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Short Communication

Cryptosporidium parvum Initiates Inflammatory Bowel Disease in Germfree T Cell Receptor-α-Deficient Mice

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Flora-bearing mice with targeted disruption of T cell receptor (TCR)-α or -β genes spontaneously develop intestinal inflammation with features similar to ulcerative colitis in humans. TCR-α-deficient mice maintained germfree or colonized with a limited number of intestinal bacteria failed to develop inflammatory bowel disease (IBD)-like lesions. Evidently, inflammation in these mice does not develop spontaneously or result from a generalized antigenic stimulation, but rather requires induction by a heretofore unidentified specific stimulus. We describe the development of IBD-like lesions in germfree TCR-α-deficient mice monoassociated with the protozoan *Cryptosporidium parvum*. Lesions were seen in distal ileum, cecum, and colon and were most severe in the cecum. A prominent leukocytic infiltrate within the lamina propria was a common characteristic of the lesions observed in the *C. parvum*-infected germfree TCR-α-deficient mice. The leukocytic infiltrate was composed of aggregates of B220$^+$ cells, the majority of which expressed surface IgD (ie, conventional B lymphocytes). It has been proposed that antigenic stimulation by a microorganism(s) is needed to initiate intestinal inflammation in TCR-α-deficient mice. Our results indicate that a single microbial species, *C. parvum*, is capable of triggering the development of IBD-like lesions in germfree TCR-α-deficient mice. *(Am J Pathol 1998, 153:1717–1722)*

Inflammatory bowel disease (IBD) of humans is primarily characterized by Crohn's disease (CD) and ulcerative colitis (UC). As is the case with other chronic diseases of unknown etiology, the etiology of IBD is believed to be multifactorial, with genetic susceptibility, environmental factors, dysregulated acquired and innate immunity, and nonspecific inflammation likely contributing to the complex clinical manifestations. Although the pathogenesis of IBD is not completely understood, an immunological and/or infectious etiology has been postulated to contribute to the onset of disease. In this regard, it is of particular interest that several immunodeficient mouse strains created via targeted deletion of cytokine, major histocompatibility complex (MHC) class II, or T cell receptor (TCR)-α or -β genes spontaneously develop IBD-like lesions as they age.[1] The chronic colitis observed to spontaneously develop in TCR-α- or TCR-β-deficient mice resembles UC, beginning after approximately 3 to 4 months of age in a majority of the knockout mice. These mice gradually develop chronic diarrhea, leading to progressive wasting that is often associated with anorectal prolapse. We have recently reported the earlier and more predictable development of IBD-like lesions in flora-bearing TCR-α-deficient mice infected with *Cryptosporidium parvum*.[2] In this model, TCR-α-deficient mice infected at 1 week of age with *C. parvum* develop IBD-like lesions within 3 weeks after infection.

C. parvum is a ubiquitous protozoan parasite that is a common cause of gastroenteritis and diarrhea worldwide. *C. parvum* is now recognized as the most important waterborne contaminant in the United States. Although the disease is generally self-limiting in immunocompetent individuals, it can be severe and life-threatening in immunocompromised individuals, such as AIDS patients. As a case in point, a cryptosporidiosis outbreak occurred in

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Milwaukee during the spring of 1993 that was traced to contamination of a municipal water-treatment facility and resulted in devastating consequences in HIV-infected individuals and those with severely impaired cellular immunity.[3] Of particular interest, among the patients treated, were 12 individuals with stable UC or CD who suffered abrupt clinical relapse during the waterborne outbreak. Eventually, these patients recovered to their clinical baseline after standard IBD therapy and administration of intravenous fluid. Thus, it is evident that *C. parvum* can induce an acute relapse in patients with pre-existing IBD and an earlier induction of intestinal inflammation in knockout mice predisposed to developing IBD-like lesions.

Previously, it was shown that TCR-α-deficient mice maintained under germfree conditions do not develop IBD-like lesions.[4] Moreover, in that study, no intestinal inflammation was observed in gnotobiotic TCR-α-deficient mice colonized with *Lactobacillus plantarum*, *Streptococcus faecalis*, *S. faecium*, and/or *Escherichia coli*. Thus, as has been proposed, inflammation is likely initiated by a specific environmental organism or group of organisms that have yet to be identified. Given our previous observation that flora-bearing TCR-α-deficient mice rapidly develop IBD-like lesions after *C. parvum* infection, the primary objective of the present experiments was to determine whether germfree TCR-α-deficient mice monoassociated with *C. parvum* would develop intestinal inflammation. Furthermore, as germfree immunocompetent and immunodeficient mice are more susceptible to *C. parvum* infection than are flora-bearing mice, a secondary objective was to examine whether germfree TCR-α-deficient mice are more susceptible to *C. parvum* than are flora-bearing TCR-α-deficient mice.

Materials and Methods

Mice

Adult flora-bearing TCR-α-deficient mice[1] were obtained from Jackson Laboratories (Bar Harbor, ME). A breeding colony of these mice was established and maintained at Iowa State University. Germfree TCR-α-deficient mice were derived at the University of Wisconsin Gnotobiotic Laboratory, Madison, WI, and a breeding colony was established and maintained at Iowa State University. Mice maintained under germfree conditions were monitored for contamination by culturing fecal samples on blood agar plates under aerobic or anaerobic conditions. Furthermore, swabs were periodically taken from random locations inside the isolators for both aerobic and anaerobic bacteriological culture.

Cryptosporidium parvum

Purified oocysts were isolated from feces collected from calves experimentally inoculated with *C. parvum* oocysts using a previously described method.[5] Before use, oocysts were incubated for 30 minutes with 2.5% peracetic acid to kill any contaminant bacteria and then washed three times with PBS. Adult germfree and flora-bearing mice were challenged with 10^6 *C. parvum* oocysts via gastric intubation. Additional flora-bearing and germfree mice were maintained as noninfected controls. Fecal pellets were collected weekly from individual mice. Fecal impression smears were prepared, stained with carbol fuschin, and examined for the presence of *C. parvum* oocysts.

Histology

At 6 to 12 weeks after challenge, mice were euthanized, and the entire intestinal tract of each mouse was infused with 10% neutral buffered formalin (NBF). In addition, the pancreas, liver, lungs, and gall bladder from individual mice were fixed in NBF. After fixation, samples of these tissues were processed and stained with hematoxylin and eosin (H&E) for routine histological evaluation. In addition, tissue sections were stained with Warthin-Starry silver stain and examined for the presence of bacterial populations.

Antibodies

The following monoclonal antibodies (MAbs) were used in these experiments: GL3, hamster IgG anti-mouse $\gamma\delta$ TCR; H57-597, hamster IgG anti-mouse TCR-β; 217-170, mouse IgG1 anti-mouse IgD^b (Igh-5b); RA3-6B2, rat IgG2a anti-mouse B220; and 53-7.313, rat IgG2a anti-mouse CD5 (Ly-1). RA3-6B2 was purified from supernatants of hybridoma culture and biotinylated using standard procedures. The other MAbs were purchased as biotinylated conjugates from PharMingen (San Diego, CA).

Immunohistochemistry

To assess the cellular composition of the mucosal lesions, immunohistochemical staining was performed on 8-μm frozen cecal sections. Briefly, tissue was snap-frozen in OCT medium (Miles, Elkhart, IN) and stored at -70°C. Sections were then cut and placed on poly-L-lysine-coated slides, fixed with 95% ethanol, and stored frozen at -70°C for later use. Frozen sections were washed in 0.05 mol/L Tris, pH 7.6, and blocked with 5% serum. Sections were incubated overnight at 4°C with biotinylated primary antibodies. Sections were incubated with streptavidin-peroxidase and developed with diaminobenzidine substrate/chromogen (Biomeda, Foster City, CA).

Results

C. parvum *Oocyst Shedding*

The number of oocysts in feces of germfree and flora-bearing TCR-α-deficient mice were examined each week as a measure of susceptibility to *C. parvum*. Fecal samples collected from germfree TCR-α-deficient mice infected with *C. parvum* were typically soft and contained

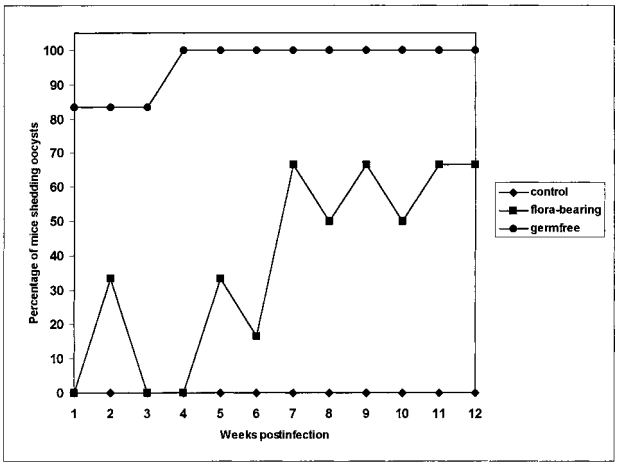

Figure 1. Percentage of TCR-α-deficient mice shedding *C. parvum* oocysts after oral inoculation. Fecal pellets were collected weekly on individual mice, smeared onto glass slides, stained with carbol fucshin, and examined for the presence of *C. parvum* oocysts.

mucus. As shown in Figure 1, greater than 80% of germfree TCR-α-deficient mice were shedding *C. parvum* oocysts at 1 week postinfection (PI). In contrast, none of the flora-bearing TCR-α-deficient mice were shedding oocysts at 1 week PI. In fact, the number of oocysts in flora-bearing *C. parvum*-infected TCR-α-deficient mice was relatively low until 6 to 7 weeks PI, when greater than 50% of the mice were found to be shedding oocysts. In contrast, by 4 weeks PI, 100% of germfree mice infected with *C. parvum* were shedding oocysts, and they continued to do so throughout the 12-week course of the experiment. Moreover, the number of oocysts shed per mouse was greater for germfree TCR-α-deficient mice than flora-bearing mice (data not shown). Thus, it is readily apparent that in comparison with flora-bearing TCR-α-deficient mice, germfree mice were more heavily infected with *C. parvum* as assessed by oocyst shedding.

Lesions in Germfree TCR-α-Deficient Mice Infected with C. parvum

Previously, lesions were observed in liver, gall bladder, and bile ducts of immunodeficient NIH III-*nu/nu* or SCID mice after infection with *C. parvum*.[6] In addition, involvement of respiratory epithelium, bile ducts, gall bladder, and pancreas due to *C. parvum* in AIDS patients has been reported.[7–13] Therefore, we examined sections of pancreas, gall bladder, and lungs from germfree TCR-α-deficient mice infected with *C. parvum*. No parasites or significant lesions were seen in these tissues at 6 or 12 weeks PI. However, aggregates of lymphocytes with or without plasma cells or macrophages were observed in the liver parenchyma of mice at 12 weeks PI.

Given our previous observation that flora-bearing TCR-α-deficient mice develop IBD-like lesions after *C. parvum* infection, we examined the ability of *C. parvum* to induce intestinal inflammation in germfree TCR-α mutant mice. There was no inflammation present in any of the intestinal sections of control adult germfree TCR-α-deficient mice. In addition, no bacterial populations were observed in intestinal tissue sections of germfree TCR-α-deficient mice based on Warthin-Starry silver staining. As shown in Figure 2A, the cecal histoarchitecture was normal with few mononuclear cells, which is similar to that previously described for the colonic architecture of germfree TCR-α mutant mice.[4] At 6 weeks PI, no lesions were observed in the duodenum of germfree TCR-α-deficient mice infected with *C. parvum*. However, in the distal jejunum and ileum, there was mild crypt epithelial cell hyperplasia. Mild to moderate diffuse infiltration of lymphocytes and plasma cells was observed in the jejunal and ileal lamina propria of these mice. As shown in Figure 2B, cecal mucosa of germfree TCR-α-deficient mice infected for 6 weeks with *C. parvum* were moderately thickened due to proliferative glands and hyperplasia of surface epithelium. There was a diffuse infiltrate of lymphocytes, macrophages, and plasma cells in the cecal lamina propria. Colonic mucosa was thickened due to proliferation of glands and mild infiltration of lymphocytes in the lamina propria.

At 12 weeks PI, there were no significant lesions in the duodenum or jejunum of germfree TCR-α-deficient mice infected with *C. parvum*. However, the ileal epithelium was hyperproliferative, and there were a moderate number of apoptotic cells. Ileal crypts of germfree TCR-α-deficient mice infected with *C. parvum* were hyperplastic. There was a mild to moderate diffuse infiltrate of lymphocytes and plasma cells in the lamina propria. In general, lesions in the small intestine were less severe than observed in the large intestine. In the cecum, mucosa, and submucosa were thickened (Figure 2C). The cecal mucosal epithelium was focally eroded and contained a moderate number of necrotic cells. The epithelium was thickened and immature. There was a marked infiltrate of lymphocytes, plasma cells, macrophages, and polymorphonuclear leukocytes. Numerous *C. parvum* were observed along the apical surface of the epithelium (Figure 2D). Submucosal lymphatics were dilated. There were a number of lymphoid aggregates in the lamina propria. Occasional crypt abscesses were characterized by gland lumina containing neutrophils and necrotic debris. The colonic mucosa was thickened due to proliferation of glands and surface epithelium. A diffuse, moderate infiltration of lymphocytes and plasma cells was seen in the colonic lamina propria.

Immunohistochemical Staining of Leukocytes in Cecal Tissue of Germfree TCR-α-Deficient Mice Infected with C. parvum

We used immunohistochemical staining of cecal tissues isolated at 12 weeks PI from germfree TCR-α-deficient mice to phenotype the leukocytes infiltrating the inflamed lamina propria of these mice. There were few TCR-β^+ cells, γ/δ TCR$^+$ cells, or NK1.1$^+$ cells in the cecal lamina propria of germfree TCR-α-deficient mice infected with *C. parvum* (not shown). However, as shown in Figure 3, A and B, there were a striking number of aggregates of B220$^+$ lymphocytes. The predominant B cell population

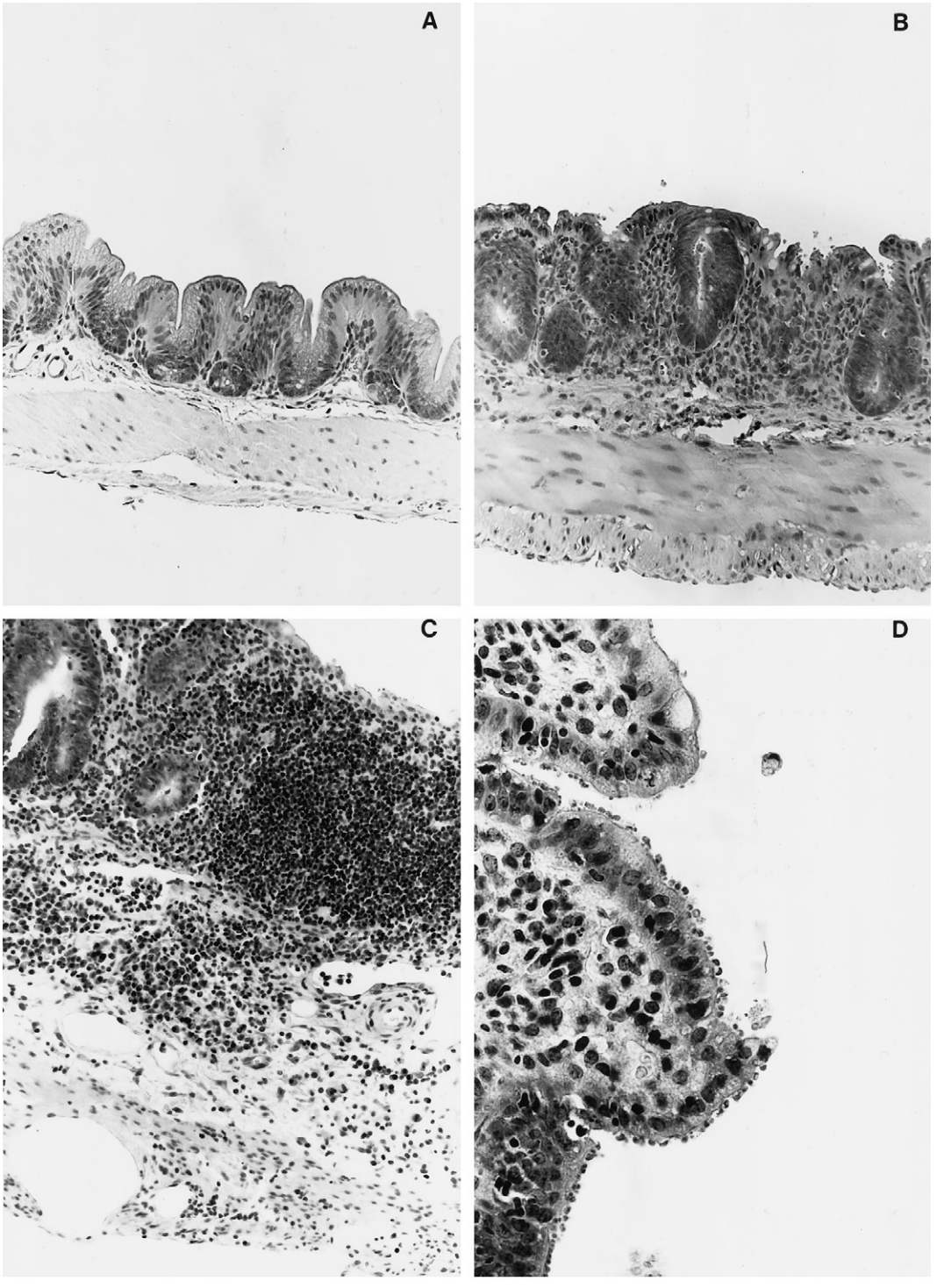

Figure 2. Histopathological features of intestinal inflammation in *C. parvum*-infected germfree TCR-α mutant mice. A: Cecal tissue from a noninfected adult germfree TCR-α-deficient mouse showing few mononuclear cells in the lamina propria. H&E; magnification, ×64. B: Cecal tissue from an adult germfree TCR-α mutant mouse infected for 6 weeks with *C. parvum* showing a moderately thickened mucosa with increased numbers of leukocytes in the lamina propria. H&E; magnification, ×64. C: Cecal tissue from an adult germfree TCR-α mutant mouse infected for 12 weeks with *C. parvum*. There is a marked thickening of cecal mucosa and submucosa, which is characteristic of the lesions observed in these mice. Note the striking number of lymphocytes infiltrating the lamina propria. H&E; magnification, ×64. D: Numerous *C. parvum* can be seen attached to the apical surface of the cecal epithelial cells in this germfree TCR-α-deficient mouse infected for 12 weeks with the parasite. H&E; magnification, ×160.

in the lamina propria of mice is the Ly-1$^+$ B cell (B-1) subset as opposed to conventional B cells (B-2).[14] As mature B-2 B cells are surface (s)IgD^{high}, whereas sIgD expression on B-1 B cells ranges from negative to dull positive,[14] we examined the expression of sIgD to distinguish these B lymphocyte subpopulations. Interestingly, the aggregates of B220$^+$ cells were predominantly composed of cells intensely stained by MAb to sIgD (Figure

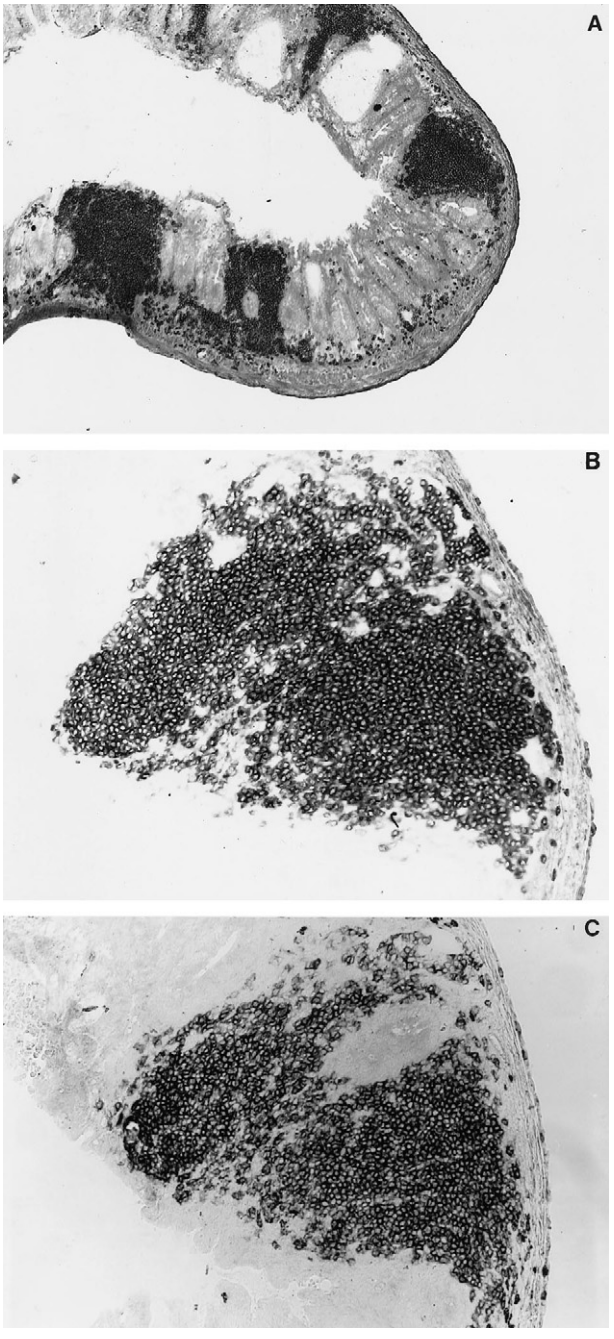

Figure 3. Immunohistochemical staining of lymphocytes infiltrating the cecal lamina propria of germfree TCR-α-deficient mice 12 weeks after oral challenge with *C. parvum*. A: Numerous aggregates of B220$^+$ cells in the lamina propria stained with monoclonal antibody RA3-6B2. Immunoperoxidase procedure; original magnification, ×25. B: Higher-power magnification of an aggregate of lymphocytes stained with RA3-6B2. Immunoperoxidase procedure; magnification, ×64. C: Serial section stained for sIgD with monoclonal antibody 217-170. Note intense staining of a majority of these cells. Immunoperoxidase procedure; magnification, ×64.

3C), indicating that these cells are likely representative of the conventional (ie, B-2) B cell subset.

Discussion

The chronic colitis observed to develop spontaneously in TCR-α- or TCR-β-deficient mice, or TCR-$\beta \times \delta$-double-deficient mice resembles UC in humans. Lesions develop in a majority of these TCR-deficient mice after approximately 3 to 4 months of age. These mice gradually develop chronic diarrhea, leading to progressive wasting that is often associated with anorectal prolapse.[1] It has been hypothesized that the disease in these immunodeficient mice is mediated by chronic stimulation of lymphoid cells via an environmental agent that would normally be cleared in immunocompetent animals. In support of this latter hypothesis, it was recently shown that germfree TCR-α-deficient mice do not develop IBD-like lesions.[4] In fact, no intestinal inflammation was observed in TCR-α-deficient mice colonized with one or two microbial species from the normal intestinal flora. We have extended these observations to show that germfree TCR-α-deficient mice monoassociated with *C. parvum* develop intestinal inflammation. Our results provide a mechanism for the induction of colitis in TCR-α-deficient mice, in that antigenic stimulation by a single microbial species, such as that provided by *C. parvum*, is sufficient to trigger intestinal inflammation. Furthermore, the chronicity of this antigenic stimulation evidently leads to the progression and perpetuation of the inflammation in these mice.

Resistance to *C. parvum* infection has been shown to be dependent on T lymphocyte function, especially that of CD4$^+$ T cells.[15–17] TCR-α-deficient mice are known to possess functional B cells, γ/δ T cells, and TCR-$\alpha^-\beta^+$ T cells.[18–19] In fact, TCR-$\alpha^-\beta^+$, and in some cases γ/δ T cells, in TCR-α mutant mice are known to express CD4.[20] In contrast to immunocompetent mice, germfree TCR-α-deficient mice, as well as flora-bearing TCR-α mutant mice, develop chronic *C. parvum* infection and intestinal inflammation. Our results expand on previous research by refining the critical role for CD4$^+$ T cells in resistance to *C. parvum* infection to further suggest a requirement for CD4$^+$ T cells possessing heterodimeric α and β chains of the TCR.

A striking feature of the intestinal lesions in germfree TCR-α-deficient mice monoassociated with *C. parvum* were the numerous aggregates of B220$^+$, sIgD$^+$ B cells in the intestine. Intense expression of sIgD would suggest that these B cells are conventional, or B-2, B cells, as opposed to B-1 (Ly-1) B cells. As TCR-α-deficient, TCR-β-deficient, TCR-$\beta \times \delta$-double-deficient mice, or MHC-class-II-deficient mice, but not RAG-1-deficient mice, develop colitis spontaneously, it has been speculated that the disease is caused by an inappropriate regulation of B cells due to the lack of class-II-MHC-restricted CD4$^+$ $\alpha\beta$ T cells. Moreover, it has been implied that IBD in TCR-deficient mice could result from an autoimmune attack against the mucosal epithelium, possibly due to cross-reactive antibodies and thus may be regarded as an autoimmune disease. Interestingly, B-2 B cells are known to produce pathogenic autoantibodies.[14] Thus, it remains to be determined whether the B-2 B lymphocytes in the lamina propria of germfree TCR-α-deficient mice monoassociated with *C. parvum* produce autoantibodies and whether these autoantibodies cross-react with host intestinal epithelium.

The previously described mucosal lesions in *C. parvum*-infected flora-bearing TCR-α-deficient mice[2] were more severe than were observed in *C. parvum*-infected germfree TCR-α-deficient mice in the present study. Moreover, the leukocytic infiltrate in flora-bearing mice contained a significant number of γ/δ T cells as well as B cells.[2] Thus, one could speculate that once the intestinal mucosa has been breached in flora-bearing TCR-α-deficient mice, other microbial organisms, including members of the normal intestinal flora, could potentiate the inflammation. The enhanced inflammatory response observed in flora-bearing TCR-α-deficient mice may result in the local proliferation or recruitment of γ/δ T cells or may be a consequence of their local proliferation or trafficking to the inflammatory site.

The results presented herein support the previous hypothesis that the inability of an immunocompromised host to regulate the immune response to chronic antigenic stimulation in the intestinal mucosa results in inflammation. Previous research had failed to identify a single microbial species capable of inducing intestinal inflammation in TCR-α-deficient mice. We have demonstrated that the ubiquitous protozoan parasite *C. parvum* is capable of inducing IBD in TCR-α-deficient mice that are predisposed to intestinal inflammation. In at least one case report, clinical relapse after exposure to *C. parvum* has been documented to occur in patients with previously stable IBD.[3] Thus, patients with certain types of transient or more permanent immunodeficiency or with a history of intestinal disease may be at risk for developing IBD-like lesions after exposure to *C. parvum* or other enteric pathogens.

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