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NOTES

Localization of α/β and γ/δ T Lymphocytes in *Cryptosporidium parvum*-Infected Tissues in Naive and Immune Calves

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The nature of the host's T-lymphocyte population within the intestinal villi following *Cryptosporidium parvum* infection was characterized with a bovine model of cryptosporidiosis. In naive animals, infection with *C. parvum* resulted in substantial increases in the numbers of α/β T cells, both $CD4^+$ (150%) and $CD8^+$ (60%), and of γ/δ T cells (70%) present within the intestinal villi of the infected ileum. In immune animals, the host T-lymphocyte response to a challenge infection with *C. parvum* was restricted to α/β T cells. The number of $CD4^+$ T cells within the Peyer's patch of the ileum increased dramatically; however, there was little change in the number or localization of $CD4^+$ T cells within the intestinal villi. In contrast, the number of $CD8^+$ T cells within the intestinal villi increased following a challenge infection. In addition, the $CD8^+$ T cells were found to be intimately associated with the epithelial cells of the intestinal villi. The precise correlation between the accumulation of $CD8^+$ T cells and the normal site of parasite development suggests an important role for $CD8^+$ T cells in the immune animal.

Cryptosporidium parvum infection is now a well-recognized cause of diarrhea in immunologically healthy and immunocompromised humans and in animals of agricultural interest throughout the world (8–10). Cryptosporidiosis is currently receiving much public attention due to the occurrence of life-threatening infections in patients with AIDS and several recent waterborne outbreaks, including one in Milwaukee, Wis., where an estimated 403,000 residents became ill (21). Currently there is no effective therapy for treating cryptosporidiosis, and prevention has been difficult, as the parasite is resistant to conventional water treatment procedures and common disinfectants.

In immunocompetent hosts active cryptosporidiosis is self limiting and results in partial or complete protection against subsequent challenge. Although a variety of immune responses are known to be induced by infection with *Cryptosporidium*, little is known of the specific mechanisms by which the immune system is able to recognize and clear initial infections or provide protection against subsequent infections. It is clear from previous studies that resistance to and recovery from cryptosporidiosis is dependent on T-cell activity (1, 6, 16, 22–26, 31, 33). In laboratory rodent models, $CD4^+$ T cells, but not $CD8^+$ or γ/δ T cells, are required for clearing and providing protection against infection with *Cryptosporidium* (1, 6, 23, 31, 33, 35). In addition, the cytokines gamma interferon and interleukin 12 have been demonstrated to be important factors for providing protection against cryptosporidiosis (6, 34, 37).

Although the systemic responses to *Cryptosporidium* infection have been well characterized (reviewed in references 7 and 32), limited information concerning the nature of the immune response at the actual site of infection is available. To better understand the immune mechanisms important for clearing of the primary infection and the subsequent development of resistance, it is necessary to identify the specific populations of host lymphocytes that respond to *Cryptosporidium* infection in vivo. In particular, it is critical to identify those lymphocytes which localize to the specific sites of infection and may serve as effector cells in controlling disease. Even in the intensely studied rodent models of cryptosporidiosis, the specific populations of host T cells that localize within the infected tissues have yet to be determined.

To address this critical issue in the present study, we used a bovine *C. parvum* infection model to identify the specific T-cell subsets that localize at the actual sites of infection. Both naive and previously infected animals were examined to identify any key differences between the primary and the secondary responses to infection. Until recently, this type of phenotypic analysis of bovine lymphocyte populations was difficult due to the lack of monoclonal antibodies (MAb) against lineage-specific differentiation antigens. This restricted the vast majority of immunological studies on *Cryptosporidium* infection to rodent models. It remains to be determined whether conclusions based solely on rodent models of cryptosporidiosis will be entirely valid for other species. In the present study we demonstrated that, in contrast to the lack of an apparent role for $CD8^+$ T cells in the rodent models, $CD8^+$ T cells are intimately associated with the epithelial cells of the intestinal villi following a challenge infection in immune calves. The precise correlation between the accumulation of $CD8^+$ T cells and the normal site of parasite development suggests an important role for $CD8^+$ T cells in immune calves.

The Iowa isolate of *C. parvum* (originally obtained from C. Sterling, University of Arizona, Tucson) was used for these

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studies. The parasite was propagated by passage in holstein calves, and oocysts were purified from the collected feces via discontinuous sucrose gradient methods (2). Oocysts were stored in 2.5% potassium dichromate at 4°C for a period no longer than 2 months. Healthy, colostrum-fed holstein bull calves were purchased from local dairies at 24 to 48 h of age. Calves were placed in isolation and monitored for signs of disease. All noninfected calves remained free of diarrhea and did not shed any *C. parvum* oocysts during the course of the study, as determined by daily examination of the feces by indirect immunofluorescence (11). For primary experimental infections, healthy calves were orally infected at 3 days of age with $\sim 2 \times 10^8$ *C. parvum* oocysts. Immune animals used in the studies were previously experimentally infected with *C. parvum* oocysts, which resulted in the shedding of large numbers of oocysts in the feces (2×10^9 to 5×10^9/animal). Two weeks after naturally clearing the primary infection, ~30 days postinoculation (p.i.), animals were challenged with 2×10^8 oocysts of the same isolate of *C. parvum.*

Four groups of three calves each were used in the studies: noninfected naive calves, *C. parvum*-infected naive calves, control immune calves, and *C. parvum*-challenged immune calves. Animals were euthanized by lethal injection with sodium pentabarbital, and harvested gut tissues were frozen in OCT compound (Tissue-Tek; Baxter Scientific). Tissues were harvested from noninfected naive animals and control immune animals sacrificed at ages identical to those of the *C. parvum*-inoculated animals. Serial sections prepared from the frozen tissue sections were air dried for 30 min, fixed in acetone for 10 min, and blocked in 5% donor goat serum (Quad Five, Ryegate, Mont.) in phosphate-buffered saline (PBS) for 30 min. Following washing with PBS, the tissue sections were incubated with hybridoma supernatants for 1 h at room temperature in a humidified chamber and washed with PBS. MAb reactivity was detected with a biotinylated goat anti-mouse $F(ab')_2$ secondary antibody and visualized by using a three-stage avidin-biotin immunoperoxidase staining kit as recommended by the manufacturer (BioSource International, Camarillo, Calif.). Slides were counterstained with hematoxylin 1 (Richard-Allan; Richland, Mich.) and mounted in an aqueous mounting medium. No reactivity was detected in the tissue sections when the biotinylated goat anti-mouse $F(ab')_2$ secondary antibody was used alone.

In order to characterize changes in the lymphocyte populations within the *C. parvum*-infected tissues, it was necessary to identify the specific sites within the gut where host cells were parasitized. In preliminary experiments, tissues were prepared from calves orally infected with *C. parvum* oocysts for various lengths of time. Staining with the antiparasite MAb Cp 65.10 (an anti-*C. parvum* MAb generated against *C. parvum* sporozoites that recognizes all development stages) (19a) revealed that by 72 h p.i. large numbers of infected cells could be identified throughout the entire gut, with the highest concentrations occurring in the three centimeters of the ileum immediately adjacent to the junction with the cecum. Based on these data, all experimentally infected animals subsequently used in these studies were analyzed at 72 h p.i. This time point precedes the peak of oocyst shedding, which occurred on either day 4 or day 5 p.i. in experimental infections in our laboratory.

For cell counting, serial sections prepared from three different frozen blocks of ileal tissue from each naive animal were stained as described above. For each section, five nonoverlapping microscope fields of the intestinal villi were visualized with a 20× objective on an Olympus B-Max (BX-60) microscope. The five microscope fields were randomly selected by starting at one end of the tissue section and moving toward the other end, with approximately one microscope field between each selected field. The images were captured by using a low-light charge-coupled device camera (Optronics TEC-470) and Image 1/MetaMorph software (West Chester, Pa.). A bright-field image was used to calculate total tissue area in pixels. The values were subsequently converted to square millimeters by using a pixel-to-millimeter calibration. Without moving the microscope stage, a digital color (RGB) image was captured and used for counting the number of positively staining cells. The following murine MAb were used in the phenotypic characterization of the lymphocyte populations in the prepared tissue sections: cc30, anti-bovine CD4 (3), and cc58, anti-bovine CD8 (20), which are preferentially expressed by α/β T cells, and GD3.8, which is a pan-anti-bovine γ/δ T-cell receptor (19, 36a).

Analysis of T-cell populations in the ilea of naive calves infected with *C. parvum*. Tissues were harvested from three calves orally infected with *C. parvum* oocysts 72 h earlier, and serial sections prepared from the distal ileum were stained with MAb Cp 65.10 to confirm infection within the specific tissue (Fig. 1B). At 72 h following infection, a dramatic increase in the number of α/β T cells ($CD4^+$ and $CD8^+$ T cells) was seen within the lamina propria and the perifollicular area of the Peyer's patch (data not shown) of the infected ileum (Fig. 1D and F) compared to tissues from control animals (Fig. 1C and E). A small portion of the large ileal Peyer's patch is visible in Fig. 1. At day 13 p.i., by which time oocyst production had naturally ceased, the populations of $CD4^+$ and $CD8^+$ T cells had returned to levels comparable to those found in control animals (data not shown). These results are significant in light of previous studies showing only minimal changes in the numbers of $CD4^+$ and $CD8^+$ T cells within the ruminant ileal Peyer's patch, which is thought to function as a primary lymphoid organ (12).

γ/δ T cells have recently been shown to be involved in the host response against a variety of intracellular pathogens (5, 13, 18, 27–29); therefore, we examined the response of this cell type in calves infected with *C. parvum*. A dramatic increase in the number of γ/δ T cells was detected in the lamina propria of infected ileum at 72 h (Fig. 1H), similar to that observed for α/β T cells. However, this increase in γ/δ T cells was restricted to the intestinal villi and lamina propria. Negligible levels of γ/δ T cells were detected in the Peyer's patches of either control or infected animals (data not shown). Consistent with the α/β T cells, the numbers of γ/δ T cells declined during the course of infection and at day 13 were similar to those in controls (data not shown).

Quantification of the T-cell response to *C. parvum* infection in naive calves. To quantify the accumulation of the specific T-cell populations within the lamina propria in response to *C. parvum* infection, the numbers of T cells per square millimeter present within the intestinal villi of control and infected animals were determined. As seen in Fig. 2, at 72 h p.i. there was an increase in the numbers of both α/β and γ/δ T cells present within the intestinal villi of the ileum in response to infection with *C. parvum*. $CD4^+$ T cells, which were present at relatively low levels within the villi of healthy calves, increased an average of 150% compared to the level in the group of control animals. The percent increase would have been substantially greater if not for the relatively high number of $CD4^+$ T cells present in the gut tissues of one of the control calves. $CD8^+$ T cells, which increased an average of 60%, were present in the villi of infected calves at levels similar to those of $CD4^+$ T cells. γ/δ T cells, which increased an average of 70% in response to infection, were present at higher levels within the intestinal villi of both control and infected calves compared to either $CD4^+$

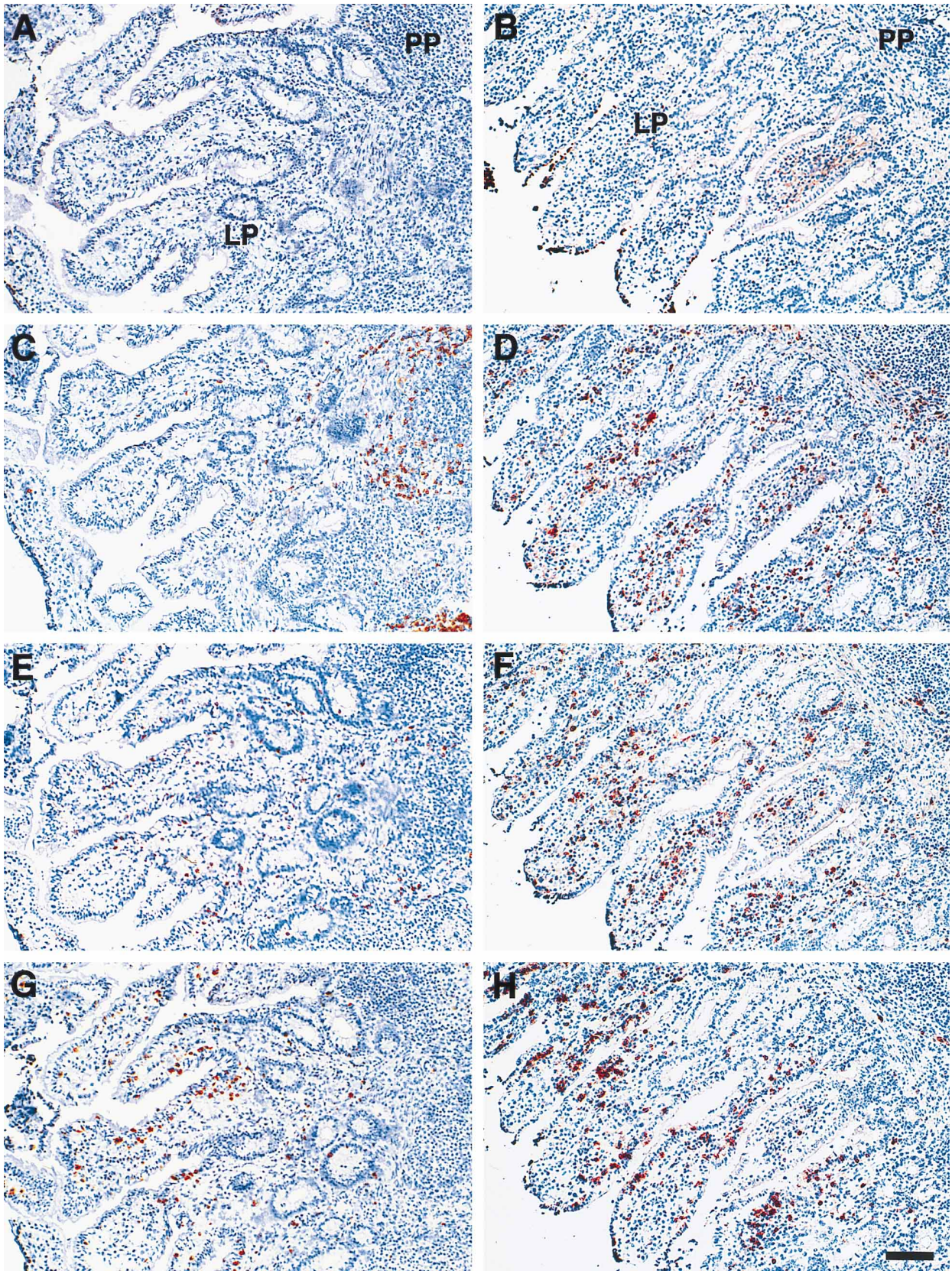

FIG. 1. Immunohistochemical analysis of lymphocyte populations in the ilea of naive calves. Serial sections were prepared from frozen ileal tissues harvested from control calves (A, C, E, and G) and from calves inoculated with *C. parvum* 72 h earlier (B, D, F, and H). Sections were incubated with the anti-*C. parvum* MAb Cp 65.10 to identify infected tissues (A and B). The developing parasite is seen on the epithelial surface of the intestinal villi of the infected animal (B) but not on that of the control animal (A). Specific lymphocyte populations were identified by using a primary MAb which recognizes $CD4^+$ (C and D) and $CD8^+$ (E and F) α/β T cells and γ/δ T cells (G and H). LP, lamina propria; PP, Peyer's patch. (Only a small portion of the large ileal Peyer's patch is visible on the right side of each of the photos.) Bar = 100 μm.

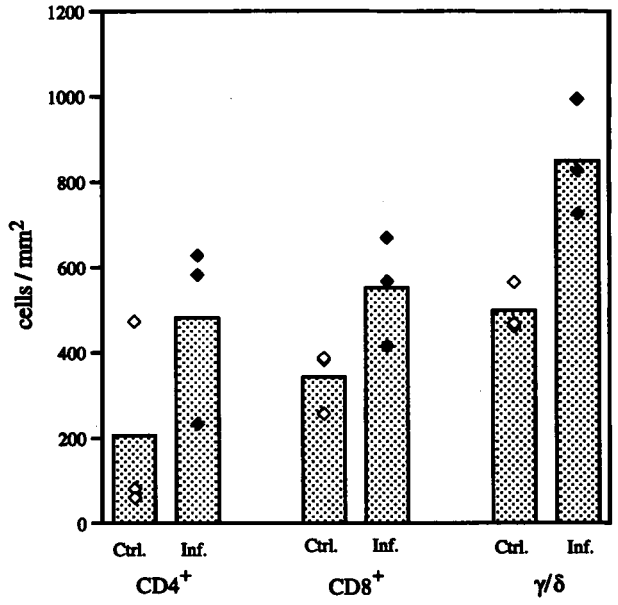

FIG. 2. Quantification of specific lymphocyte populations in the intestinal villi of the ilea of noninfected (Ctrl.) and 72-h *C. parvum*-infected (Inf.) calves. Each data point shown represents the average of 15 measurements made for each animal. Each bar represents the average value for the three animals in the indicated experimental group.

or $CD8^+$ α/β T cells. Following infection γ/δ T cells outnumbered $CD4^+$ T cells almost 2 to 1, suggesting that they may be an important component of the primary immune response to *C. parvum* infection.

Analysis of T-cell subsets in the ilea of immune calves challenged with *C. parvum*. Calves infected with *C. parvum* typically clear the infection within 2 weeks and are resistant to a second challenge with the same isolate (14). To identify any key differences in T-cell populations between the primary and secondary immune responses to *C. parvum* infection in calves, the same type of immunohistological analysis following infection that was described above for naive calves was performed with immune animals. At 72 h following challenge of immune animals, a large increase in the populations of $CD4^+$ and $CD8^+$ T cells was seen in the ileum (Fig. 3). However, the response of $CD4^+$ and $CD8^+$ T cells was more localized in nature in the immune animals, compared to the generalized changes seen during the primary infection in naive animals. In immune animals, the increase in $CD4^+$ T cells following challenge was largely restricted to the perifollicular area of the Peyer's patch (of which only a small portion is visible in Fig. 3), with little change in the number or localization of $CD4^+$ T cells within the intestinal villi (Fig. 3A and B). In contrast, the response of $CD8^+$ T cells was most apparent within the intestinal villi of challenged immune animals, where the vast majority of $CD8^+$ T cells were closely associated with epithelial cells of the ileum (Fig. 3D). This intimate association of $CD8^+$ T cells with the epithelium was not seen in the tissues of control immune animals (Fig. 3C) or in the infected tissues of naive animals (Fig. 1F).

Unlike α/β T cells, there was little change in the number of γ/δ T cells present in the gut tissues of the immune animal following a challenge infection (data not shown). The levels of γ/δ T cells in the challenged animals were similar to those found in the noninfected naive animals (Fig. 1G) and the control immune animals (data not shown). This indicates that γ/δ T cells may be important only for the primary immune response to *C. parvum* infection in a naive animal. It should be noted that even with the large challenge dose given to the immune animals, we were unable to identify any *C. parvum*-infected cells by using MAb Cp 65.10 at 72 h p.i. This suggests that the secondary immune response of the host is very rapid and effective at eliminating the parasite or preventing it from infecting epithelial cells. In addition, this absence of parasite development may explain the lack of a γ/δ T-cell response.

Previous studies support the hypothesis that lymphocytes are important for the immune response to *Cryptosporidium* infection in calves. A study of the kinetics of specific serum and local antibody responses during natural and experimental infections in calves indicated that secretory antibodies alone are not sufficient to control reinfection (30). This led the authors to conclude that cell-mediated immune mechanisms are important for clearance of parasites from the infected mucosa and for rendering immunocompetent animals resistant to reinfection. In addition, lymphocytes isolated from various tissues harvested from experimentally infected calves respond to *C. parvum* antigen preparations in vitro (15, 36).

The present analyses demonstrated that in naive calves, *C. parvum* infection resulted in a substantial increase in α/β T cells, both $CD4^+$ and $CD8^+$, and γ/δ T cells within the intestinal villi shown to be infected. This increase in the numbers of lymphocytes in the intestinal villi following infection may be important for limiting the extent of the primary infection. In the rodent models of cryptosporidiosis, a generalized increase in the number of intraepithelial lymphocytes, including $CD8^+$ and γ/δ T cells, was observed in the gastrointestinal tracts of mice during infection with *C. parvum* (4, 17). In studies with the closely related parasite *Cryptosporidium muris*, the transfer of intraepithelial lymphocytes from immune mice to immunodeficient mice was sufficient to overcome the infection (24). Recently, it has been demonstrated in calves that changes occur in the phenotype of the ileal intraepithelial lymphocyte population during infection with *C. parvum* (37). Taken together, these data support the conclusion that intraepithelial lymphocytes play an important role in providing protection against cryptosporidiosis; however, it remains to be determined in any of these experimental models whether the increased number of lymphocytes in the intestinal villi is due to localized proliferation or is the result of increased recruitment of cells from the circulation in response to inflammatory mediators.

In immune calves, the large increase in the number of $CD4^+$ T cells present in the Peyer's patch following a challenge infection was accompanied by the specific localization of $CD8^+$ T cells to the epithelium of the intestinal villi. The precise correlation between the accumulation of $CD8^+$ T cells at the epithelium and the normal site of parasite development suggests that $CD8^+$ T cells could be the effector cells directly responsible for eliminating the parasite from an immune calf. Others have observed increased numbers of $CD8^+$ T cells in the spleens of calves (15) and within ileal intraepithelial lymphocyte populations following *C. parvum* infection (37). Furthermore, approximately 70% of the $CD8^+$ intraepithelial lymphocytes coexpressed the activation marker CD25 during *C. parvum* infection (37). Together, these observations are in conflict with the apparent lack of a significant role for $CD8^+$ T cells in clearing or providing protection against *C. parvum* infections in murine models (1, 6, 31, 33). In SCID mice resolution of *C. parvum* infection after reconstitution of the immune system with spleen cells was unaffected by depletion of the $CD8^+$ T cells (31), and major histocompatibility complex class I-deficient mice were no more susceptible to infection

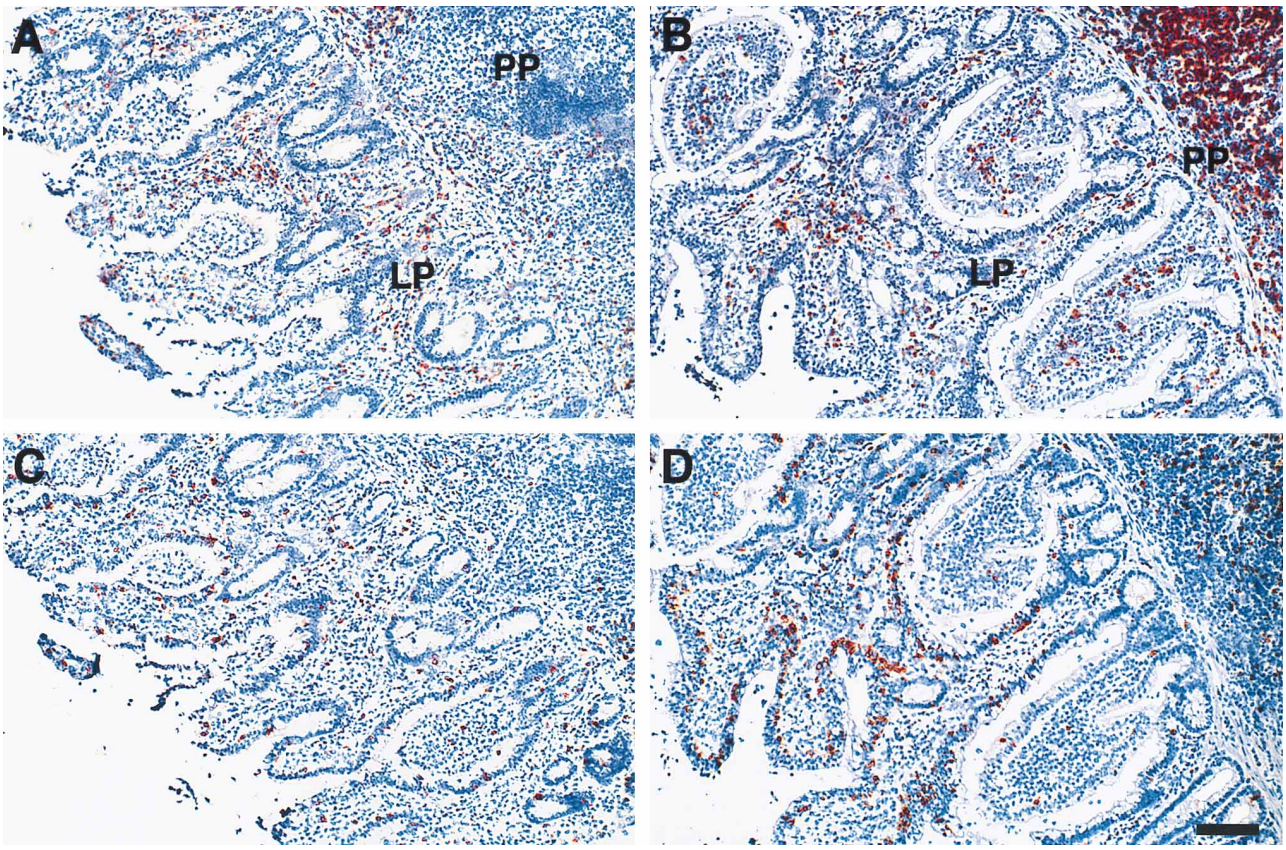

FIG. 3. Immunohistochemical analysis of lymphocyte populations in the ilea of immune calves. Serial sections were prepared from frozen ileal tissues harvested from control immune animals (A and C) and from immune animals challenged with *C. parvum* 72 h earlier (B and D). Sections were stained with a primary MAb which recognizes $CD4^+$ (A and B) and $CD8^+$ (C and D) α/β T cells. LP, lamina propria; PP, Peyer's patch. (Only a small portion of the large ileal Peyer's patch is visible in the upper right of each of the photos.) Bar = 100 μm.

than were control mice (1). This apparent discrepancy in the importance of $CD8^+$ T cells between mice and cattle may be the result of fundamental differences in the immune systems, suggesting that conclusions based on rodent models of cryptosporidiosis may not be valid for ruminants and by extension, humans.

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