Role of Appendix in the Development of Inflammatory Bowel Disease in TCR-α Mutant Mice

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Summary

T cell receptor-α mutant mice (TCR-$\alpha^{-/-}$), created by gene targeting of the TCR-α gene in embryonic stem cells, spontaneously develop inflammatory bowel disease (IBD) resembling human ulcerative colitis. Since gut-associated lymphoid tissue is likely to play an important role in the development of chronic intestinal inflammation, we examined the changes in the appendix lymphoid follicle (ALF) and Peyer's patches (PP) in these mice. We found the structure of the ALF to be remarkably similar to that of the PP in the small intestine; in both instances, lymphoid follicles covered by surface epithelium (dome-formation) were found. The amount of proliferation in the lymphoid follicles of the appendix estimated by in vivo incorporation of 5-bromo-2′deoxyuridine was more than two times that of PP in TCR-$\alpha^{-/-}$ mice. ELISPOT assay showed an increase of IgA, IgG1, and IgG2a, but not IgM-secreting B cells in ALF of TCR-$\alpha^{-/-}$ mice compared to TCR-$\alpha^{+/-}$ control mice. Furthermore, TCR-$\alpha^{-/-}$ mice revealed an increase of autoantibody-producing B cells against the cytoskeletal protein tropomyosin in ALF as compared to PP. When TCR-$\alpha^{-/-}$ mice underwent appendectomy at a young age (3–5 wk), the number of mesenteric lymph nodes cells at 6–7 mo were markedly less than in the sham-operated TCR-$\alpha^{-/-}$ mice. Furthermore, appendectomy at 1 mo of age suppressed the development of IBD, with only 3.3% of these mice developing IBD in the 6–7-mo period of observation. In contrast, ~80% of controls, including the sham-operated TCR-$\alpha^{-/-}$ mice, developed IBD during this period. These results suggest that ALF, rather than PP, is the priming site of cells involved in the disease process and plays an important role in the development of IBD in TCR-$\alpha^{-/-}$ mice.

Gut-associated lymphoid tissue, consisting of Peyer's patches (PP), intraepithelial lymphocytes (IEL)[1], and lamina propria lymphocytes in the small and large intestine, mesenteric lymph nodes (MLN), and the appendix lymphoid follicle (ALF), act as the inductive and effector sites of the mucosal immune system (1, 2, 3). The structure of PP is characterized by the follicle-associated epithelium containing specialized epithelial cells (M cells) overlying lymphoid follicles (4, 5). M cells, which transport luminal antigens to the mucosal immune cells, also have been identified in the colonic epithelium over the lymphoid follicles in mice and in the appendix of rabbits (5, 6). The PP are involved in the infection and subsequent immune response against enteropathogens, including the group of microbes capable of selective attachment to M cells, such as *Salmonella typhi*, *Yersinia enterocolitica*, and *reovirus* (4, 7). M cells overlying PP are also capable of taking up non-invasive bacteria such as *Vibrio cholerae* and *Escherichia coli-RDEC* (4, 8, 9). In contrast, the immunological function of the appendix has not yet been defined.

Although mucosal immune mechanisms are considered to be important in the development of human IBD (ulcerative colitis [UC] and Crohn's disease), the exact cause of these disorders has remained unknown (10). Recently, a number of experimental models of IBD have been described (11–15). These models include the development of spontaneous chronic intestinal inflammation in TCR-α and -β mutant mice and mice deficient in IL-2 and IL-10 (16–18). The IBD is more severe and present more consistently in TCR-$\alpha^{-/-}$ mice than in TCR-$\beta^{-/-}$ mice (16). No known pathogenic organisms have been identified as a cause of IBD in these mice (11, 16). In TCR-$\alpha^{-/-}$ mice, the T cell population is composed of TCR-γδ T cells and a unique population of T cells bearing TCR-β chain on the cell surface in the absence of a TCR-α chain (TCR-$\alpha^{-}\beta^{+}$ T cells)(19, 20). The TCR-γδ T cells in TCR-$\alpha^{-/-}$ mice increase with age (19) and after pathogenic infection (21).

[1] *Abbreviations used in this paper:* ALF, appendix-lymphoid follicle; BrdU, 5-bromo-2′deoxyuridine; ELISPOT, enzyme-linked immunospot; IBD, inflammatory bowel disease; MLN, mesenteric lymph nodes; PP, Peyer's patch; SFC, spot-forming cells; UC, ulcerative colitis.

TCR-$\alpha^-\beta^+$ T cells are able to respond appropriately to stimulation with minor lymphocyte stimulating superantigen 1 (22). Although B cells can develop and differentiate in these mice, the capacity to regulate local T cell and B cell proliferation may be lost in the absence of TCR-$\alpha^+\beta^+$ T cells (21, 23, 24).

Mucosal immune responses are most likely to be involved in the development of IBD in TCR-$\alpha^{-/-}$ mice (16, 25). A possible role of the appendix in the development of human IBD has been suggested (26). Since immune responses to enteric bacteria have been postulated to play an important role in the pathogenesis of IBD in the experimental animals (11, 12, 17, 27), and since appendix and cecum act as a reservoir of enteric bacteria, we examined the role of appendix in the development of IBD in TCR-$\alpha^{-/-}$ mice.

Materials and Methods

Mice. Colonies of TCR-$\alpha^{-/-}$ mutant mice (19) were developed at the Massachusetts General Hospital from mice provided by Drs. P. Mombaerts and S. Tonegawa (Massachusetts Institute of Technology). These mice are derived genotypically from two (H-2^b) strains, 129/Sv and C57BL/6. C57BL/6 were purchased from The Jackson Laboratory (Bar Harbor, ME). TCR-α heterozygous mice (TCR-$\alpha^{+/-}$) were generated by crossing TCR-$\alpha^{-/-}$ mice with C57BL/6, background mice of TCR-$\alpha^{-/-}$ mice. The mice were housed in specific pathogen-free facility of Massachusetts General Hospital. Due to the presence of *Pneumocystis carinii* infection of lungs in older mice (16), TCR-$\alpha^{-/-}$ breeder mice were treated with oral administration of sulfamethoxazole and trimethoprim (Schein Pharmaceutical, Port Washington, NY) to increase breeding of TCR-$\alpha^{-/-}$ mice. This treatment did not effect development of IBD.

Antibodies. For flow cytometry, CD3ϵ (145-2C11)-FITC (Boehringer Mannheim, Indianapolis, IN.), B220 (RA3-6B2), CD4 (RM4-5), CD8α (53-6.7), CD8β (53-5.8), αE (M290)-FITC, TCR-β (H57-597), TCR-δ (GL3), CD23 (B3B4), and CD5 (53-7.3)-PE (PharMingen, San Diego, CA) mAbs were purchased. For immunohistochemical analysis, biotin-anti I-$A^{b,d}$ (25-9-17; PharMingen) and purified anti-BrdU (Sera-Lab, Crawley Down, England) mAbs, and mAbs to B220, TCR-β, TCR-δ, IgM, and IgD (PharMingen) were used.

Histological and Immunohistochemical Analysis. For histological examination, specimens were obtained from five intestinal regions: rectum, distal and proximal colon, cecum, and appendix in every mouse tested. Specimens were fixed in 3% buffered formalin and embedded in paraffin. Multiple 4-μm sections were stained with hematoxylin and eosin. The severity of UC-like disease was determined according to the diagnostic criteria previously described (16).

For immunohistochemical analysis, tissue specimens were embedded in OCT compound, snap frozen in liquid nitrogen, and then subsequently stored at -80°C. 4-μm cryo-sections were stained by an avidin-biotin complex method (16).

Cell Preparation. PP cells were extracted as described previously (28). Grossly, ALF was found near the tip of the appendix and the appearance was similar to PP, forming a gray-white bulging on the peritoneal surface (Fig. 1 *A*). PP and ALF were excised from the outside by a blade and crushed gently by 25-gauge needles. Cell suspensions in medium (RPMI-1640 containing amphotericin B, penicillin, streptomycin, and 2% FCS) were passed through glass wool columns under sterile conditions.

MLN cells were extracted according to the method previously described (25). After removal of fat, all grossly recognizable MLN were dispersed gently using 25-gauge needles and the resulting cell suspension was passed through a nylon membrane (60 μm).

Flow Cytometry. Between 2×10^5 and 5×10^5 cells were washed with FACS® buffer (PBS containing 0.2% BSA and 0.1% sodium azide), blocked with 50 μl of FACS® buffer containing 10% normal rat and hamster serum (Jackson ImmunoResearch Labs., Inc., West Grove, PA) at 4°C for 20 min, and incubated with the FITC– and PE– mAbs listed above at 4°C for 30 min. After washing with FACS® buffer, cells were analyzed using Lysis II software on a FACScan® (Becton Dickinson, Mountain View, CA). In some experiments, anti-FcγII/III was used to block Fc-receptor.

In Vivo BrdU Incorporation. For labeling of proliferative cells in vivo, 5-bromo-2′-deoxyuridine (BrdU)(Sigma chemical Co., St. Louis, MO) was injected intraperitoneally (100 μg/g in PBS) 1 h before killing. BrdU incorporated cells were detected by anti-BrdU mAb as described previously (29) using frozen and paraffin-embedded sections. Deparaffinized paraffin-embedded sections were treated with 0.1% trypsin 37°C for 20 min before staining for BrdU. After incubation with 3N HCl at room temperature for 15 min to denature double-strand DNA, frozen tissue or paraffin-embedded sections were washed in PBS. Sections were then incubated with anti-BrdU mAb at an optimal dilution for 1 h followed by sequential incubations with biotinylated rabbit anti-rat Ig (Vector, Burlingame, CA) and avidin-biotinylated peroxidase complexes (Dako, Santa Barbara, CA). After development with 3-amino-9 ethylcarbazole (AEC)(Aldrich, Milwaukee, WI), sections were post-fixed with 2% paraformaldehyde and mounted with Glycergel. Each incubation was followed by three washes in PBS. Incubation with 0.3% H_2O_2 in PBS was used to block endogenous peroxidase activity, while sequential incubations with avidin and biotin (Vector) were used to block endogenous biotin. After staining, sections were observed at $\times$400 under microscopy. The BrdU-labeling index in ALF and PP was calculated as positive cells/field ($\times$400) and averaged. For counting BrdU-incorporated cells of colonic epithelium, one high power field ($\times$200) of each of these areas was counted. We counted 20 different fields and the average was calculated as the total number of BrdU labeled cells per 1-mm length of colonic mucosa.

Enzyme-Linked Immunospot (ELISPOT) Assay for Detection of Ig Isotype and Autoantibody. The ELISPOT assay was performed as described previously (25, 30). A 96-well plate with a nitrocellulose base (Millititer HA; Millipore Corp., Bedford, MA) was coated overnight at 4°C with 100 μl of tropomyosin (5 μg/ml; Sigma)(31) in 0.1 M carbonate buffer (pH 9.6) or with 100 μl of goat anti–mouse Ig (5 μg/ml; Dako) in D-PBS. After the wash, each well was blocked with 1% BSA/PBS at 37°C for at least for 1 h. Cells (2×10^5 to 5×10^2 cells/well) in 100 μl of complete medium (RPMI-1640 containing sodium bicarbonate, nonessential amino acids, sodium pyruvate, L-glutamine, Hepes, penicillin, streptomycin, and 10% FCS) were added to each well and incubated at 37°C for 16 h. To block de novo synthesis of Ig, cells in some experiments were treated with 80 μg/ml cycloheximide (Sigma) for 3 h, washed and suspended in complete medium containing 80 μg/ml cycloheximide before being added to each well. After wash with 0.05% Tween-PBS at least five times, alkaline phosphatase (AP)-anti-Ig, -IgG2a, or horseradish peroxidase (HRP)-anti-IgG1 (Southern Biotechnology, Birmingham, AL), HRP-anti IgM or IgA (Sera-Lab) were added to each well. After

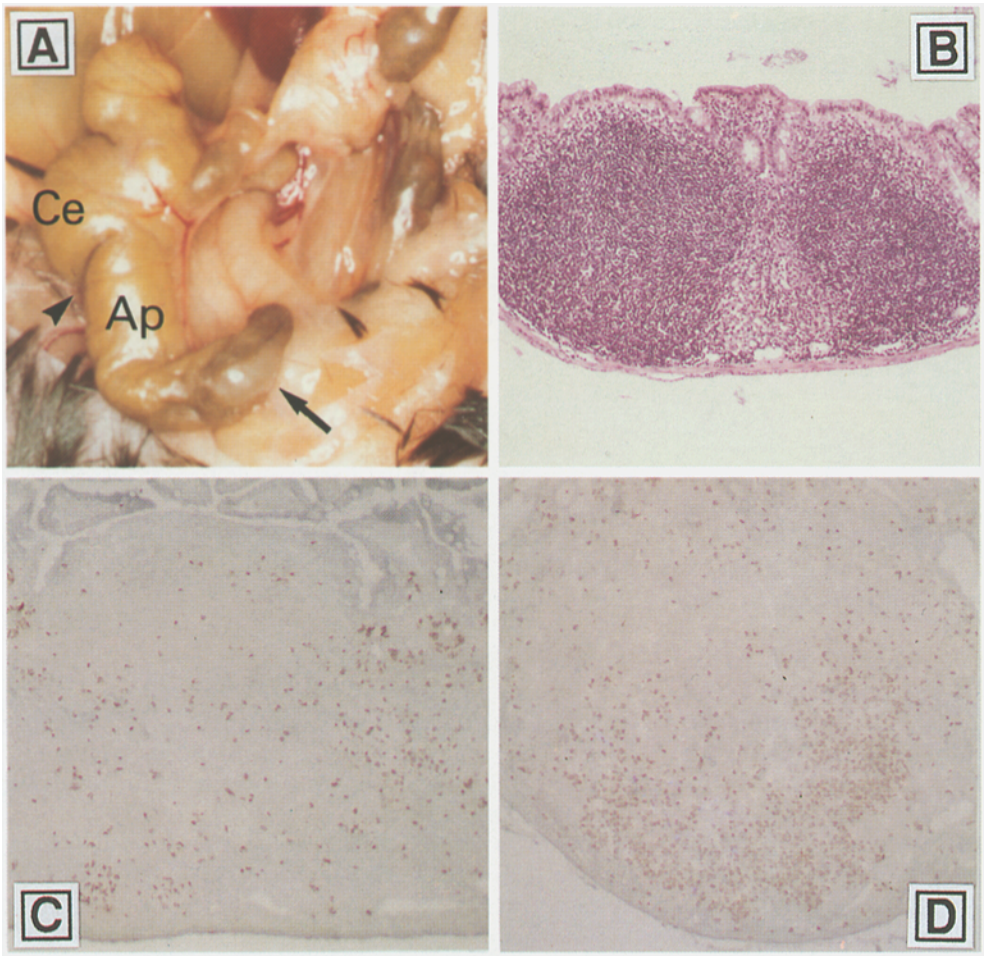

Figure 1. (*A*) Appendix (*Ap*), appendix lymphoid follicle (*arrow*), and cecum (*Ce*) of a TCR-$\alpha^{-/-}$ mouse are shown. Arrow head indicates the border between cecum and appendix. (*B*) Histology of the appendix-lymphoid follicle shows dome-formation (×4). (*C* and *D*) Immunohistochemical analysis of frozen tissue sections shows in vivo BrdU-incorporated cells in PP (*C*) and ALF (*D*) of TCR-$\alpha^{-/-}$ mice (20 weeks of age). There are more stained cells in ALF as compared to PP.

incubation at room temperature for 2 h, each well was washed and developed with nitro blue tetrazolium (NBT) and 5-bromo-4 chloro-3-indolyl phosphate (BCIP; Promega Biotec, Madison, WI) or AEC. After wash with tap water, all the spots in the wells were counted under a dissecting microscope. Results were statistically analyzed using Mann-Whitney U-test.

Appendectomy. TCR-$\alpha^{-/-}$ (3–15 weeks of age) mice were anesthetized by inhalation of isoflurane and after a 1-cm incision on abdomen, the appendix was gently pulled out and ligated with polypropylene suture at the border between appendix and cecum, and resected. The border between cecum and appendix is determined by a slight constriction as shown in Fig. 1 *A*. In sham-operated group, TCR-$\alpha^{-/-}$ mice were anesthetized and underwent 1-cm abdominal incision without excision of the appendix. These mice were killed at 6–7 months of age.

Results

Structure of Appendix Lymphoid Follicle. There are two major differences between the appendix in mice and humans. Grossly, the appendix in mice (Fig. 1 *A*) is relatively larger with a wide lumen continuous with cecum as compared to cord-like appearance of human appendix. Microscopically, a single large lymphoid follicle is present near the tip of the mouse appendix, whereas lymphoid follicles exist as clusters in the human appendix.

In mice, the structure of ALF is remarkably similar to PP showing a monolayer of mucosal epithelium without goblet cells, termed follicle-associated epithelium, overlying the lymphoid follicle (Fig. 1 *B*). ALF also shows glandular follicle-formation, a characteristic feature of colonic lymphoid follicle. Immunohistochemical analysis showed the presence of Ia-positive macrophage-like cells between the epithelium and lymphoid follicle in the appendix (data not shown), indicating the presence of antigen-presenting cells.

Phenotypic Analysis of ALF and PP Cells. The proportions of B cells and T cells in the PP and ALF of TCR-$\alpha^{-/-}$ mice and TCR-$\alpha^{+/-}$ mice were similar (Table 1). In TCR-$\alpha^{-/-}$ mice (20 wk old), maintained under pathogen-free conditions, PP and ALF were composed of 86.2 ± 2.1% and 85.4 ± 4.2% of $B220^+CD23^+$ cells, respectively. $CD3^+$ T cells were 11.3 ± 3.2% and 12.4 ± 5.3% in PP and ALF, respectively. The proportions of TCR-$\alpha^-\beta^+$ cells in the $CD3^+$ T cell population in PP (41.4 ± 5.4%) and ALF (51.7 ± 2.5%) were markedly higher than in peripheral lymphoid tissue (Fig. 2). Among the T cells isolated from spleen and MLN in TCR-$\alpha^{-/-}$ mice, TCR-$\alpha^-\beta^+$ T cells were less than 25% of $CD3^+$ T cells. In ALF of TCR-$\alpha^{-/-}$ mice, 50.0 ± 11.1 and 44.3 ± 14.0 of TCR-$\alpha^-\beta^+$ T cells were $CD4^+$ $CD8\alpha^-$ and $CD4^-$ $CD8\alpha^-$ (DN), respectively. In addition, over 80% and 10% of TCR-$\alpha^-\beta^+$

Table 1. *Phenotype of Cells from PP and ALF**

		$B220^+CD23^+$	$CD3\epsilon^+$	TCR-β/CD3‡	CD4/TCR-β‡
TCR-$\alpha^{+/-}$	PP	80.3±6.2	19.5±3.2	90.5±2.4	64.0±6.6
	ALF	81.6±4.2	16.2±1.4	92.6±1.6	63.4±3.7
TCR-$\alpha^{-/-}$	PP	86.2±2.1	11.3±3.2	41.4±5.4	44.4±8.4
	ALF	85.4±2.1	12.4±5.3	51.7±2.5	50.0±11.1

*Phenotype of PP and ALF lymphocytes in TCR-$\alpha^{-/-}$ mice and TCR-$\alpha^{+/-}$ mice was determined by flow cytometric analysis. All mice were maintained under pathogen free conditions. The age of mice was between 20 and 26 wk.
‡Percentages of TCR-β^+ cells witin $CD3\epsilon^+$ cells and $CD4^+$ cells within TCR-β^+ cells.
§Data averaged from 7 TCR-$\alpha^{+/-}$ and 18 TCR-$\alpha^{-/-}$ mice represent mean±SEM.

T cells expressed CD5 and M290, respectively, indicating that most of TCR-$\alpha^-\beta^+$ T cells were thymic dependent. Immunohistochemical analysis of MLN, ALF and PP using anti-CD3, TCR-δ, IgM, IgD, and B220 confirmed the results obtained with FACScan® analysis (data not shown).

Increase of Proliferative Cells in ALF. To compare the frequency of proliferative lymphoid cells in PP and ALF in TCR-$\alpha^{-/-}$ and TCR-$\alpha^{+/-}$ mice, in vivo BrdU incorporation in lymphoid cells was analyzed. The results are shown in Figs. 1, *C* and *D* and 3. In TCR-$\alpha^{-/-}$ mice, the number of BrdU-incorporated cells was more than twofold higher in ALF than in PP; the labeling index was 59.9 ± 4.7 and 25.4 ± 2.1 cells/field in ALF and PP, respectively ($P < 0.005$). Furthermore, in ALF, the BrdU-incorporated cells increased about threefold in TCR-$\alpha^{-/-}$ mice as compared to TCR-$\alpha^{+/-}$ mice (Fig. 3). However, in PP, there was no difference in the number of BrdU-incorporated cells between TCR-$\alpha^{-/-}$ and TCR-$\alpha^{+/-}$ mice (Fig. 3). The proliferative cells were found not only in B cell areas (follicles), but also in the interfollicular areas in ALF of TCR-$\alpha^{-/-}$ mice. This finding suggests that both B and T cells proliferate.

Activation of and Autoantibody Production by B cells in ALF. In TCR-$\alpha^{+/-}$ mice, the number of IgA-, IgG1-, and IgG2a-producing B cells in PP was higher than in ALF (Fig. 4 *A*), indicating that, in normal mice, PP seems to be more reactive to foreign antigens than ALF. However, in TCR-$\alpha^{-/-}$ mice, the numbers of IgA-, IgG1-, IgG2a-, and IgM-secreting B cells in ALF were similar or more than that in PP (Fig. 4 *A*). These findings indicate that activation and Ig-switching of B cells in ALF of TCR-$\alpha^{-/-}$ mice is more than that present in TCR-$\alpha^{+/-}$ mice. Pretreatment of these cells with 80 μg/ml of cycloheximide, a protein synthesis inhibitor, reduced Ig producing spots to 5–10% of their level, indicating that the spots were a result of de novo protein synthesis.

Antibodies to the cytoskeletal protein tropomyosin have been reported to be present in patients with UC (31). We have recently found that TCR-$\alpha^{-/-}$ mice with IBD also produce autoantibodies to tropomyosin (25). The development of antibodies to tropomyosin may be due to molecular mimicry by viral or bacterial antigens for tropomyosin (32, 33). In the present study, we investigated the presence of B cells secreting autoantibodies to tropomyosin by ELISPOT assay. ELISPOT assay showed over twofold increase in B cells secreting autoantibodies to tropomyosin in ALF as compared to PP of TCR-$\alpha^{-/-}$ mice. Furthermore, the number of anti-tropomyosin secreting B cells was more in ALF of TCR-$\alpha^{-/-}$ mice as compared to TCR-$\alpha^{+/-}$ mice (Fig. 4 *B*).

Effect of Appendectomy. To investigate the role of appendix in the development of IBD, we performed appendectomy in 49 TCR-$\alpha^{-/-}$ mice at different ages. The littermates which underwent sham-operation were used as controls. 12 (80%) out of 15 control TCR-$\alpha^{-/-}$ mice (6–7 months of age) revealed gross and microscopic features characteristic of IBD in TCR-$\alpha^{-/-}$ mice. These findings are consistent with our previous report that 83% of TCR-$\alpha^{-/-}$ mice at 24–30 weeks of age develop IBD spontane-

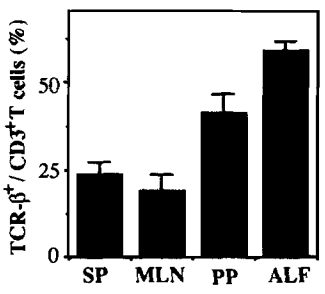

Figure 2. Frequency of TCR-β^+ cells in the $CD3^+$ T cell population of ALF, PP, MLN, and spleen from 20 TCR-$\alpha^{-/-}$ mice (20 wk old). Cells were incubated with FITC-anti-CD3ε (145-2C11), and PE-anti-TCR-δ (GL3), or PE-anti-TCR-β (H57-597) mAbs and then subjected to two color flow cytometric analysis.

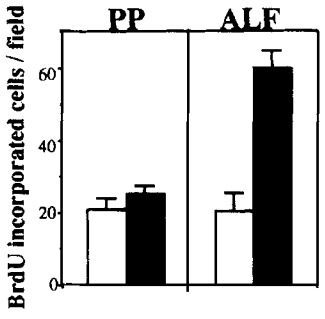

Figure 3. In vivo BrdU-incorporated cells in the ALF and PP of TCR-$\alpha^{-/-}$ and TCR-$\alpha^{+/-}$ mice. The numbers indicate averages of BrdU-incorporated cells/field (×400) from groups of 7 mice (20 wk old). Open bars represent TCR-$\alpha^{+/-}$ and solid bars indicate TCR-$\alpha^{-/-}$ mice. There are significantly more ($P < 0.005$) proliferative cells in the ALF of TCR-$\alpha^{-/-}$ mice as compared to that in PP of TCR-$\alpha^{-/-}$ mice or PP and ALF of TCR-$\alpha^{+/-}$ mice.

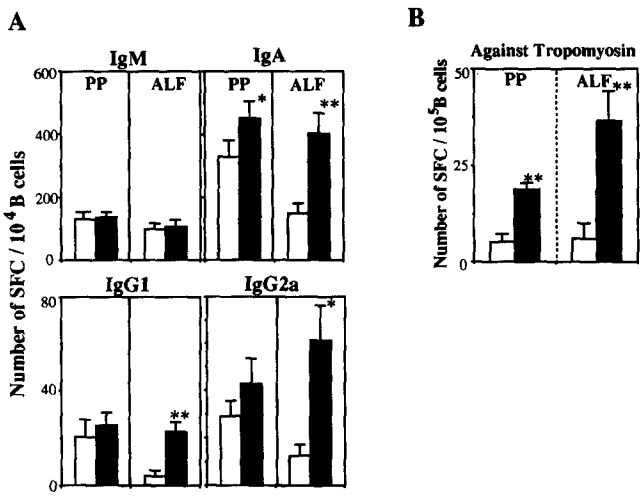

Figure 4. Quantification of Ig and autoantibody-secreting cells in ALF and PP of age-matched (20–24 wk) TCR-$\alpha^{-/-}$ and TCR-$\alpha^{+/-}$ mice by ELISPOT assay. (*A*) The frequency of IgM, IgA, IgG1, and IgG2a secreting B cells in PP and ALF are shown. (*B*) The numbers of B cells secreting autoantibody (*Ig*) against tropomyosin are shown. Open bars indicate TCR-$\alpha^{+/-}$ and solid bars indicate TCR-$\alpha^{-/-}$ mice. The values are mean of spot-forming cells (*SFC*) ± SEM from 18 TCR-$\alpha^{+/-}$ and 24 TCR-$\alpha^{-/-}$ mice. Significant differences between the values for TCR-$\alpha^{-/-}$ and TCR-$a^{+/-}$ mice are indicated: $^{*}P < 0.05$, $^{**}P < 0.005$.

ously (16, 25). The disease involved the entire colon including cecum and appendix, and was characterized by a thickened colonic wall with marked elongation of crypts, depletion of goblet cells , occasional crypt abscesses and the presence of mixed inflammatory cell infiltrate in the lamina propria (Fig. 5)(16). In contrast, only one (3.3%) out of 30 TCR-$\alpha^{-/-}$ mice, appendectomized at 3–5 weeks of age, showed the findings of IBD (Fig. 6). However, the frequency of IBD development in the appendectomized mice increased depending on the period when appendectomy was performed; histological evidence of IBD was detected in 4 (33%) out of 12 TCR-$\alpha^{-/-}$ mice and 6 (75%) out of

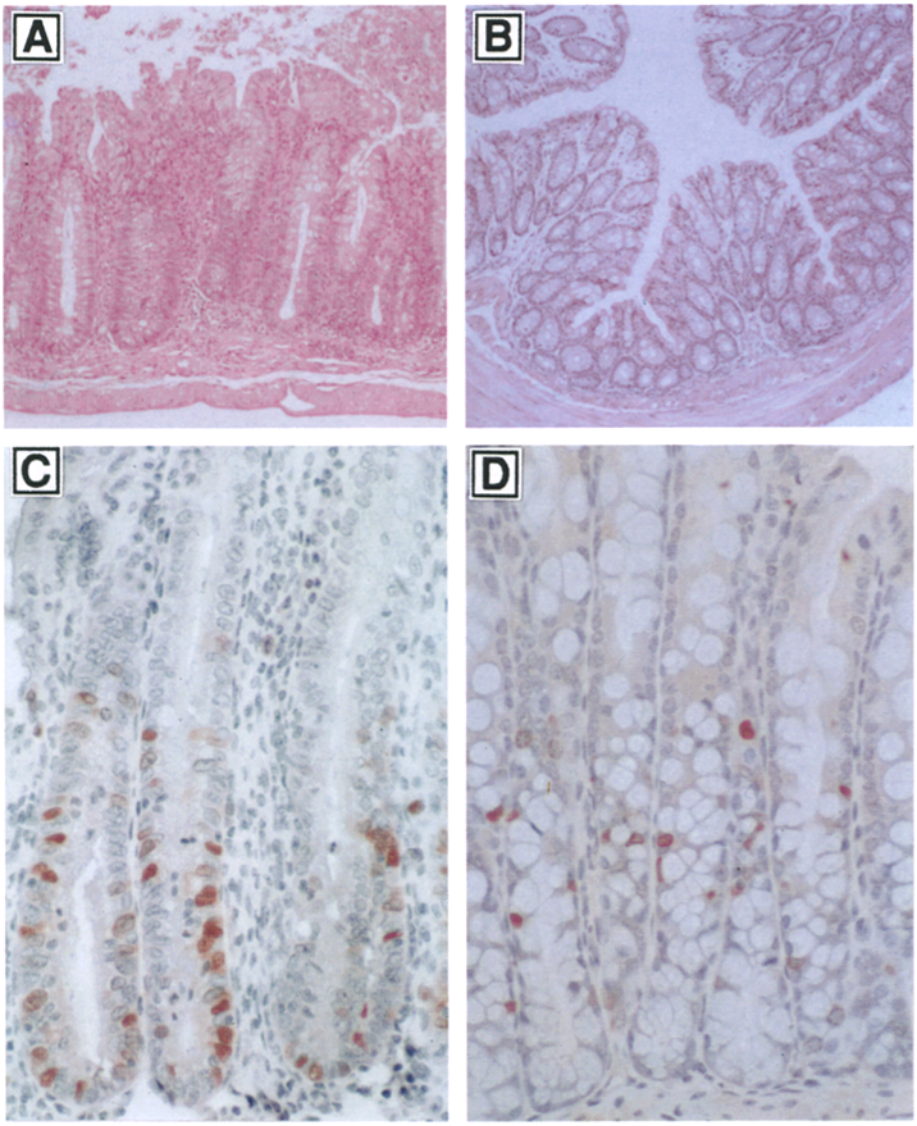

Figure 5. Histological findings and proliferative epithelial cells in the rectum of TCR-$\alpha^{-/-}$ mice after sham-operation (*A* and *C*) or appendectomy at 3 weeks of age (*B* and *D*). These mice were maintained in the same cage under pathogen-free conditions and killed at 7 months of age. *A* and *B* show histological examination from the rectum. *C* and *D* show in vivo BrdU-incorporated epithelial cells in the rectum as revealed by immunoperoxidase staining of paraffin-embedded sections. The rectum of the sham-operated mouse shows histological features of IBD (*A*) and many more stained crypt epithelial cells (*C*) as compared to the normal histological features (*B*) and normal numbers of stained crypt epithelial cells (*D*) in the appendectomized mouse.

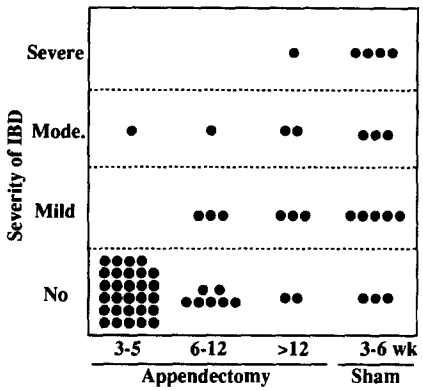

Figure 6. Effect of appendectomy on the development of IBD. Groups of TCR-$\alpha^{-/-}$ mice were sham-operated or appendectomized at different ages (3–5 wk, 6–12 wk, and over 12 wk), and were killed at 6–7 months of age. The severity of IBD (no, mild, moderate, and severe disease) was determined by histological examination as previously described (16).

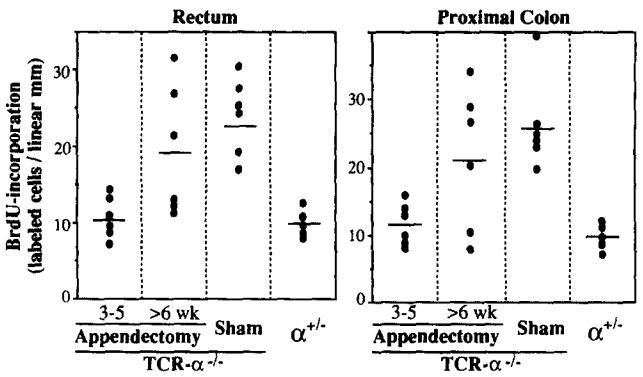

Figure 7. Effect of appendectomy on proliferation of epithelial cells in rectum and proximal colon of TCR-$\alpha^{-/-}$ mice was assessed by detection of in vivo BrdU-incorporated epithelial crypt cells. TCR-$\alpha^{-/-}$ mice were sham-operated (at 3–5 weeks of age) or appendectomized at different ages (3–5 wk and over 6 wk), and were killed at 6–7 months of age. TCR-$\alpha^{+/-}$ mice were used as normal control. Dots indicate the average (BrdU-labeling cells/1 mm of mucosa) from 20 different fields in an individual mouse. The average from each group of mice is indicated as a bar. There is significant difference ($P < 0.005$) between the values for mice appendectomized at young age (3–5 wk) and sham-operated TCR-$\alpha^{-/-}$ mice.

12 TCR-$\alpha^{-/-}$ mice which underwent appendectomy at 6–12 weeks of age and over 12 weeks of age, respectively (Fig. 6). Analysis of the proliferative index of colonic epithelial cells by in vivo incorporation of BrdU can be a useful method for the detection of colonic epithelial proliferation, a feature of IBD (34). Therefore, we also examined the proliferative index of epithelium in the rectum and proximal colon of the TCR-$\alpha^{-/-}$ mice with appendectomy or sham-operation and the TCR-$\alpha^{+/-}$ mice. The proliferative index of colonic epithelium in TCR-$\alpha^{-/-}$ mice appendectomized at 3–5 wk was similar to that of TCR-$\alpha^{+/-}$ mice (Figs. 5 and 7). In contrast, there was increased colonic epithelial proliferation in sham-operated mice and in the mice which underwent appendectomy after 6 weeks of age. These results support the histological findings of absence of IBD in the appendectomized TCR-$\alpha^{-/-}$ mice.

Decrease of MLN Cells in Appendectomized TCR-$\alpha^{-/-}$ Mice. Both B and T lymphocytes, primed by gut-lumenal antigenic stimulation, migrate first into MLN, enter the systemic circulation via the thoracic duct, and then preferentially recirculate back to intestinal mucosa (35, 36). Since the increase of MLN cells relates to the development of IBD in TCR-$\alpha^{-/-}$ mice (16, 25), we examined the changes of cell number in MLN. The total numbers of MLN cells in TCR-$\alpha^{-/-}$ mice, which underwent appendectomy at young age (3–5 wk), were significantly less ($2770 \pm 322 \times 10^4$, $P < 0.001$) than those of sham-operated TCR-$\alpha^{-/-}$ mice ($6191 \pm 649 \times 10^4$). The number of MLN cells from TCR-$\alpha^{-/-}$ mice, appendectomized at an older age (over 6 wk), showed no significant difference ($5027 \pm 468 \times 10^4$) as compared to the sham-operated TCR-$\alpha^{-/-}$ mice (Fig. 8).

Flow cytometric analysis showed that there was no difference in the proportion of B cells and T cells of MLN cells between the appendectomized TCR-$\alpha^{-/-}$ mice and the sham-operated TCR-$\alpha^{-/-}$ mice; MLN of the appendectomized TCR-$\alpha^{-/-}$ mice consisted of $79.5 \pm 1.8\%$ of B cells ($CD3^-B220^+$) and $16.4 \pm 1.2\%$ of $CD3^+$ T cells, and the percentage of B cells and T cells in the sham-operated TCR-$\alpha^{-/-}$ mice was $80.7 \pm 1.7\%$ and $17.7 \pm 1.7\%$, respectively.

Discussion

Although the lymphoid tissue of the appendix forms a part of the mucosal associated lymphoid system, the function of appendix is not known. We found the structure of ALF to be similar to that of PP in the mouse and suggest that ALF, like PP, participates in the recognition of lume-

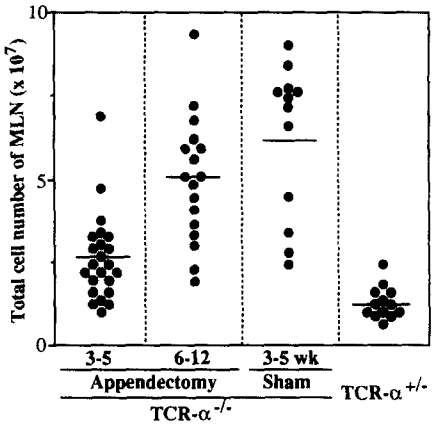

Figure 8. Decrease of MLN cells in TCR-$\alpha^{-/-}$ mice appendectomized at young age (3–5 wk). TCR-$\alpha^{-/-}$ mice after sham-operation or appendectomy at different ages (3–5 wk and over 6 wk) were maintained under pathogen-free conditions and killed at 6–7 months of age. TCR-$\alpha^{+/-}$ mice (6–7 months of age) were used as normal controls. Dots indicate the number of total MLN cells. The average from each group of mice tested is indicated as a bar. There is significant difference ($P < 0.001$) between the TCR-$\alpha^{-/-}$ mice appendectomized at young age (3–5 wk) and sham-operated mice.

nal antigens. In contrast to small intestine, where the lumenal contents are continuously pushed forward, the contents in the appendix are retained over a period of time and provide a place for colonization and proliferation of enteric bacterial flora. Although PP has been reported to play an important role for defense against lumenal pathogens, such as *Salmonella typhi*, *Yersinia enterocolitica*, reovirus, *Vibrio cholerae*, and *E. coli-RDEC* (4, 7, 8, 9), the role of ALF in defense against these or other pathogens is not known. It is possible that PP may be involved in the recognition of foreign lumenal antigens (dietary and enteric pathogens), whereas ALF may recognize the normal enteric bacterial flora, which reside mainly in the cecum and appendix.

The studies so far have not identified any pathogenic organisms as the cause of IBD in TCR-$\alpha^{-/-}$ mice (16). Similarly, pathogenic organisms have not been identified in other models of IBD (11, 12). Interestingly, the IBD in IL-2–deficient mice and HLA-B27 transgenic rats does not occur when the animals are maintained in a germ free environment (17, 27). These findings strongly suggest that nonpathogenic enteric bacterial flora play an important role in the development of IBD (12, 17, 27). Since the cecum and appendix are usually a reservoir of enteric bacterial flora, it is possible that the ALF may be involved in the generation of the immune response to enteric bacterial flora. In TCR-$\alpha^{-/-}$ mice, the proliferative response of ALF cells to lipopolysaccharide obtained from *E. coli* serotype 026:B6 (37) is greater than that of PP cells (our unpublished observations). Thus, ALF may also be important in the development of autoantibodies through molecular mimicry. In TCR-$\alpha^{-/-}$ mice, the predominant T cells population is TCR-$\gamma\delta^+$ cells (more than 70%) in peripheral blood, spleen and MLN (16, 20, 21). However, the predominant T cells population in ALF of TCR-$\alpha^{-/-}$ mice is TCR-$\alpha^-\beta^+$ cells (51.7 $\pm$ 2.5%), which express CD5 (a marker of thymus-dependence). This predominance of TCR-$\alpha^-\beta^+$ in ALF may reflect changes due to recognition of lumenal antigens. We have found that the humoral response to aerobic cecal bacteria (*E. coli*) antigens changes from polyclonal to oligoclonal with age and development of IBD in TCR-$\alpha^{-/-}$ mice (38). The polyclonal response seems to be preserved at 7 months of age in TCR-$\alpha^{-/-}$ mice appendectomized at 5 wk (our unpublished results). It is possible that CD4$^+$ TCR-$\alpha^-\beta^+$ cells may be involved in this alteration of humoral response, and in the development of IBD in TCR-$\alpha^{-/-}$ mice. Recently, thymus-dependent CD4$^+$ T cells have been reported to be important in the development of IBD in mice transgenic for the human CD3ϵ gene (39).

B cells in the human appendix express the IL-6 receptor, indicating that the appendix is a site for B cell–differentiation (40). In the current studies, we have found an increase of IgA-, IgG1-, and IgG2a-secreting B cells in ALF of TCR-$\alpha^{-/-}$ mice compared to TCR-$\alpha^{+/-}$ (controls), indicating that activation of B cells in ALF occurs selectively in the TCR-$\alpha^{-/-}$ mice. Furthermore, anti-tropomyosin secreting B cells were found to be increased in ALF of TCR-$\alpha^{-/-}$ mice. Taken together, these findings support the importance of the appendix as a site for the activation of B cells and the generation of immune responses to bacterial antigens in TCR-$\alpha^{-/-}$ mice. The development of IBD in TCR-$\alpha^{-/-}$ mice might reflect loss of oral tolerance to microbial antigens as a result of the lack of TCR-$\alpha^+\beta^+$ T cell–mediated suppression of B cells; this could lead to an autoimmune attack against the colonic epithelium, possibly due to cross-reactive antibodies (16). It is also possible that lack of regulatory T cells and cytokines allows development of inflammatory responses to enteric microbial antigens (11, 12).

Recently, it has been postulated that appendectomy may protect development of UC in humans (26). In the present study, we found that appendectomy at a young age (3–5 wk), but not at older age (>6 wk) suppressed the development of IBD in TCR-$\alpha^{-/-}$ mice. In addition to histological examination, the absence of disease in appendectomized mice was also confirmed by the lack of increase in colonic epithelial proliferation as compared to sham-operated TCR-$\alpha^{-/-}$ mice. In mice appendectomized at 3–5 weeks of age, only 1 out of 30 (3.3%) developed IBD. Since the frequency of IBD was greater in TCR-$\alpha^{-/-}$ mice appendectomized after 5 weeks of age, it is possible that the cells involved in the development of IBD expand beyond appendix and populate MLN and other mucosal lymphoid tissues after 5 weeks of age. Colonic lymphoid follicles are more frequently detected in the appendectomized TCR-$\alpha^{-/-}$ mice compared to controls (unpublished observations). Therefore, it is likely that after appendectomy the other lymphoid follicles in gut, such as colonic lymphoid follicles, can perform the function of ALF. A study of a much larger number of animals over a long period of time will help determine whether appendectomy at a young age (3 wk) completely prevents or only delays development of IBD in TCR-$\alpha^{-/-}$ mice.

Appendectomy at young age (3–5 wk) was associated with lower numbers of MLN cells as compared to the sham-operated TCR-$\alpha^{-/-}$ mice. We have previously found that the increase of MLN cells was related to development of IBD in TCR-$\alpha^{-/-}$ mice (16, 25). The number of MLN cells in TCR-$\alpha^{-/-}$ mice appendectomized at young age was similar to those of age-matched TCR-$\alpha^{-/-}$ mice without IBD, as described previously (25). Since the cells primed in the gut migrate to MLN (35, 36), our findings support the possibility that appendix is an important site for priming of the cells involved in the development of IBD in TCR-$\alpha^{-/-}$ mice.

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