

# Cytokine Imbalance and Autoantibody Production in T Cell Receptor- $\alpha$ Mutant Mice with Inflammatory Bowel Disease

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## Summary

Spontaneous inflammatory bowel disease (IBD) resembling human ulcerative colitis develops in mice mutant for the T cell receptor  $\alpha$  gene (TCR- $\alpha^{-/-}$ ). TCR- $\alpha^{-/-}$  mice lack TCR- $\alpha/\beta^+$  cells but contain TCR- $\gamma/\delta^+$  cells and a small population of a unique CD4<sup>+</sup>, TCR- $\alpha^{-}/\beta^{+}$  (low) cells. Since all the immunoglobulin (Ig) classes are present in these mice, help to B cells must be provided by cells other than TCR- $\alpha/\beta^+$  cells. In the present study, we found serum levels of IgG1 and IgG2 to be markedly increased in TCR- $\alpha^{-/-}$  mice with IBD as compared to TCR- $\alpha^{-/-}$  mice without IBD or TCR- $\alpha^{+/-}$  controls. An increase in IgG1-, IgG2a- and IgA- but not IgM-secreting mesenteric lymph node (MLN) B cells was detected in TCR- $\alpha^{-/-}$  mutant mice. There was also a marked increase in MLN B cells secreting autoantibody (IgG) to tropomyosin, a cytoskeletal protein. Examination of the hyperplastic MLN showed a marked increase in the number of B, TCR- $\delta^+$ , and CD4<sup>+</sup> TCR- $\alpha^{-}/\beta^+$  cells, similar to the cell population observed at the site of colonic inflammation. Analysis of spontaneous cytokine production by MLN cells using an enzyme-linked immunospot assay, immunohistochemistry, and reverse transcription-polymerase chain reaction showed a decrease of interleukin 2 (IL-2) but a marked increase of IL-4 and interferon  $\gamma$  (IFN- $\gamma$ ) production in TCR- $\alpha^{-/-}$  mice with IBD as compared to TCR- $\alpha^{-/-}$  mice without IBD and TCR- $\alpha^{+/-}$  control mice. Both TCR- $\alpha^{-}/\beta^+$  and TCR- $\delta^+$  cells were found to be capable of producing IL-4; IFN- $\gamma$  was produced mostly by non-T cells, many of which were shown to be CD3<sup>-</sup> NK 1.1<sup>+</sup> cells. We propose that the cytokine imbalance present in these mice results in expansion of B cells, production and switching of autoantibodies to IgG2 subclass, and development of IBD. It is possible that the unusual CD4<sup>+</sup> TCR- $\alpha^{-}/\beta^+$  population and expanded TCR- $\gamma/\delta^+$  population present in TCR- $\alpha^{-/-}$  mice plays a central role in this abnormal immune response.

Help for antibody responses is typically provided by CD4<sup>+</sup> TCR- $\alpha/\beta$  T cells. CD4<sup>+</sup> T cells can be divided into two functional subsets based on their production of cytokines. Th1 cells secrete IL-2, IFN- $\gamma$ , and TNF and are the primary effector T cells involved in delayed-type hypersensitivity responses and cellular immunity to intracellular pathogens. Th1-derived cytokines (IFN- $\gamma$ ) also support switching to the IgG2a isotype. By contrast, Th2 cells are primarily involved in B cell activation and maturation, mediated by their secretion of IL-4, IL-5, IL-6, IL-10, and IL-13. Th2-derived cytokines direct switching to IgG1 and

IgE isotypes (1–3). In TCR- $\alpha^{-/-}$  mice, created by targeting TCR- $\alpha$  genes in embryonic stem cells, the population of T cells is composed of TCR- $\gamma/\delta^+$  cells and unique T cells bearing a TCR- $\beta$  chain on the cell surface in the absence of a TCR- $\alpha$  chain (4, 5). The TCR- $\gamma/\delta^+$  T cells normally develop and increase with age (4) or pathogenic infection (6). These mice can produce antibodies of all Ig subclasses (7, 8), suggesting that other cell types can drive antibody responses and isotype switching in the absence of TCR- $\alpha/\beta$  T cells (9). However, mucosal immunoregulatory mechanisms are also apparently affected by the absence

of TCR- $\alpha/\beta$  T cells since these mice spontaneously develop an ulcerative colitis (UC)<sup>1</sup>-like inflammatory bowel disease (IBD) (7, 10). Compared to other autoimmune diseases, the etiological factors of UC, an organ-specific autoimmune disease, remain unknown because of a lack of appropriate animal models (11). We have examined production of Th1- and Th2-derived cytokines in TCR- $\alpha$  mutant mice with and without disease. We show here that this IBD is associated with the production of autoantibodies of both Th1- and Th2-dependent isotypes. The hyperplastic mesenteric lymph nodes (MLN) found in diseased mice spontaneously produce increased levels of both Th1 (IFN- $\gamma$ ) and Th2 (IL-4) cytokines. IFN- $\gamma$  is produced mostly by non-T cells, whereas IL-4 production can be attributed to both TCR- $\gamma/\delta^+$  T cells and an unusual population of CD4<sup>+</sup> TCR- $\alpha^-/\beta^+$  T cells. We suggest that the resulting cytokine imbalance in the MLN of diseased TCR- $\alpha^-/-$  mice, perhaps exacerbated by a unique population of CD4<sup>+</sup> TCR- $\alpha^-/\beta^+$  cells, can promote excessive expansion of B cells. In the absence of immunoregulatory controls normally provided by TCR- $\alpha/\beta$  T cells, autoantibody-producing B cells may contribute to the development of IBD in these mice.

## Materials and Methods

**Mice.** Colonies of TCR- $\alpha^-/-$  mutant mice (4) were developed at 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<sup>b</sup>) strains, 129/Sv and C57BL/6. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TCR- $\alpha$  heterozygous mice (TCR- $\alpha^{+/-}$ ) were generated by crossing TCR- $\alpha^-/-$  mice with C57BL/6. All of the mice were housed in microisolator cages in a specific viral pathogen-free facility at Massachusetts General Hospital. Food, distilled water, and cages were autoclaved at 250°F for 25 min and changed weekly.

**Antibodies.** For flow cytometry, CD3 $\epsilon$  (145-2C11)-FITC (Boehringer Mannheim Corp., Indianapolis, IN); and B220 (RA3-6B2), CD4 (RM4-5), CD8 $\alpha$  (53-6.7), CD8 $\beta$  (53-5.8)-FITC, TCR- $\beta$  (H57-597), TCR- $\delta$  (GL3), and CD5 (53-7.3)-PE (PharMingen, San Diego, CA) were purchased. For cell sorting, bioin-NK-1.1 (PK136) was purchased from PharMingen. Antibodies for Fc-blocking, anti-Fc $\gamma$ II/III, and for *in vitro* stimulation, anti-TCR- $\beta$  (H57-597) and TCR- $\delta$  (UC7) (all from PharMingen) were used. For capture of enzyme-linked immunospot (ELISPOT) assay, anti-IL-2 (JES6-1A12), and IL-4 (BVD4-1D11) (PharMingen), IFN- $\gamma$  (RMMG-1; Biosource, Camarillo, CA); and anti-immunoglobulin (Ig; Dako Corp., Santa Barbara, CA) were used. For detection of ELISPOT, biotinylated anti-IL-2 (JES6-5H4), IL-4 (BVD6-24G2), and IFN- $\gamma$  (XMG1.2) (PharMingen); horseradish peroxidase (HRP)-anti-biotin (Vector Laboratories, Inc., Burlingame, CA), alkaline phosphatase (AP)-anti-Ig and -IgG2a and HRP-anti-IgG1 (Southern Biotechnology Associates, Birmingham, AL); and HRP-anti-IgM, -IgA, and IgG (Sera-Lab, Sussex, UK) were used.

<sup>1</sup>Abbreviations used in this paper: ELISPOT, enzyme-linked immunospot; HRP, horseradish peroxidase; IBD, inflammatory bowel disease; MLN, mesenteric lymph node; SFC, spot-forming cells; UC, ulcerative colitis.

**Histological Analysis.** For histological examination, specimens were obtained from four regions: rectum, distal and proximal colon, and cecum in every mouse tested. Specimens were fixed in 10% buffered formalin and embedded in paraffin. Multiple 4- $\mu$ m sections were stained with hematoxylin and eosin. The severity of IBD was determined according to the diagnostic basis previously described (7).

**Cell Preparation.** MLN were obtained from 20–26-wk-old TCR- $\alpha^-/-$  mice with or without IBD and age-matched TCR- $\alpha^{+/-}$  mice. After removal of fat, MLN were dispersed gently using a 25G needle, and the cell suspensions obtained were passed through a 60- $\mu$ m nylon membrane. In some experiments, adherent cells were depleted by incubating the cells in complete medium (RPMI-1640 containing sodium bicarbonate, nonessential amino acids, sodium pyruvate, L-glutamine, penicillin, streptomycin, and 5% FCS) on tissue culture plates, PRIMARIA<sup>TM</sup> (Becton Dickinson Labware, Oxnard, CA), at 37°C with 5% CO<sub>2</sub> for 90 min. After depletion of adherent cells, B and Fc receptor positive cells were depleted by panning.  $5 \times 10^7$  cells were incubated at 4°C for 30 min in a plate coated with 10  $\mu$ g/ml of goat anti-mouse Ig or 10  $\mu$ g/ml of anti-Fc $\gamma$ II/III in 0.1 M bicarbonate buffer, pH 9.4, overnight at 4°C. Nonadherent cells were collected and analyzed for purity by flow cytometry. The cell population was >93% CD3<sup>+</sup> T cells.

For purification of NK-1.1<sup>+</sup> cells, freshly isolated MLN cells were incubated with biotin-anti-NK-1.1 mAb for 30 min at 4°C followed by a 15-min incubation at 4°C with streptavidin conjugated with magnetically activated cell sorting (MACS<sup>®</sup>) superparamagnetic microbeads (Miltenyi, Auburn, CA). These complexes were separated on the VarioMACS system (Miltenyi). Both positive and negative fractions were analyzed by flow cytometry. The positive fraction typically contained ~80% NK-1.1<sup>+</sup> cells.

Colonic mucosal lymphocytes were extracted according to the method for extraction of lamina-propria lymphocytes in intestine previously described (12). Large intestine including rectum and colon but not cecum was dissected free from connective tissue. Large intestine was opened longitudinally, cut into small pieces, and incubated and rotated in Joklik's medium (Sigma Chemical Co., St. Louis, MO) containing 0.5  $\mu$ g/ml collagenase type 2 (GIBCO BRL, Gaithersburg, MD) and 1.5  $\mu$ g/ml Dispase for 45 min at 37°C. The tubes were vortexed for 10 s and cell suspensions were passed through a 70- $\mu$ m nylon membrane to remove incompletely dissociated epithelial tissue sheets. After washing with complete medium three times, the cell suspensions were passed through a glass-wool column by gravity filtration to remove debris and epithelial cells. Colonic mucosal lymphocytes were then separated using discontinuous 40/72% Percoll gradient centrifugation for 20 min at 900 g. After washing with complete medium three times, the cells were used for flow cytometric analysis.

**In Vitro Stimulation.** 96-well plates (Corning Glass Inc., Corning, NY) were coated with 20  $\mu$ g/ml anti-TCR- $\beta$  or anti-TCR- $\delta$  mAb in 100  $\mu$ l of D-PBS at 4°C overnight. After washing with PBS followed by complete medium,  $2 \times 10^5$  CD3<sup>+</sup> enriched cells (described above) in 100  $\mu$ l of complete medium were added to each well. After 3 h at 37°C, the nonadherent cells were collected and pooled, and the adherent cells were harvested by incubation with 1 mM EDTA in Ca<sup>2+</sup>, Mg<sup>2+</sup>-free HBSS followed by pipetting. After three washes with complete medium, the pooled cells were mixed.

**Single and Two-color Immunohistochemical Analysis.** Tissue specimens were embedded in OCT compound, snap frozen in liquid nitrogen, and subsequently stored at -80°C. Single staining was

performed by an avidin–biotin complex method as described previously (7).

Two-color immunohistochemical analysis was performed as previously described with the following modification (13). 4- $\mu$ m-thick specimens were fixed in acetone for 7 min, air-dried, and incubated with anti-IL-4 for 1 h at room temperature. For detection, biotinylated rabbit anti-rat Ig was used, followed by a 1:100 dilution of avidin–biotinylated peroxidase complex (Dako). Each step was followed by four washes with PBS. The specimens were developed in a solution of 3-amino-9 ethylcarbazole (AEC; Aldrich Chemical Co., Milwaukee, WI); the reaction was stopped by dipping the specimen in distilled water for 5 min and washing it in PBS for 10 min. The specimens were then incubated with anti-TCR- $\delta$  at room temperature for 1 h. For detection, biotinylated goat anti-hamster Ig was used, followed by incubation with ABC-alkaline phosphatase reagent (Vector Laboratories, Inc.) for 30 min. After development with alkaline phosphatase substrate kit III (Vector Laboratories, Inc.) for 15 min, the specimens were postfixed with 2% paraformaldehyde and mounted with Glycer-gel (Dako). Incubation with 0.3% H<sub>2</sub>O<sub>2</sub> in PBS was used to block endogenous peroxidase activity, whereas sequential incubations with avidin and biotin (Vector Laboratories, Inc.) were used to block endogenous biotin.

**Flow Cytometry.** 2–5  $\times$  10<sup>5</sup> MLN or mucosal cells were washed with flow cytometry buffer (PBS containing 0.2% BSA and 0.1% sodium azide), blocked with 50  $\mu$ l of flow cytometry buffer containing 10% normal rat and hamster serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 4°C for 20 min, and incubated with the FITC and PE mAbs listed earlier at 4°C for 40 min. After washing with flow cytometry buffer, cells were analyzed using Lysis II software on a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA). In some experiments, anti-Fc $\gamma$  II/III was used to block Fc-receptor (14).

**ELISPOT Assay for Detection of Cytokine-secreting Cells.** To quantify the frequency of IL-2, IL-4, or IFN- $\gamma$  spot-forming cell (SFC), an ELISPOT assay was performed by a modification of a previously described method (15). A 96-well plate with a nitrocellulose base (Millititer HA; Millipore Corp., Bedford, MA) was coated overnight at 4°C with 50  $\mu$ l of anti-IL-2 (10  $\mu$ g/ml), anti-IL-4 (4  $\mu$ g/ml), or anti-IFN- $\gamma$  (10  $\mu$ g/ml) mAbs diluted with 0.1 M bicarbonate buffer, pH 8.4, or D-PBS. After washing, all wells were blocked at first with 3% BSA/PBS at 37°C for 1 h, and then with complete medium at 37°C for 1 h. In some experiments, de novo synthesis of cytokines was blocked by treatment with 50  $\mu$ g/ml cycloheximide (Sigma Chemical Co.). As a positive control for IL-2-secreting cells, a  $\gamma/\delta$  T cell hybridoma stimulated with the pan  $\delta$  antibody UC7-13D5 was used. Dilutions of 10<sup>6</sup>–10<sup>4</sup> cells in 100  $\mu$ l of complete medium with or without 5  $\mu$ g/ml Con A (Sigma Chemical Co.) were added to each well and incubated at 37°C for 14 h. After sequential washes with PBS (three times), distilled water (two times), 0.25% Tween-PBS (two times), and with 0.05% Tween-PBS (three times), each well was blocked again with 3% BSA-PBS at 37°C for 1 h and incubated overnight at 4°C with 100  $\mu$ l of biotinylated mAbs, 2  $\mu$ g/ml anti-IL-2, 1.5  $\mu$ g/ml IL-4, or 1  $\mu$ g/ml IFN- $\gamma$  in PBS containing 0.05% Tween and 1% BSA. After washing with 0.05% Tween-PBS, wells were incubated with 5  $\mu$ g/ml HRP-anti-biotin at room temperature for 2 h. The spots were developed by AEC and counted under a dissecting microscope.

**ELISPOT Assay for Detection of Ig Isotype and of Autoantibody.** A two- and single-color ELISPOT assay was performed as previously described (16). A 96-well plate with a nitrocellulose base

was coated overnight at 4°C with 100  $\mu$ l of tropomyosin (5  $\mu$ g/ml) (Sigma Chemical Co.) in 0.1 M carbonate buffer, pH 9.6, (17) or with 100  $\mu$ l of goat anti-mouse Ig (5  $\mu$ g/ml) in PBS. After washing, each well was blocked with 1% BSA-PBS at 37°C for 1 h. Dilutions of 2  $\times$  10<sup>5</sup> to 5  $\times$  10<sup>2</sup> cells in 100  $\mu$ l of complete medium were added to each well and incubated at 37°C for 14 h. After washing with 0.05% Tween-PBS (five times), HRP or AP antibodies to various Ig isotypes were added to each well for single-color ELISPOT, or both HRP and AP various Ig isotype antibodies listed above were added simultaneously for a two-color ELISPOT assay. After incubation at room temperature for 2 h, each well was washed and developed with nitroblue tetrazolium and 5-bromo-4 chloro-3-indolyl phosphate (Promega Biotech, Madison, WI), followed by development with AEC.

**RT-PCR.** Total RNA was extracted from MLN cells by the acid guanidiniumthiocyanate-phenol-chloroform method (18) with the addition of a treatment with 4 M LiCl to remove degraded RNA (19). IL-2, IL-4, and IFN- $\gamma$ -specific mRNA were examined by a modified standard RT-PCR amplification procedure (20). 2  $\mu$ g total RNA was converted to cDNA in 20  $\mu$ l of reaction buffer (50 mM Tris, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 500  $\mu$ M dNTP, 50  $\mu$ g/ml oligo(dT)primer, and 200 U murine-leukemia virus reverse transcriptase; GIBCO BRL) at 43°C. After 45 min, each tube was heated at 95°C for 5 min, cooled briefly on ice, and incubated with 2 U RNase H (GIBCO BRL) at 37°C for 20 min. The reactions were then diluted five times. Samples of 3  $\mu$ l of RT reactions were amplified in 50  $\mu$ l of PCR mix (10 mM Tris, pH 8.3, 50 mM KCl, 1–2 mM MgCl<sub>2</sub>, 0.01% Triton X, 0.2 mM dNTP, and 1 U Taq DNA polymerase; Perkin Elmer Cetus, Norwalk, CT) with hot start (21); 1  $\mu$ M of each primer was added at 80°C. The primers for murine IL-2, IFN- $\gamma$ , and  $\beta$ -actin (Stratagene Inc., La Jolla, CA) and IL-4 (Clontech Laboratories, Inc., Palo Alto, CA) were used in this study. PCR cycling conditions were 36 s at 96°C, 1 min at 60°C, and 1 min at 72°C for 35 cycles after 5 min at 94°C. At the end of 35 cycles, samples were held at 72°C for 10 min and then stored at 4°C until analyzed. PCR products were electrophoresed in a 3% Nusieve agarose gel containing 0.5  $\mu$ g/ml ethidium bromide and were visualized by UV light illumination.

**ELISA Using Mouse Sera.** Ig levels in sera were measured by using 1:100 diluted mouse sera and mouse Ig isotyping ELISA kit (PharMingen), according to the manufacturer's instructions.

## Results

**Phenotypic Analysis of Mucosal Lymphoid Tissue in TCR- $\alpha^{-/-}$  Mice.** Histological examination of colon from 211 TCR- $\alpha^{-/-}$  mice confirmed our previously reported findings (7) that IBD in TCR- $\alpha^{-/-}$  mice develops by 20 wk of age. The frequency of IBD increased with age and was 60% by 20–25 wk, 83% by 25–30 wk, and 93% in mice over 30 wk of age. Therefore, 20–26-wk-old TCR- $\alpha^{-/-}$  mice were used for the analysis of changes in the lymphoid tissue during the development of IBD.

Since the inflammation noted in the colons of TCR- $\alpha^{-/-}$  mice is apparently the result of dysregulation of the mucosal immune system, we were interested in performing a detailed phenotypic and functional characterization of the gut-associated lymphoid tissue in these mice (Table 1). About 50% of the lymphocytes isolated from the colon of TCR- $\alpha^{+/+}$  control mice were TCR- $\alpha/\beta^{+}$ ; 25% of these cells

**Table 1.** Phenotype of Mesenteric Lymph Node Cells and Colonic Lymphocytes

		IBD	No. mice	Percent B220 <sup>+</sup> CD3 <sup>-</sup>	Percent TCR- $\delta^+$	Ratio TCR- $\delta^-/\beta^-$
TCR- $\alpha^{+/-}$	-	Colon	8	31.8 $\pm$ 4.4* (19 $\pm$ 7) <sup>‡</sup>	15.7 $\pm$ 2.1 (9 $\pm$ 3)	0.52 $\pm$ 0.05
		MLN	18	39.0 $\pm$ 3.4 (346 $\pm$ 35)	1.2 $\pm$ 0.2 (346 $\pm$ 35)	0.04 $\pm$ 0.01
TCR- $\alpha^{-/-}$	-	Colon	6	25.6 $\pm$ 3.5 (23 $\pm$ 9)	44.8 $\pm$ 8.5 (40 $\pm$ 13)	2.8 $\pm$ 1.5
		MLN	16	82.9 $\pm$ 1.5 (1,738 $\pm$ 382)	9.8 $\pm$ 1.9 (205 $\pm$ 45)	2.4 $\pm$ 0.4
TCR- $\alpha^{-/-}$	+	Colon	13	40.9 $\pm$ 5.5 (108 $\pm$ 48)	50.7 $\pm$ 6.6 (135 $\pm$ 60)	5.7 $\pm$ 0.9
		MLN	21	79.9 $\pm$ 1.4 (4,123 $\pm$ 477)	15.7 $\pm$ 2.3 (810 $\pm$ 93)	6.9 $\pm$ 0.9

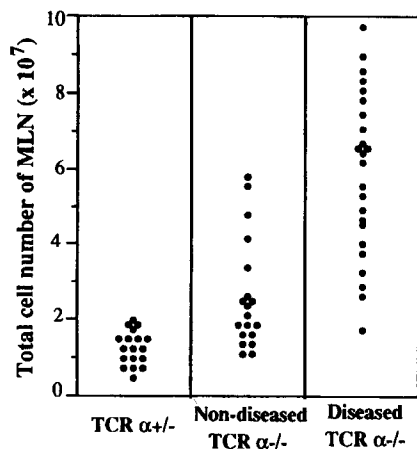
Phenotype of colonic and MLN lymphocytes in diseased and nondiseased TCR- $\alpha^{-/-}$  and TCR- $\alpha^{+/-}$  mice was determined by flow cytometric analysis. All mice were maintained under pathogen-free conditions. Mice are between 20 and 26 wk of age.

\*Data represent mean  $\pm$  SEM.

<sup>‡</sup>The numbers in parentheses indicate the absolute numbers ( $\times 10^4$ ) of positive cells in MLN.

were CD4<sup>+</sup>CD8<sup>-</sup> and the remainder were CD4<sup>-</sup>CD8 $\alpha^+$ / $\beta^+$ . TCR- $\alpha^-/\beta^+$  lymphocytes comprise a small (11–13%) population of colonic lymphocytes in TCR- $\alpha^{-/-}$  mice (the ratio of TCR- $\delta^-/\beta^-$  lymphocytes is about 5). In diseased mice most (>90%) of these TCR- $\alpha^-/\beta^+$  cells were CD4<sup>+</sup> (in nondiseased TCR- $\alpha^{-/-}$  mice, 70% TCR- $\alpha^-/\beta^+$  cells express CD4). The expanded TCR- $\gamma/\delta^+$  T cell population in the diseased TCR- $\alpha^{-/-}$  mice was predominantly (70%) CD8 $\alpha^+$ / $\beta^-$ CD4<sup>-</sup> with a smaller population of CD4<sup>-</sup>CD8<sup>-</sup> cells. We did not detect any CD4<sup>+</sup> TCR- $\gamma/\delta^+$  cells in the colons of these mice.

The MLN were markedly hyperplastic in mice with IBD and the number of cells obtained from the nodes increased dramatically with development of IBD in TCR- $\alpha^{-/-}$  mice

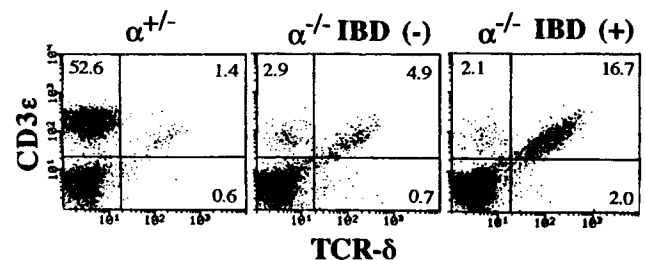


**Figure 1.** Total cell number of MLN from diseased and nondiseased TCR- $\alpha^{-/-}$  and TCR- $\alpha^{+/-}$  mice. All mice were maintained under pathogen-free conditions. Mice are between 20 and 26 wk of age. IBD was diagnosed by histological analysis.

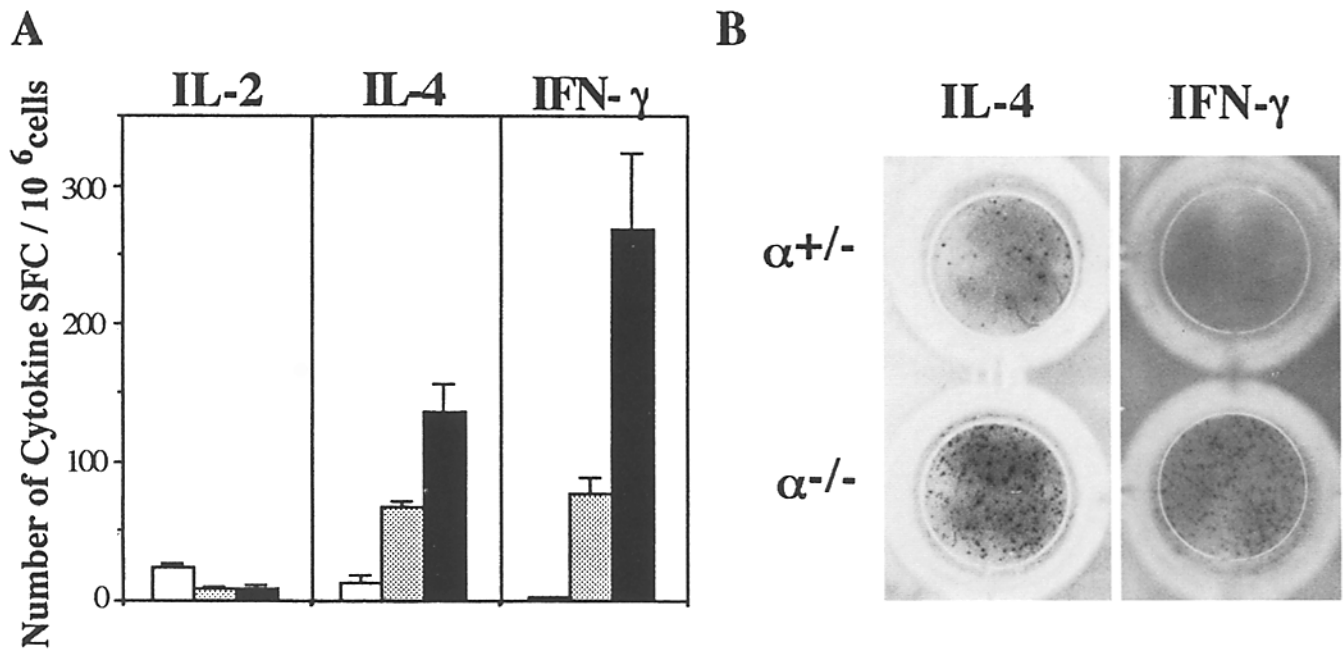
(Fig. 1). The average number of cells from TCR- $\alpha^{+/-}$  age-matched control and TCR- $\alpha^{-/-}$  mice without or with IBD was 1,083  $\pm$  90, 2,096  $\pm$  461, and 5,160  $\pm$  597  $\times 10^4$  cells, respectively.

Like the colon, TCR- $\gamma/\delta^+$  T cells were increased in the MLN of TCR- $\alpha^{-/-}$  mice both with and without IBD. It is interesting to note that there was a greater increase of TCR- $\gamma/\delta^+$  T cells in TCR- $\alpha^{-/-}$  mice with IBD compared to TCR- $\alpha^{-/-}$  mice without IBD (Fig. 2, Table 1). Furthermore, the ratio of TCR- $\gamma/\delta^+$ /TCR- $\alpha^-/\beta^+$  T cells was markedly increased in diseased TCR- $\alpha^{-/-}$  mice (6.9  $\pm$  0.9) compared to nondiseased mice (2.4  $\pm$  0.4) (Table 1). The TCR- $\gamma/\delta^+$  T cell population was 63.5  $\pm$  5.2% CD4<sup>-</sup>CD8<sup>-</sup> and 27.5  $\pm$  3.2% CD4<sup>-</sup>CD8 $\alpha^+$ . Only 2.3  $\pm$  0.2% of the MLN cells in TCR- $\alpha^{-/-}$  mice were TCR- $\alpha^-/\beta^+$ ; most (>90%) of these were CD4<sup>+</sup>CD8<sup>-</sup>.

The low numbers of lymphocytes present in the colons of diseased mice precluded their use for our initial functional studies of T cell subsets in TCR- $\alpha^{-/-}$  mice. How-



**Figure 2.** Frequency of TCR- $\gamma/\delta^+$  T cells in MLN from diseased and nondiseased TCR- $\alpha^{-/-}$  and TCR- $\alpha^{+/-}$  mice. Mice are 20 wk of age. MLN cells were incubated with FITC-anti-CD3 $\epsilon$  (145-2C11), and PE-anti-TCR- $\delta$  (GL3) and then subjected to two-color flow cytometry analysis. Percentages of cells in quadrants 1, 2, and 4 are indicated in the corners.



**Figure 3.** Comparison of the frequency of spontaneous IL-2-, IL-4-, and IFN- $\gamma$ -producing MLN cells freshly extracted from diseased TCR- $\alpha^{-/-}$  and TCR- $\alpha^{+/-}$  mice. (A) Total MLN cells were subjected to the cytokine-specific IL-2, IL-4, and IFN- $\gamma$  ELISPOT assay. Values are the mean of SFC  $\pm$  SEM from 8 separate experiments using 10 diseased TCR- $\alpha^{-/-}$  (solid bar), 4 TCR- $\alpha^{-/-}$  mice without evidence of disease (dotted bar), and 8 TCR- $\alpha^{+/-}$  mice (open bar). (B) Representative photograph of ELISPOT assay showing IL-4- and IFN- $\gamma$ -specific spot production by freshly isolated MLN cells ( $2 \times 10^5$  cells/well) from TCR- $\alpha^{+/-}$  mice and TCR- $\alpha^{-/-}$  mice with IBD.

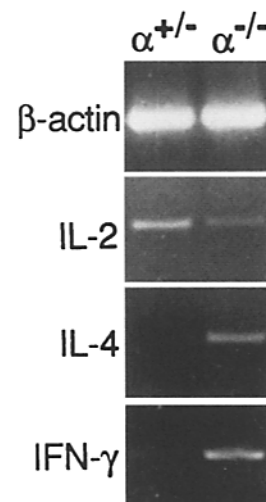
ever, as shown in Table 1, each of the phenotypic subsets of lymphocytes detected in the colon was also present in the MLN, the first site of migration for cells sensitized in the intestinal epithelium, and this tissue became the focus of our functional studies.

**Increase of Spontaneous IL-4- and IFN- $\gamma$ -, but not IL-2-secreting Cells in MLN of Diseased TCR- $\alpha^{-/-}$  Mice.** To examine the roles of TCR- $\alpha^{-/}\beta^{+}$  and TCR- $\gamma/\delta^{+}$  T cells in providing help for Ig production and promoting isotype switching in TCR- $\alpha^{-/-}$  mice, we examined their ability to produce both Th1 (IL-2, IFN- $\gamma$ ) and Th2 (IL-4) cytokines using an ELISPOT assay as described in Materials and Methods. As shown (Fig. 3, A and B), MLN cells from normal TCR- $\alpha^{+/-}$  mice secrete small amounts of IL-4. The number of spontaneous IL-4 SFC increased with the development of IBD from  $8.6 \pm 3.7$  per  $10^6$  cells in normal TCR- $\alpha^{+/-}$  mice to  $137.0 \pm 19.7$  per  $10^6$  cells in TCR- $\alpha^{-/-}$  mice with IBD, a 15-fold increase ( $p = 0.001$ ). An even more dramatic increase was observed for IFN- $\gamma$ -producing SFC that were barely detectable ( $1.2 \pm 0.7$ ) in the MLN of TCR- $\alpha^{+/-}$  mice and increased to  $261.6 \pm 53.7$  in the MLN of diseased TCR- $\alpha^{-/-}$  mice. In contrast, spontaneous IL-2-secreting cells in MLN of TCR- $\alpha^{-/-}$  mice were less compared to TCR- $\alpha^{+/-}$  mice. To confirm that our ELISPOT assay was capable of detecting IL-2-producing cells, a TCR- $\gamma/\delta^{+}$  T cell hybridoma (1E6.11.D7; 22) stimulated with plate-bound pan  $\delta$  antibody UC7-13D5, was used as a positive control. Almost all of these cells could make spots (>80%).

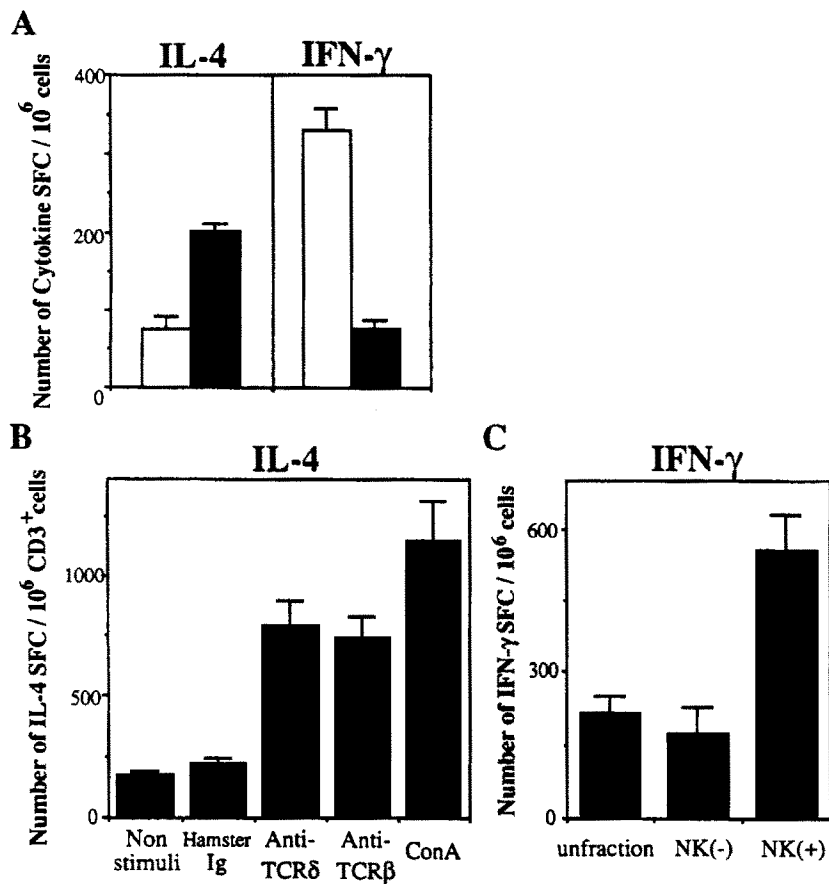
Pretreatment of MLN cells with 50  $\mu$ g/ml of cyclohex-

imide, a protein synthesis inhibitor, reduced IL-4-producing spots to 20% of their level in diseased TCR- $\alpha^{-/-}$  mice, indicating that the spots were the result of de novo protein synthesis during the 14-h assay incubation.

These observations were confirmed by examining the expression of cytokine mRNA in the MLN by RT-PCR. When mRNA extracted from unfractionated MLN cells from diseased TCR- $\alpha^{-/-}$  mice was used, high density IL-4 and IFN- $\gamma$  bands were noted. However, IL-4 and IFN- $\gamma$  mRNA extracted from unfractionated MLN of TCR- $\alpha^{+/-}$  mice was undetectable and slightly positive, respectively. In contrast with IL-4 and IFN- $\gamma$  mRNA, the expression level



**Figure 4.** IL-2-, IL-4-, and IFN- $\gamma$ -specific PCR analysis of freshly isolated total MLN cells from diseased TCR- $\alpha^{-/-}$  and normal TCR- $\alpha^{+/-}$  mice. Isolated mRNA was reverse transcribed and then amplified by IL-2, IL-4, IFN- $\gamma$  and  $\beta$ -actin 5' and 3' primers using PCR. The length of amplified cDNA by IL-2, IL-4, IFN- $\gamma$  and  $\beta$ -actin primers is 451, 399, 405, and 245 bp, respectively.

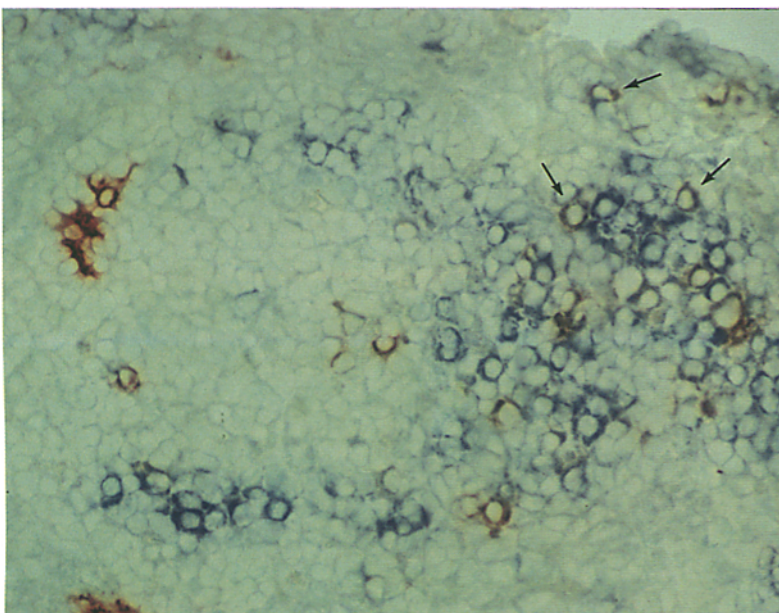


**Figure 5.** (A) Comparison of the frequency of spontaneous IL-4 and IFN- $\gamma$ -producing cells in total MLN cells and CD3<sup>+</sup> MLN cells (depleted of adherent and B cells) from diseased TCR- $\alpha^{-/-}$  mice. (Open bars) Total MLN cells (CD3<sup>+</sup>, 10–20%); (solid bars) CD3<sup>+</sup> MLN cells (CD3<sup>+</sup>, >93%). Values are the mean of SFC  $\pm$  SEM from seven diseased TCR- $\alpha^{-/-}$  mice (five separate experiments). (B) Enumeration of IL-4 SFC in anti-TCR- $\beta$  (H57-597), anti-TCR- $\delta$  (UC7), Con A (5  $\mu$ g/ml), or hamster Ig (control) in vitro-stimulated or nonstimulated CD3<sup>+</sup> cells prepared from MLN of diseased TCR- $\alpha^{-/-}$  mice (20 wk of age). Values are the mean of IL-4 SFC  $\pm$  SEM in cells pooled from five diseased TCR- $\alpha^{-/-}$  mice (three separate experiments). (C) Comparison of spontaneous IFN- $\gamma$  production in unfractionated MLN cells (total), NK-1.1-depleted fraction (NK-1.1-), and NK-1.1-enriched fraction (NK-1.1+) prepared from MLN of diseased TCR- $\alpha^{-/-}$  mice. Values are the mean of IFN- $\gamma$ -SFC  $\pm$  SEM in cells pooled from five diseased TCR- $\alpha^{-/-}$  mice. Similar results were obtained from two additional experiments.

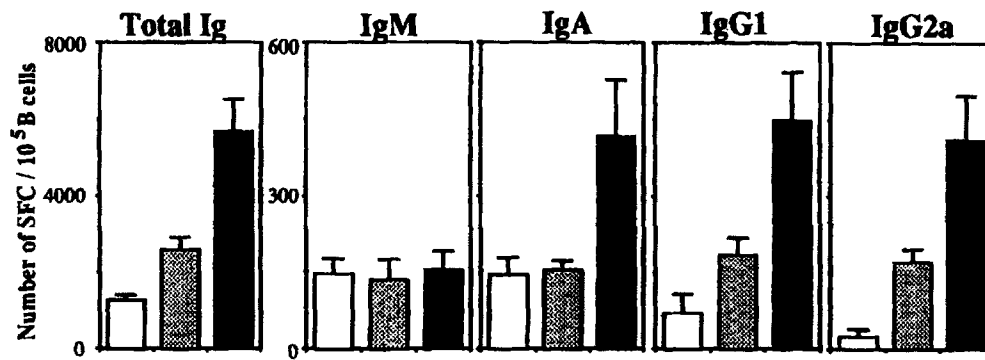
of IL-2 mRNA by TCR- $\alpha^{-/-}$  mice was reduced compared to TCR- $\alpha^{+/+}$  mice (Fig. 4).

*Spontaneous Secretion of IL-4 by Both TCR- $\alpha^{-}/\beta^{+}$  and TCR- $\gamma/\delta^{+}$  T Cells, and Predominant Secretion of IFN- $\gamma$  by Non-T Cells in TCR- $\alpha^{-/-}$  Mice.* The cytokine imbalance

revealed in the MLN of diseased TCR- $\alpha^{-/-}$  mice cannot, of course, be explained by Th1/Th2 regulation because these mice do not carry TCR- $\alpha/\beta$  T cells. Experiments were performed to investigate the cell type responsible for secreting these cytokines in MLN of TCR- $\alpha^{-/-}$  mice. Af-



**Figure 6.** Two-color immunohistochemical analysis shows TCR- $\gamma/\delta$ -positive cells (blue), IL-4-positive cells (red), and TCR- $\gamma/\delta^{+}$ , IL-4<sup>+</sup> (purple; arrow) in MLN from diseased TCR- $\alpha^{-/-}$  mice.



**Figure 7.** Comparison of frequency of total Ig, IgM, IgA, IgG1, and IgG2a secreting B220<sup>+</sup> CD3<sup>-</sup> B cells in MLN from diseased TCR- $\alpha^{-/-}$  and TCR- $\alpha^{+/-}$  mice. Values are the mean of Ig, IgM, IgA, IgG1, and IgG2a SFC  $\pm$  SEM from 17 diseased TCR- $\alpha^{-/-}$  (solid bars), 8 nondiseased TCR- $\alpha^{-/-}$  (dotted bar), and 14 TCR- $\alpha^{+/-}$  mice (open bars).

ter depletion of B cells and adherent cells (CD3<sup>+</sup> T cells >93%), the ELISPOT assay showed an increase of IL-4-secreting cells and a decrease of IFN- $\gamma$ -secreting cells as compared to unfractionated MLN cells (Fig. 5 A). Among depleted cells, distinct CD3 or TCR- $\gamma/\delta$  positive cells could not be found by immunohistochemical analysis of cytopspins from these preparations. This result suggests that IL-4 is secreted by T cells, whereas IFN- $\gamma$  appears to be predominantly secreted by non-T cells.

To investigate whether either TCR- $\alpha^{-}/\beta^{+}$  or TCR- $\gamma/\delta^{+}$  T cells or both secrete IL-4, B- and adherent cell-depleted MLN cells (CD3<sup>+</sup> T cells >93%) were stimulated for 3 h in vitro with plate-bound anti-TCR- $\beta$  (H57-597) or anti-TCR- $\delta$  (UC7-13D5) before performing the ELISPOT assay. Both anti-TCR- $\beta$  and anti-TCR- $\delta$  were able to enhance the production of IL-4 by fivefold as compared to nonstimulated cells ( $p = 0.001$ ; Fig. 5 B). This finding suggests that both TCR- $\alpha^{-}/\beta^{+}$  and TCR- $\gamma/\delta^{+}$  T cells can secrete IL-4 upon TCR triggering. Although the T cell population in diseased MLN was composed of  $\sim 80\%$  TCR- $\gamma/\delta^{+}$  and  $\leq 20\%$  TCR- $\alpha^{-}/\beta^{+}$  T cells, the degree of increase was similar for anti-TCR- $\beta$  and anti-TCR- $\gamma/\delta$  stimulation.

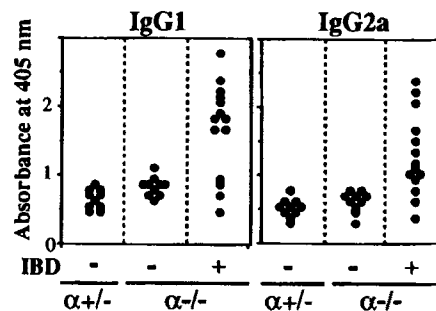
To obtain additional evidence for spontaneous secretion of IL-4 by TCR- $\alpha^{-}/\beta^{+}$  and TCR- $\gamma/\delta^{+}$  cells in situ we performed two-color immunohistochemical analysis. Staining with both anti-TCR- $\gamma/\delta$  (GL-3) and anti-IL-4 antibodies showed not only double positive cells (TCR- $\gamma/\delta^{+}$  IL-4<sup>+</sup>), but also TCR- $\gamma/\delta^{-}$  IL-4<sup>+</sup> cells in T cell areas (containing both TCR- $\alpha^{-}/\beta^{+}$  and TCR- $\gamma/\delta^{+}$  cells) of MLN in diseased TCR- $\alpha^{-/-}$  mice (Fig. 6). We were unable to directly demonstrate double positive TCR- $\alpha^{-}/\beta^{+}$  IL-4<sup>+</sup> cells using this technique because of the low expression level of the TCR- $\beta$  molecule in TCR- $\alpha^{-/-}$  mice. Nevertheless, this finding supports the result obtained using the ELISPOT assay that suggests that both TCR- $\alpha^{-}/\beta^{+}$  and TCR- $\gamma/\delta^{+}$  T cells spontaneously secrete IL-4.

To examine which population among non-T cells produce IFN- $\gamma$ , NK-1.1<sup>+</sup> cells (2–5% of MLN cells were NK-1.1<sup>+</sup> in diseased TCR- $\alpha^{-/-}$  mice) were analyzed by ELISPOT assay after magnetic sorting. The NK-1.1-enriched fraction showed increased IFN- $\gamma$  production compared to unfractionated MLN cells (Fig. 5 C), suggesting that NK-1.1<sup>+</sup> cells are a major source of IFN- $\gamma$  in diseased TCR- $\alpha^{-/-}$  mice. IFN- $\gamma$  production was also detectable in the NK-1.1-depleted fraction, indicating that cells other than NK-

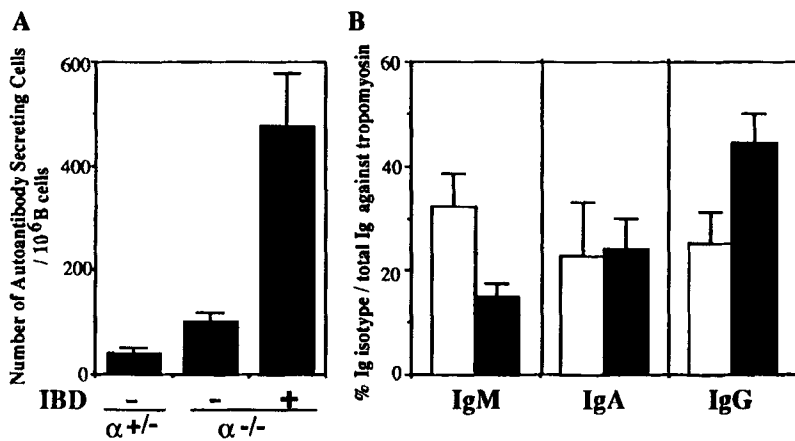
1.1<sup>+</sup> cells can also produce IFN- $\gamma$  in diseased TCR- $\alpha^{-/-}$  mice.

**Isotype of Ig Secreted by MLN B Cells.** To examine the effect of the increased secretion of IL-4 and IFN- $\gamma$  in MLN of TCR- $\alpha^{-/-}$  mice on Ig synthesis, the frequency and the Ig isotypes were quantitated by ELISPOT assay using various Ig isotype antibodies listed in Materials and Methods. We found a marked increase of B cells secreting Ig in MLN of TCR- $\alpha^{-/-}$  mice with IBD as compared to TCR- $\alpha^{-/-}$  mice without IBD and TCR- $\alpha^{+/-}$  mice ( $p = 0.001$ ). An ELISPOT assay using Ig isotype antibodies also showed that, in contrast to TCR- $\alpha^{+/-}$  mice, IgA-, IgG1-, and IgG2a-secreting B cells (B220<sup>+</sup>CD3<sup>-</sup>) were increased in MLN of TCR- $\alpha^{-/-}$  mice with IBD 3, 40, and 80 times, respectively (Fig. 7). However, the frequency of IgM-secreting B cells revealed no obvious difference between these mice. These findings indicate that B cells in MLN of TCR- $\alpha^{-/-}$  mice with IBD have been activated resulting in Ig switching from IgM to IgA, IgG1, or IgG2a. The increase in IgG1- and IgG2a-secreting B cells in the MLN of diseased mice parallels the increases in both IL-4 and IFN- $\gamma$ , suggesting that, in the absence of TCR- $\alpha/\beta$  T cells, TCR- $\gamma/\delta$  and TCR- $\alpha^{-}/\beta^{+}$  T cells (IL-4), as well as non-T cells (IFN- $\gamma$ ) can support switching to classically Th1 (IgG1)- and Th2 (Ig2a)-dependent isotypes in the MLN of diseased TCR- $\alpha^{-/-}$  mice.

We investigated whether the increase of IgG1- and IgG2a-secreting B cells in the MLN correlates with elevated serum Ig levels. ELISA showed significantly higher concentrations of IgG1 and IgG2a in the sera of diseased TCR- $\alpha^{-/-}$  mice



**Figure 8.** IgG1 and IgG2a concentrations in sera (1:100 dilution) from diseased TCR- $\alpha^{-/-}$ , nondiseased TCR- $\alpha^{-/-}$ , and TCR- $\alpha^{+/-}$  mice as determined by ELISA.



**Figure 9.** (A) Comparison of the frequency of B220<sup>+</sup> CD3<sup>-</sup> cells producing an autoantibody against tropomyosin, a colonic cytoskeletal protein, in MLN from diseased TCR- $\alpha^{-/-}$  and TCR- $\alpha^{+/-}$  mice. Values are the mean of SFC  $\pm$  SEM from 15 diseased TCR- $\alpha^{-/-}$  (solid bars), 6 nondiseased TCR- $\alpha^{-/-}$  (dotted bar), and 11 TCR- $\alpha^{+/-}$  mice (open bars). (B) Isotype of autoantibody against tropomyosin secreted by B cells in MLN from nondiseased (open bars) and diseased (solid bars) TCR- $\alpha^{-/-}$  mice (20–24 wk of age).

compared with TCR- $\alpha^{+/-}$  or TCR- $\alpha^{-/-}$  mice without disease (Fig. 8).

**Switching of Autoantibodies against Tropomyosin.** If the IBD observed in TCR- $\alpha^{-/-}$  mice is the result of uncontrolled reactivity to self-antigens (or luminal antigens cross-reactive with self-antigens), one might expect to find autoantibodies to cellular constituents in the sera of diseased mice. Antibodies to the cytoskeletal protein tropomyosin have been reported in patients with UC (17). We therefore examined autoreactivity to tropomyosin in both normal (TCR- $\alpha^{+/-}$ ) and TCR- $\alpha^{-/-}$  mice with and without IBD. An average of  $38.1 \pm 10.2$  anti-tropomyosin-secreting cells/ $10^6$  B cells (B220<sup>+</sup>CD3<sup>-</sup>) was detectable in the MLN of TCR- $\alpha^{+/-}$  mice. In MLN of diseased TCR- $\alpha^{-/-}$  mice, anti-tropomyosin-secreting B cells increased to  $478.6 \pm 104.0$ , a 15-fold increase over the number of cells in TCR- $\alpha^{+/-}$  mice (Fig. 9,  $p = 0.001$ ). Moreover, this increase correlated with the development of disease since autoantibody production was only modestly increased in TCR- $\alpha^{-/-}$  mice without histological evidence of IBD. The predominant isotype composition of the autoantibody was IgM in TCR- $\alpha^{-/-}$  mice without IBD; however, the isotype of the autoantibody was changed to IgG, especially IgG2a, in TCR- $\alpha^{-/-}$  with IBD, indicating that switching of autoantibodies had been induced (Fig. 9). Therefore, this finding also supports the notion that the cytokine profile of MLN in TCR- $\alpha^{-/-}$  mice also induces the switching of autoantibodies from IgM to IgG (predominantly IgG2a).

## Discussion

Most antibody responses to protein antigens are T cell dependent because they require help, in the form of cytokines, from CD4<sup>+</sup> T cells. Some antigens have been classified as “T-independent” in that they elicit antibody responses in athymic mice. TCR- $\alpha^{-/-}$  mice respond weakly to classical “T-dependent” antigens, such as ovalbumin but respond well to prototypical T independent antigens, such as phosphorylcholine and TNP-LPS (8). It is clear that, whereas responses to T-independent antigens do not require TCR- $\alpha/\beta$  T cells, they do require T cell-derived cytokines (23). However, many non-T cells can also secrete cytokines that may play a

role in B cell maturation and class switching (24). We have found that, although IL-2 production is greatly decreased in the absence of TCR- $\alpha/\beta$  T cells in the MLN of diseased TCR- $\alpha^{-/-}$  mice, spontaneous secretion of both IL-4 and IFN- $\gamma$  is markedly increased. Both the proportion and absolute number of  $\gamma/\delta$  T cells is increased in the MLN of TCR- $\alpha^{-/-}$  mice (Table 1). Whereas they are apparently unable to substitute for TCR- $\alpha/\beta$  T cells in a T-dependent antibody response,  $\gamma/\delta$  T cells are likely to play a role in the T-independent responses noted in TCR- $\alpha^{-/-}$  mice. The general rules for antigen recognition by TCR- $\gamma/\delta$  T cells and their role in immune responsiveness in normal mice are not clear. However, a recent report (25) suggests that infection of mice with the mucosal pathogen *Nippostrongylus brasiliensis*, known to be a potent inducer of Th2 cells, is characterized by the early appearance of IL-4-secreting TCR- $\gamma/\delta$  T cells. By contrast, IFN- $\gamma$ -secreting TCR- $\gamma/\delta$  T cells predominate in response to the Th1-stimulating pathogen *Listeria monocytogenes* (25). These results suggest that TCR- $\gamma/\delta$  T cells can function in the establishment of an immune response and that their secretion of cytokines may influence the subsequent development of Th1 and/or Th2 subsets of TCR- $\alpha/\beta$  T cells later in infection.

TCR- $\alpha^{-}/\beta^{+}$  T cells seem to play an important role in the development of IBD since TCR- $\beta^{-/-}$  mice (which lack any residual population of TCR- $\alpha^{-}/\beta^{+}$  or TCR- $\alpha^{+}/\beta^{-}$  cells) have a milder form of this disease (7). The surface expression of a TCR- $\beta$  chain in the absence of a TCR- $\alpha$  chain (TCR- $\alpha^{-}/\beta^{+}$ ) has been reported in the thymus of recombination activating gene (RAG) 1 and 2 mutant mice with TCR- $\beta$  transgene (26–28). However, the function of TCR- $\alpha^{-}/\beta^{+}$  T cells in these mice remains unclear since these cells are unstable and undetectable in peripheral organs. An unusual population of TCR- $\alpha^{-}/\beta^{+}$  cells is present in the thymus and peripheral organs of TCR- $\alpha^{-/-}$  mice (8). The TCR- $\beta$  chain on the TCR- $\alpha^{-}/\beta^{+}$  subset of cells in the thymus is found in association with a previously undescribed 33-kD protein that may play a role in early T cell development (29). Other work has shown that TCR- $\alpha^{-}/\beta^{+}$  cells derived from bronchoalveolar lavage of influenza virus-infected TCR- $\alpha^{-/-}$  mice can demonstrate functional



responses to both endogenous (Mls-1) and bacterial (Staphylococcus enterotoxin B) superantigens in the absence of a detectable response to viral antigens (30). This dichotomy may reflect differences in the way that superantigens and conventional antigens stimulate T cells. However, we show here that TCR- $\alpha^{-}/\beta^{+}$  cells are also responsive (as measured by IL-4 secretion) to TCR cross-linking with plate-bound anti-TCR- $\beta$  antibody (Fig. 5 B), a form of TCR stimulation often used as a model for conventional antigen-MHC interactions. The ternary TCR- $\alpha/\beta$ -antigen-MHC complex is involved in determining Th development (31, 32). It is possible that the lack of a TCR- $\alpha$  chain on T cells results in the skewed development of Th subsets (Th2 but not Th1) in TCR- $\alpha^{-/-}$  mice. Alternatively, by analogy with the *Nippostrongylus* model noted above, the early induction of IL-4-secreting TCR- $\gamma/\delta$  T cells may drive the smaller population of CD4<sup>+</sup> TCR- $\alpha^{-}/\beta^{+}$  cells toward a Th2 (IL-4)-secreting phenotype. In the absence of TCR- $\alpha/\beta$  T cells, autoantigen recognition may proceed unchecked and the resultant continued IL-4 secretion may function both in B cell activation and switching to drive a Th2-dependent autoantibody response (7). IL-4-activated B cells can induce IFN- $\gamma$  production by NK cells (33) which are unimpaired in TCR- $\alpha^{-/-}$  mice (8, and Fig. 5 C). IFN- $\gamma$  released by activated NK cells can induce IgG2a class switching (34). In TCR- $\alpha^{-/-}$  mice, both NK-1.1<sup>+</sup> and NK-1.1<sup>-</sup> cells can produce IFN- $\gamma$ . This induced (and unregulated) IFN- $\gamma$  production might then drive switching of a Th1-dependent autoantibody response. In support of

this scenario, we have noted that IL-4 secretion by MLN cells from TCR- $\alpha^{-/-}$  mice tends to increase earlier than IFN- $\gamma$  (our unpublished observations).

Indeed, we present evidence for autoantibody production of both Th1 (IgG2a)- and Th2 (IgG1)-dependent Ig isotypes in diseased TCR- $\alpha^{-/-}$  mice (Fig. 9). In a recent study (9), autoantibodies of IgG subclass were demonstrated in ~50% of sera from another line of TCR- $\alpha^{-/-}$  mice after 5 wk of age. The antibodies included anti-DNA, antinuclear antibody, and antibodies reactive with human cell proteins and murine small nuclear ribonucleoproteins. We chose to focus on the humoral immune response to tropomyosin because of the prevalence of antitropomyosin autoantibodies in a large percentage of patients with UC (17, 35). Cross-reactivity for tropomyosin with a bacterial protein has been demonstrated for group A streptococci (36), suggesting the interesting possibility that an autoimmune response to colonic epithelial tropomyosin is initiated by a cross-reactive antibody response elicited by antigens on the surface of bacteria (e.g., bacterial polysaccharides or LPS) in the intestinal lumen. The development of IBD in mice in which the mucosal immune response is dysregulated by a mutation in the IL-2 gene is abrogated by rearing the mice in a germ-free environment (37). If this observation is also true for TCR- $\alpha^{-/-}$  mice raised in a germ-free environment, it would provide further support for the idea that cross-reactivity to constituents of the bacterial flora play a role in driving this disease.

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