP) from the worms (23, 24). Lack of GC and B cells of any class other than IgM in LGCs of pigs with *T. suis* alone indicated that whatever ESPs did reach these LGCs were not highly stimulatory. In *C. jejuni*-infected pigs, a significantly different pattern of morphology and lymphocyte distribution was seen. The LGC follicles formed dome structures, in which follicles expanded toward the mucosal surface, and follicular cells abutted directly on mucosal epithelium. The GCs were prominent, with cores demonstrating immunoglobulin class switching from IgM to IgA and, to a much lesser extent, IgG. The mantle of the GC was composed primarily of IgM^+ cells, with a significant population of $CD4^+$ and/or $CD8^+$ T-cells. This distribution is consistent with an influx of antigen-stimulated IgM^+ B cells and helper-T cells to the GC (42).

In contrast to *T. suis*-infected and control pigs, the lamina propria overlying LGCs of *C. jejuni*-infected pigs contained substantial numbers of IgA-BC. Very few $CD4^+$ or $CD8^+$ T cells were observed in the lamina propria of any group. This finding is similar to reports on jejunal and ileal lamina propria of 49-day-old germfree pigs (5). The presence of IgA^+ cells in LGC GC and the lamina propria suggests that, in the case of *Campylobacter* infection, the LGCs were an important site for the production of immunocompetent B cells in the distal colon.

In this study, LGCs were identified as submucosal secondary lymphoid structures that responded to *C. jejuni*, but not *T. suis*. The anatomy of LGCs strongly suggests them as sites for entrapment and antigenic sampling of gut bacteria. We have found support for this hypothesis in the immune response of LGCs to infection with *C. jejuni.* Also, it appears that pathogenic bacteria such as *C. jejuni* are capable of colonizing and causing pathologic changes in these structures (34). Twenty-seven days after either single or dual infection, *C. jejuni* was found mainly within the LGCs based on immunohistochemical and electron microscopic evidence, and dual-infected pigs had *C. jejuni* within cells of the follicle while singly infected pigs did not (34). There were few germinal centers in ICPP that stained positively for this bacterium, but ICPPs don't have the mucosal crypts with entrapped mucus in the lymphoid compartment. Studies have indicated that mucus is chemotactic for and supports the growth of *C. jejuni* (15). Our study, however, did not determine whether bacteria detected in LGC follicles were viable or persistent. In an in vitro study, 10% of voluntary human blood donors carried monocytes that were incapable of killing phagocytosed *C. jejuni* (48). Such macrophages displayed normal uptake, but killing was insufficient and bacterial growth was observed for all strains and mutants tested. We found that significant numbers of cells within the LGCs stain for SWC3a, a swine macrophage marker. Follow-up studies should address the ability of *C. jejuni* isolates to persist in a viable state within the LGCs and whether this takes place within macrophages or dendritic cells, or in another compartment of the follicle. Taken together these data suggest that LGCs are important sites for *C. jejuni* colonization, invasion, and immune induction, probably due to their characteristic anatomy.

In this swine model of human polymicrobial infection, significant disease and pathologic changes occurred in the colon of germfree pigs given dual infections with *T. suis* and *C. jejuni* (34). These pigs had appreciable hemorrhage and inflammatory cell infiltrates in the proximal colon where *T. suis* fourth-stage larvae were found, and abscessed LGCs in the distal colon with intracellular *C. jejuni*. The significant increase in size of LGCs in pigs with dual infection was mainly due to expanded B cell-dependent GCs committed to IgA production. Pigs given *C. jejuni*

alone had mild clinical signs of disease and pathologic changes, and bacteria only in extracellular sites (34). Response to *C. jejuni* was not as well defined in the proximal as in the distal colon. In ICPP of *C. jejuni*-infected pigs, GC and domes were present and stained similarly to those from *T. suis* and control groups. The large efflux of IgA^+ cells from follicle to lamina propria observed in the LGC was not seen in ICPP. This could indicate either a differential reaction to *C. jejuni* between the two tissues or a lower infection burden in ICPP. However, IgA^+ BC were found in the GC of LGCs, ICPPs, and MLNs of all pigs that received *C. jejuni* and were not found in LGCs of pigs that received only *T. suis*, supporting the conclusion that the LGC IgA response was largely specific for this bacterium. On the basis of lesion distribution and quantitative comparisons of IgA-specific staining between groups, the LGC was an important site for this sensitization compared with the ICPP and MLN. Furthermore, although dual immunostaining of the same sections was not performed, IgA-specific staining co-localized to the GCs of LGCs that had *C. jejuni*-specific staining. Clearly, there was a strong switch to colonic mucosal IgA secretion in response to the presence of *C. jejuni*, which occurred in both single and dual infections and was prominent in the LGCs. It remains to be determined how *T. suis* acts at a distance to enhance *C. jejuni* invasiveness.

Other pathogens have been suggested to sensitize LGCs. In eight cases of sporadic ileocecal tuberculosis, LGCs and lymphoid aggregates increased and proliferated adjacent to areas of ulceration (19). That study suggested that LGC were sites of mycobacterial antigen sampling, of T-lymphocyte and macrophage activation, and of (potential) granuloma formation. Another study examined the size of LGCs in the resected segment of aganglionic left colon of 20 children with Hirschsprung's disease (16). All the resected aganglionic segments had a nodular mucosa with significantly increased size of LGCs compared with that in controls. The authors hypothesized that this hyperplasia of LGCs in defunctionalized colonic segments may represent a reaction to stagnant contents. Also, cattle orally infected with the human pathogen *Escherichia coli* O157:H7 shed the organism for 14 days prior to necropsy and had these bacteria in feces and adherent only to the mucosal epithelium within the recto-anal junction overlying LGCs (38). As a consequence of this specific distribution, *E. coli* O157:H7 was present predominantly on the surface of the feces, providing a mechanism by which cattle serve as a reservoir for enterohemorrhagic *E. coli*. The role of LGCs in enteric pathogen invasion and transmission should be explored.

Comparative studies have indicated that LGCs are part of the immune surveillance system of the colon. In a study of young pigs, ferritin was taken up by M cells but not by enteroabsorptive cells of the small and large intestines (28). The number of M cells was highest in LGCs in the rectum and lowest on domes of the ileal Peyer's patches. High numbers of M cells with ferritin were also found on domes of the jejunal Peyer's patches, and in LGCs in the colon. Endocytosed ferritin was found in the apical tubulovesicular system, multivesicular bodies, and in a few vacuoles in the central area of M cells. Ferritin was also exocytosed into the lateral intercellular spaces next to M cells and was taken up by subepithelial macrophages. In horses where LGC drainage has been explored, lymphatic vessels do not leave the LGC follicles but intercellular pathways of the follicles are continuous with those in the surrounding interfollicular tissue and basal sinuses (31). These pathways appeared to provide the only available route for lymphocytes leaving LGCs to enter the lymphatic system. In *C. jejuni*-infected pigs of this study, individual IgA^+ and IgM^+ cells could also be seen outside the GC and in the submucosal space surrounding the LGC follicle, suggesting a similar pathway. Thus, it is highly likely that the pathogenesis of and pattern of immune reactivity to those enteric pathogens with a more passive means of cellular entry such as *C. jejuni*, may closely follow the distribution of M cells in lymphoid tissues. Because of their high M-cell content and location in the distal colon, LGCs deserve careful scrutiny.

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