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Colitis in Mice Lacking the Common Cytokine Receptor γ Chain Is Mediated by IL-6–Producing CD4+ T Cells

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Background & Aims: Mice that have a truncated mutation of the common cytokine receptor γ chain (CRγ$^{-/Y}$) are known to spontaneously develop colitis. To identify the pathologic elements responsible for triggering this localized inflammatory disease, we elucidated and characterized aberrant T cells and their enteropathogenic cytokines in CRγ$^{-/Y}$ mice with colitis. *Methods:* The histologic appearance, cell population, T-cell receptor Vβ usage, and cytokine production of lamina propria lymphocytes were assessed. CRγ$^{-/Y}$ mice were treated with anti–interleukin (IL)-6 receptor monoclonal antibody to evaluate its ability to control colitis, and splenic CD4+ T cells from the same mouse model were adoptively transferred into SCID mice to see if they spurred the appearance of colitis. *Results:* We found marked thickening of the large intestine, an increase in crypt depth, and infiltration of the colonic lamina propria and submucosa with mononuclear cells in the euthymic CRγ$^{-/Y}$ mice, but not in the athymic CRγ$^{-/Y}$ mice, starting at the age of 8 weeks. Colonic CD4+ T cells with high expressions of antiapoptotic Bcl-x and Bcl-2 were found to use selected subsets (Vβ14) of T-cell receptor and to exclusively produce IL-6. Treatment of CRγ$^{-/Y}$ mice with anti–IL-6 receptor monoclonal antibody prevented the formation of colitis via the induction of apoptosis in IL-6–producing CD4+ T cells. Adoptive transfer of pathologic CD4+ T cells induced colitis in the recipient SCID mice. *Conclusions:* Colonic IL-6–producing thymus-derived CD4+ T cells are responsible for the development of colitis in CRγ$^{-/Y}$ mice.

The gastrointestinal immune system is continuously exposed to a harsh environment of microbial and mitogenic stimulation. To maintain immunologic homeostasis, the gastrointestinal mucosa is equipped with a regulatory T-cell network formed by an array of subsets of αβ or γδ T-cell receptor (TCR)-bearing T cells (αβ or γδ T cells). A delicate balance between Th1- and Th2-type CD4+ αβ T cells is essential for the induction and regulation of a secretory immunoglobulin (Ig) A response.[1] Interferon (IFN)-γ, a well-known Th1 cell–derived cytokine, induces secretory component (or polymeric Ig receptor) production by epithelial cells for the formation and transport of secretory IgA.[2] IgA-enhancing cytokines such as interleukin (IL)-5 and IL-6, which are produced by Th2 cells, induce IgA-committed B cells to differentiate into IgA plasma cells.[1] Further, Th3 cells and/or regulatory T cells (Tr1 cells) produce transforming growth factor (TGF)-β and IL-10, 2 suppressor cytokines that prevent inflammation and induce oral tolerance.[3] γδ T cells have also been shown to be involved in the regulation of IgA responses.[4]

Immunologic diseases of the gastrointestinal tract, such as inflammatory bowel disease (IBD), may occur when the immunologic harmony of the regulatory T-cell network is disturbed, as by alterations of intestinal environments, including cytokines and their receptor-mediated signaling cascades, and the gut microflora.[5–10] Indeed, chronic IBD-like disease development has been observed in mice that have undergone various targeted disruptions of cytokine genes,[11,12] TCR components,[13–15] or a G-protein gene.[16] Further, most of these inflamma-

Abbreviations used in this paper: CRγ, common cytokine receptor γ chain; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; IL, interleukin; IL-6R, interleukin-6 receptor; LCR, LightCycler Red 640; LP, lamina propria; mAb, monoclonal antibody; PCR, polymerase chain reaction; PE, phycoerythrin; SP, spleen; TCR, T-cell receptor; TGF, transforming growth factor; TNF, tumor necrosis factor.

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tory diseases do not occur in the absence of gastrointestinal flora.[17–19]

The murine common cytokine receptor γ chain (CRγ) is a 64-kilodalton type-1 transmembrane protein of the cytokine receptor family.[20,21] CRγ alone is unable to bind to cytokines, but the γ chain is an essential component of the cell-surface receptor complexes of IL-2, IL-4, IL-7, IL-9, and IL-15.[20,22] Disruption of the CRγ has been shown to result in an absence or severe reduction in numbers of natural killer cells, decreased numbers of T cells (including thymus-independent T cells) and B cells, marked hypoplasia of the thymus and peripheral lymphoid tissues, defective formation of lymphoid follicles,[22,23] and marked splenomegaly and mesenteric lymphadenopathy.[24,25] Disruption of CRγ is also known to lead the gut-associated tissue and mucosal γδ T cells deficient in key elements, thereby disturbing the mucosal immune system,[22] and $CR\gamma^{-/Y}$ mice spontaneously develop chronic large intestinal inflammation. Thus, we decided to examine the possible pathologic elements responsible for the development of intestinal inflammation and the cellular and molecular aspects of the pathology seen in $CR\gamma^{-/Y}$ mice, focusing on aberrant T cells and their enteropathogenic cytokines.

Materials and Methods

Mice

Euthymic (nu/+) mutant $CR\gamma^{-/Y}$, euthymic (nu/+) wild-type $CR\gamma^{+/Y}$, and athymic (nu/nu) mutant $CR\gamma^{-/Y}$ mice (Japan Clea, Tokyo, Japan) were used for the generation of $CR\gamma^{-/Y}$ mutants with a BALB/c background. The original $CR\gamma^{-/x}$ mice with B6 background[26] were crossed with athymic BALB/c mice, and their heterozygous $CR\gamma^{-/x}$ progeny were backcrossed to athymic BALB/c mice to obtain euthymic (nu/+) wild-type $CR\gamma^{+/Y}$, euthymic (nu/+) mutant $CR\gamma^{-/Y}$, and athymic (nu/nu) mutant $CR\gamma^{-/Y}$ littermates. Male $CR\gamma^{-/Y}$ offspring were typed by polymerase chain reaction (PCR) analysis of tail DNA with a set of primers to the neomycin-resistant gene described elsewhere.[27] All mice used for experiments were between 6 and 20 weeks of age, and the absence of the thymus was checked at necropsy. $CR\gamma^{-/Y}$ mice on a BALB/c background were obtained from the B6 mice backcrossed more than 20 times to athymic BALB/c nude mice. The mice were housed in the Experimental Animal Facility at the Research Institute for Microbial Diseases at Osaka University. All mice were kept on a 12-hour light/dark cycle and received sterilized food and autoclaved distilled water ad libitum.

Isolation of Lymphoid Cells From Mucosa-Associated Tissues and Spleen

Spleen (SP) and mesenteric lymph nodes were aseptically removed, and single-cell suspensions were prepared by a standard mechanical disruption procedure.[4,14,15] Single-cell suspensions of Peyer's patches and lamina propria (LP) lymphocytes were prepared by an enzymatic dissociation method, using collagenase as previously described.[4,14,15] The viability of the Peyer's patches, mesenteric lymph nodes, and SP cells was 98% and that of the LP lymphocytes was 95%.

Culture Conditions for the Analysis of Cytokine Production

LP and SP T cells from $CR\gamma^{-/Y}$ and control mice were resuspended and cultured in complete medium consisting of RPMI 1640 supplemented with 3 mmol/L L-glutamine, 10 mmol/L HEPES buffer, 10 μg/mL gentamicin, 100 U/mL penicillin and 100 μg/mL streptomycin, 0.05 mmol/L 2-mercaptoethanol, and 10% fetal calf serum (Hyclone Co, Salt Lake City, UT) for the assessment of Th1 and Th2 cytokine synthesis.[4,14,15] For measurement of TGF-β production, serum-free media supplemented with 1% Nutridoma-SP (Boehringer Mannheim Biochemicals, Indianapolis, IN) were used.[28] For the analysis of spontaneous cytokine production, purified LP or SP T cells (1×10^6 cells/mL) were added to culture wells (24-well Costar plates) without exogenous stimulation and cultured for 48 hours (Th1 and Th2 cytokine) and 60 hours (TGF-β).[28] The culture supernatants were then harvested and assayed by cytokine-specific enzyme-linked immunosorbent assay (ELISA) by using the Biotrak IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-12, tumor necrosis factor (TNF)-α ELISA system (Amersham Pharmacia Biotech, Aylesbury, England) and Predica TGF-β ELISA system (Genzyme Corp, Cambridge, MA) according to the manufacturer's protocol. Detection levels of these cytokines were 37–3000 pg/mL for IFN-γ, 34–850 pg/mL for IL-2, 15–375 pg/mL for IL-4, 20–320 pg/mL for IL-5, 50–2000 pg/mL for IL-6, 47–3000 pg/mL for IL-12, 50–2450 pg/mL for TNF-α, and 31.2–2000 pg/mL for TGF-β.

Flow Cytometric Analysis and Cell Sorting

Immunofluorescent analysis was performed using FACScan flow cytometry (Becton Dickinson, Mountain View, CA). Cells stained with single-color reagent were used to set the appropriate compensation levels, and at least 10,000 events were analyzed. Cell sorting was performed on a FACStar (Becton Dickinson). The following monoclonal antibodies (mAbs) from BD PharMingen (San Diego, CA) were used: anti-CD4 (clone RM4-5), anti-CD8 (53-6.7), anti-CD3ε (145-2C11), anti-CD45R/B220 (RA3-6B2), anti-CD11b (M1/70), anti-TCRβ (H57-597), anti-TCRδ (GL3), anti-TCR Vβ2 (B20.6), anti-TCR Vβ3 (KJ25), anti-TCR Vβ4 (KT4), anti-TCR Vβ5.1/5.2 (MR9-4), anti-TCR Vβ6 (RR4-7), anti-TCR Vβ7 (TR310), anti-TCR Vβ8.1/8.2 (MR5-2), anti-TCR Vβ8.3 (1B3.3), anti-TCR Vβ9 (MR10-2), anti-TCR $V\beta10^b$ (B21.5), anti-TCR Vβ11 (RR3-15), anti-TCR Vβ12 (MR11-1), anti-TCR Vβ13 (MR12-3), and anti-TCR Vβ14 (14-2).

For 2-color flow cytometry, 1×10^6 cells in 20 μL phosphate-buffered saline (PBS) containing 2% fetal calf serum and 0.02% sodium azide were first incubated with anti-Fc receptor

mAb (BD PharMingen) to prevent nonspecific staining and then stained with the appropriate fluorescein isothiocyanate (FITC)-conjugated mAb, phycoerythrin (PE)-conjugated mAb, and/or biotinylated mAb followed by streptavidin-phycoerythrin (BD PharMingen). All mAbs were used at the saturating concentrations recommended by the manufacturer.

Staining of intracellular cytokines was performed in accordance with a modified version of the manufacturer's protocol for Fix & Perm Cell Permeabilization Kits (Caltag Laboratories, Vienna, Austria) using the following monoclonal antibodies: FITC/anti-CD11b, FITC/anti-CD4, PE/anti–IL-6, and PE/anti–IFN-γ (BD PharMingen).[29] Negative control samples were stained with irrelevant, isotype-matched PE-conjugated rat IgG1 antibody.

Quantitative Reverse-Transcription PCR

A highly sensitive, quantitative RT-PCR was performed to analyze the IL-6 receptor (IL-6R)– and the anti-apoptotic gene (Bcl-x and Bcl-2)-specific mRNA expressions by $CD4^+$ T cells isolated from the colonic LP of diseased $CR\gamma^{-/Y}$ mice.[30] Total RNA was extracted from fluorescence-activated cell sorter (FACS)-purified $CD4^+$ T cells by using TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA was reverse transcribed into complementary DNA (cDNA) using Superscript II reverse transcriptase (Invitrogen), ribonuclease inhibitor (Toyobo, Tokyo, Japan), oligo(dT)12–18 primer (Invitrogen), and deoxyribonucleoside IL-6R mAb (kindly provided by Chugai Pharmaceuticals Co, Ltd, Tokyo, Japan) triphosphates (Amersham Pharmacia Biotech, Arlington Heights, IL). The mixture was incubated at 42°C for 120 minutes and heated to 90°C for 5 minutes. After treatment with ribonuclease H (Toyobo), the synthesized cDNA was extracted by phenol/chloroform. Then, the IL-6R– and the antiapoptotic gene (Bcl-x and Bcl-2)-specific cDNA were quantified using LightCycler-DNA Master Hybridization Probes (Roche Diagnosis, Mannheim, Germany). For the amplification of cDNA, 20 μL of the PCR mix was added to each tube to give a final concentration of 0.05 μmol/L 5′ primer, 0.05 μmol/L 3′ primer, 0.2 μmol/L FITC-labeled probe, 0.2 μmol/L LightCycler Red 640 (LCR)-labeled probe, 2 mmol/L $MgCl_2$, and 1 × LightCycler-DNA Master Hybridization Probes mix (Roche Diagnosis). We used the oligo primers specific for IL-6R (sense, 5′-AAGAGTGACTTCCAGGTGCC-3′; antisense, 5′-GGTATCGGAAGCTGGAACTGC-3′), Bcl-x (sense, 5′-TGGTCGACTTTCTCTCCTAC-3′; antisense, 5′-GAGATCCACAAAAGTGTCCC-3′), Bcl-2 (sense, 5′-TGCAC-CTGACGCCCTTCAC-3′; antisense, 5′-TAGCTGATTCGAC-CATT TGCCTGA-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense, 5′-TTCACCACCATG-GAGAAGGC-3′; antisense, 5′-GGCATGGACTGTGGT-CATGA-3′). To detect the target molecule, we then followed the manufacturer's protocol to prepare an FITC-labeled hybrid probe and an LCR-labeled hybrid probe to IL-6R (FITC, 5′-TGATACCACAAGGTTGGCAGGTGG-3′; LCR, 5′-TCCGGCTGCACCATTTTTAAGCTG-3′), Bcl-x (FITC, 5′-CTCTTTCGGGATGGAGTAAACTGGGG-3′; LCR, 5′-CGCATCGTGGCCTTTTTCTCCTT-3′), Bcl-2 (FITC, 5′-CCCTGTTGACGCTCTCCACACACA-3′; LCR, 5′-GACCCCACCGAACTCAAAGAAGGC-3′), and GAPDH (FITC, 5′-TGGG TGTGAACCACCAGAAATATGAC-3′; LCR, 5′-ACTCACTCAAGATTGTCAGCAATGCA-3′). After heating at 94°C for 2 minutes, cDNA were amplified for 40 cycles, with each cycle consisting of 95°C for 10 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Once during the cycle the log-linear signal could be distinguished from the background, it was then possible to compare the target concentrations (external standard) in samples with an internal standard in the same samples. After the PCR had been completed, the LightCycler software (Roche Diagnosis) automatically converted the raw data into copies of target molecules. In this study, the relative quantitative expression of IL-6R–, Bcl-x–, or Bcl-2–specific mRNA in each sample was expressed as the quantity of the respective mRNA divided by the quantity of mRNA GAPDH.[30]

Treatment of Mice With Anti–IL-6R mAb

Rat IgG anti-mouse IL-6R mAb[31] or isotype-matched rat IgG (BD PharMingen) at a dose of 8 mg per mouse was injected intraperitoneally into euthymic (nu/+) mutant $CR\gamma^{-/Y}$ mice at the age of 6 weeks. The weekly mAb treatment was continued for 4 weeks. Rat anti-mouse IL-6R mAb was prepared from the MR16-1 hybridoma cell line according to the protocol previously described elsewhere.[31]

Analysis of Apoptosis Following Anti–IL-6R mAb Treatment

To examine the effect of anti–IL-6R mAb for the induction of apoptosis in IL-6–producing $CD4^+$ T cells from $CR\gamma^{-/Y}$ mice, a previously established in vitro apoptosis analysis protocol was used.[32] Thus, SP and colonic LP $CD4^+$ T cells were isolated from $CR\gamma^{-/Y}$ mice and then cocultured with anti–IL-6R mAb (1 mg/mL, MR16-1) or isotype control IgG (BD PharMingen) for 6 hours. $CD4^+$ T cells were harvested for FACS analysis using the Annexin V FITC Apoptosis Detection Kit I (BD PharMingen).[32]

Adoptive Transfer Experiment

SP cells were aseptically removed from euthymic (nu/+) mutant $CR\gamma^{-/Y}$ donor mice with colitis. Erythrocytes were removed by hypotonic lysis. To avoid stimulating T cells during the purification process, a negative selection procedure was used for the preparation of $CD4^+$ T cells. Initially, mononuclear cells were resuspended in complete medium and then incubated in culture plates (Millipore, Bedford, MA) for 2 hours at 37°C to remove adherent cells, including macrophages and fibroblasts.[33] The nonadherent cell suspension was then incubated in wells precoated with $F(ab')_2$ fragments of goat anti-mouse IgG (Jackson, West Grove, PA) at 4°C for 90 minutes.[33] To obtain the T-cell–enriched fraction, wells were washed gently 3 times with PBS containing 5% fetal calf serum. The T-cell–enriched fraction was then subjected to magnetic-activated cell sorting (Miltenyi Biotec, Gladbach, Germany) with anti-CD4–coated beads (Miltenyi Biotec) for

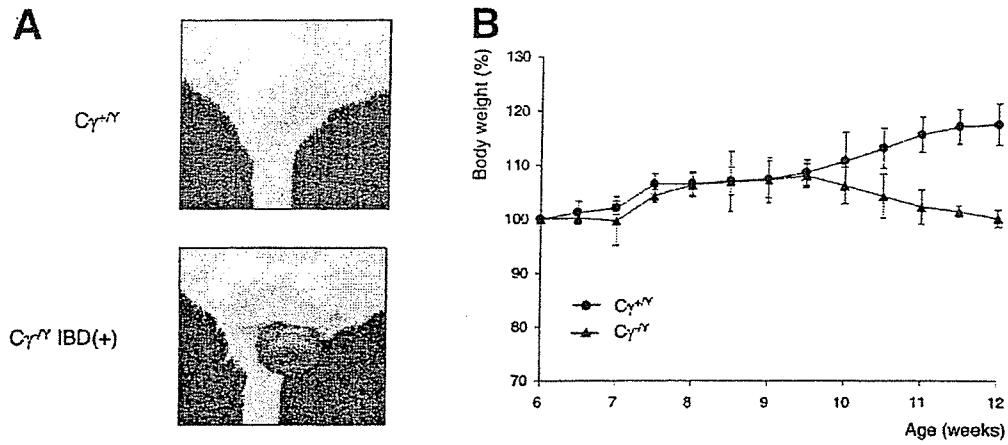


Figure 1. Gross appearance of the (*A*) anorectal prolapse and (*B*) body weight changes in CRγ$^{+/Y}$ mice, CRγ$^{-/Y}$ mice without IBD, and CRγ$^{-/Y}$mice with IBD. CRγ$^{-/Y}$ mice with IBD showed anal prolapse beginning at 8 weeks of age and obvious lack of body weight gain from 10 weeks of age. The data represent the values (mean ± SD) from 3 different experiments (4 mice per group).

preparation of CD4$^+$ T cells.[34] The purified donor CD4$^+$ T cells (3×10^6) were resuspended in sterile PBS and injected intraperitoneally (1 mL) into C.B-17 SCID mice (Clea Japan). Five weeks after the adoptive transfer, mice were examined for the presence of disease.

Histologic Analysis

Small and large intestines obtained from CRγ$^{-/Y}$ and control mice at predetermined time points were fixed in 4% paraformaldehyde in PBS for 4 hours and embedded in paraffin for the preparation of 5-μm tissue sections. The sections were stained with H&E for the assessment of disease and clinical score. Periodic acid–Schiff/alcian blue staining was performed for the identification of goblet cells.

Assessment of Disease Score

Histopathologic alterations in the colon were semiquantified according to a modified scoring system[35] using the following criteria: (1) cellular infiltration into the lamina propria of the large intestine (score from 0 to 3), (2) mucin depletion (score from 0 to 2), (3) crypt abscesses (score from 0 to 2), (4) epithelial erosion (score from 0 to 2), (5) hyperemia (score from 0 to 3), and (6) thickness of the

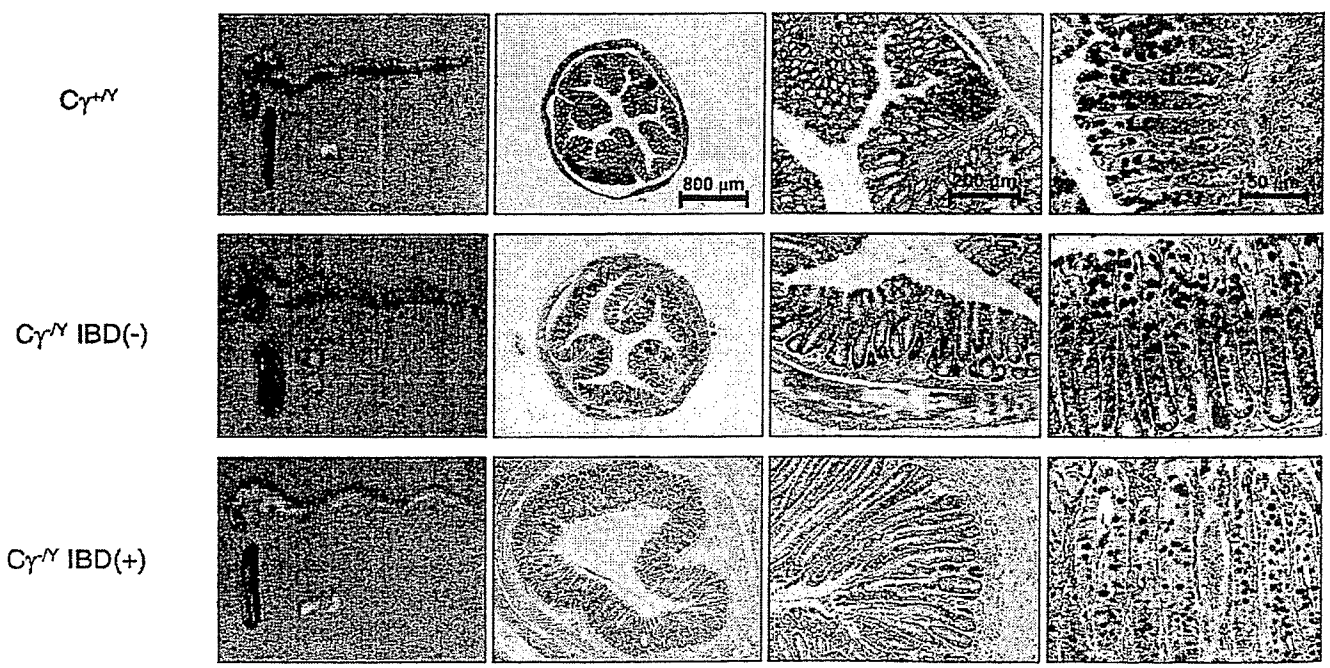


Figure 2. Macroscopic and microscopic appearance of the large intestine in CRγ$^{+/Y}$ mice, CRγ$^{-/Y}$ mice without IBD, and CRγ$^{-/Y}$ mice with IBD. The large intestine of the mice was dissected for routine histologic analysis, including fixing with 4% paraformaldehyde, embedding in paraffin, and staining with H&E. Histopathologic alteration in the colons was assessed by use of a modified clinical analysis system.

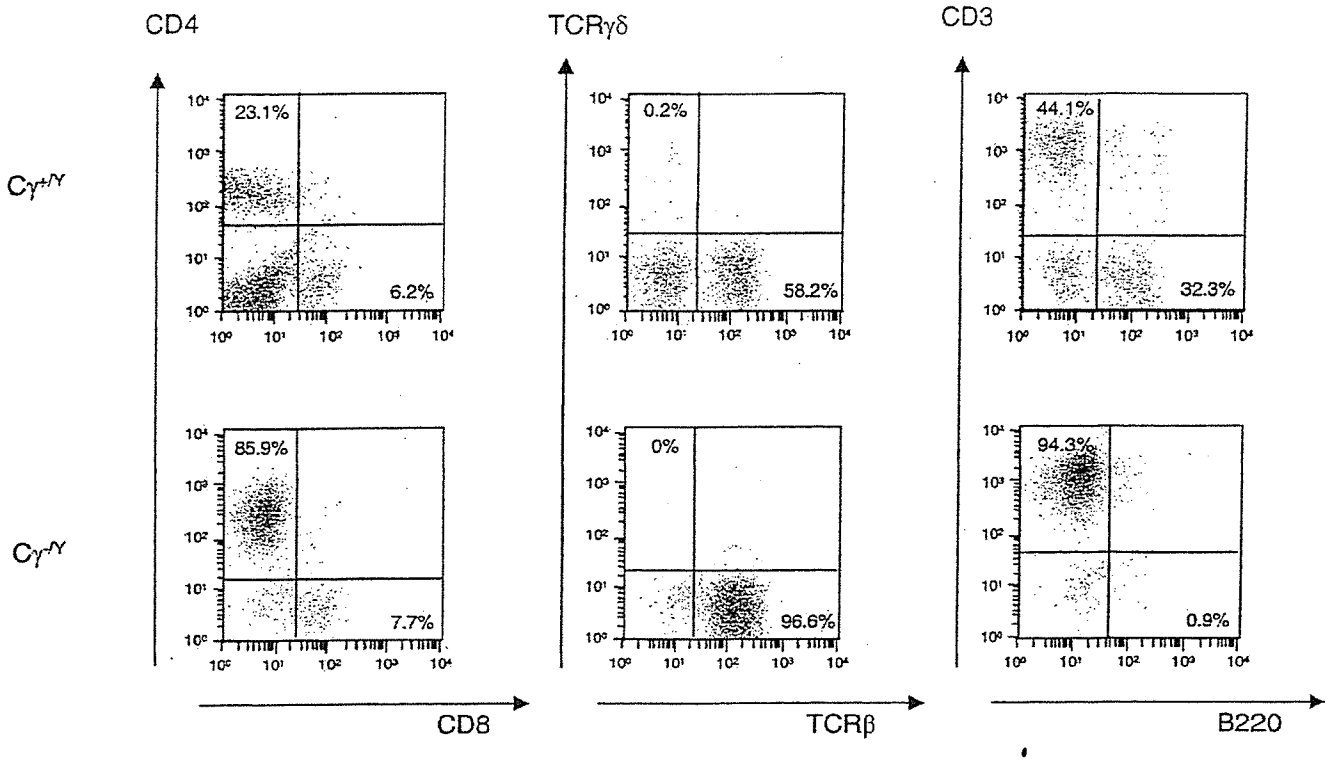


Figure 3. Flow cytometric analysis of colonic LP lymphocytes isolated from CRγ$^{-/Y}$ and CRγ$^{+/Y}$ mice. CRγ$^{-/Y}$ mice did not possess mature B cells or TCR γδ T cells but had increased numbers of CD4$^+$ T cells. The data are representative of results from 2 independent experiments.

colonic mucosa (score from 1 to 3). Hence, the range of histopathologic scores was from 1 (no alteration) to 15 (most severe colitis).

Statistical Analysis

Significant differences between mean values were determined by use of the Student *t* test. *P* values of .05 were considered statistically significant.

Results

Histologic Appearance of Colonic Inflammation in Euthymic (nu/+) Mutant CRγ$^{-/Y}$ Mice

At 8 weeks of age, all of the euthymic (nu/+) mutant CRγ$^{-/Y}$ mice developed signs of IBD, characterized by anorectal prolapse (Figure 1*A*), lack of weight gain (Figure 1*B*), and a hunched posture. Necropsy of the diseased euthymic (nu/+) mutant CRγ$^{-/Y}$ mice revealed inflammation of the large intestine and rectum and a more marked hyperplasia, dilatation, and thickening of the wall than seen in control euthymic (nu/+) wild-type CRγ$^{+/Y}$ mice and euthymic (nu/+) mutant CRγ$^{-/Y}$ mice without IBD (Figure 2). In addition, microscopic examination of the diseased euthymic (nu/+) mutant CRγ$^{-/Y}$ mice revealed elongation, hyperemia, crypt distortion, goblet cell reduction, and lymphocyte infiltration into the colonic lamina propria (Figure 2).

Cell Population of Euthymic (nu/+) Mutant CRγ$^{-/Y}$ Mice

Lack of functional CRγ has been reported to affect lymphocyte development.[22–26] In both young adult and aged CRγ$^{-/Y}$ mice, we noted the absence of natural killer cells (data not shown) and γδ T cells and a great reduction in B cells (Figure 3). Among lymphocytes isolated from colonic LP of 8-week-old CRγ$^{-/Y}$ mice, we found that increased numbers of CD4$^+$ T cells but not of CD8$^+$ T cells (Figure 3) were always associated with colitis.

TCR Vβ Repertoire Use of Colonic LP CD4$^+$ T Cells by Euthymic (nu/+) Mutant CRγ$^{-/Y}$ Mice With Colitis

Because the increase of LP CD4$^+$ T cells was associated with development of colitis, we next analyzed the qualitative alterations in the CD4$^+$ T cells. Flow cytometric analysis of TCR Vβ repertoire use in colonic LP CD4$^+$ T cells isolated from euthymic (nu/+) wild-type CRγ$^{+/Y}$ and euthymic (nu/+) mutant CRγ$^{-/Y}$ mice without IBD showed that the major TCR Vβ repertoire use was TCR Vβ8, followed by Vβ4, Vβ6, and Vβ14 (Figure 4). In contrast, CD4$^+$ T cells isolated from

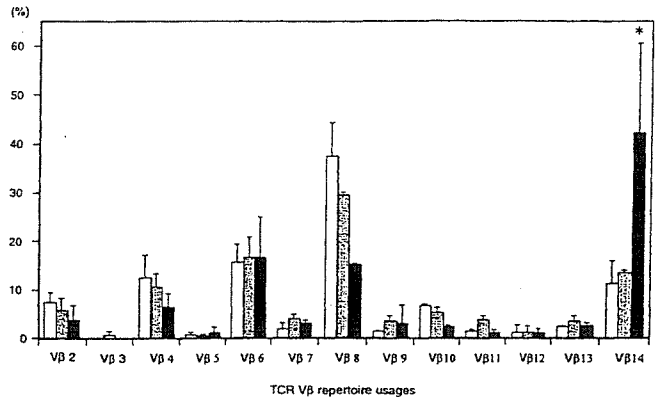


Figure 4. Flow cytometric analysis of the TCR Vβ repertoire of CD4+ T cells isolated from colonic LP of the CRγ$^{-/Y}$ and CRγ$^{+/Y}$ mice. Mononuclear cells isolated from CRγ$^{-/Y}$ mice with or without colitis and control CRγ$^{+/Y}$ mice were costained with mAbs specific for FITC/TCR Vβ and PE/CD4. The percentage of T cells bearing each TCR Vβ was calculated as 100 × (% of CD4+, Vβx+ cells)/(% of CD4+ cells). The percentages of T cells are expressed as the mean values from 3 different mice. *White, dotted,* and *black bars* represent CRγ$^{+/Y}$ mice, CRγ$^{-/Y}$ mice without IBD, and CRγ$^{-/Y}$ mice with IBD, respectively. Statistical comparisons were determined by Student *t* test (*$P < .05$).

euthymic (nu/+) mutant CRγ$^{-/Y}$ mice with IBD had a predominance of TCR Vβ14 (Figure 4). Further, under immunoscope analysis of CDR3 length to investigate the clonality of these Vβ14+ T cells, Gaussian distribution of clonotype was observed, suggesting the polyclonality of colonic CD4+ T cells in the diseased mice (data not shown). Thus, local augmentation of CD4+ T cell populations expressing TCR Vβ14 was associated with the development of colitis in the CRγ$^{-/Y}$ mice.

Characterization of IL-6–Producing LP CD4+ T Cells Isolated From Euthymic (nu/+) Mutant CRγ$^{-/Y}$ Mice With Colitis

We next examined the ability of the CD4+ T cells in colonic LP of euthymic (nu/+) mutant CRγ$^{-/Y}$ mice with IBD to produce Th1- or Th2-type cytokines. LP lymphocytes were isolated from young, anal prolapse–free (6 weeks of age) and diseased (8–12 weeks) mice. The cells were cultured in complete medium without exogenous stimulation to determine their spontaneous cytokine production. Minimum amounts of IL-6 (376 ± 3 pg per 10^6 cells) were detected in the culture supernatants harvested from the wells containing colonic LP lymphocytes of young healthy CRγ$^{-/Y}$ mice. The level of spontaneous IL-6 production was increased in the various stages of colitis from 375.9 ± 3.3 pg per 10^6 cells in the anal prolapse–free mice to 2655.1 ± 410.4 pg per 10^6 cells in the diseased mice (Figure 5). When cytokine production was examined, these colonic CD4+ T cells did not produce IL-4, IL-5, IL-12, and TNF-α. Although IFN-γ was noted in the culture containing colonic CD4+

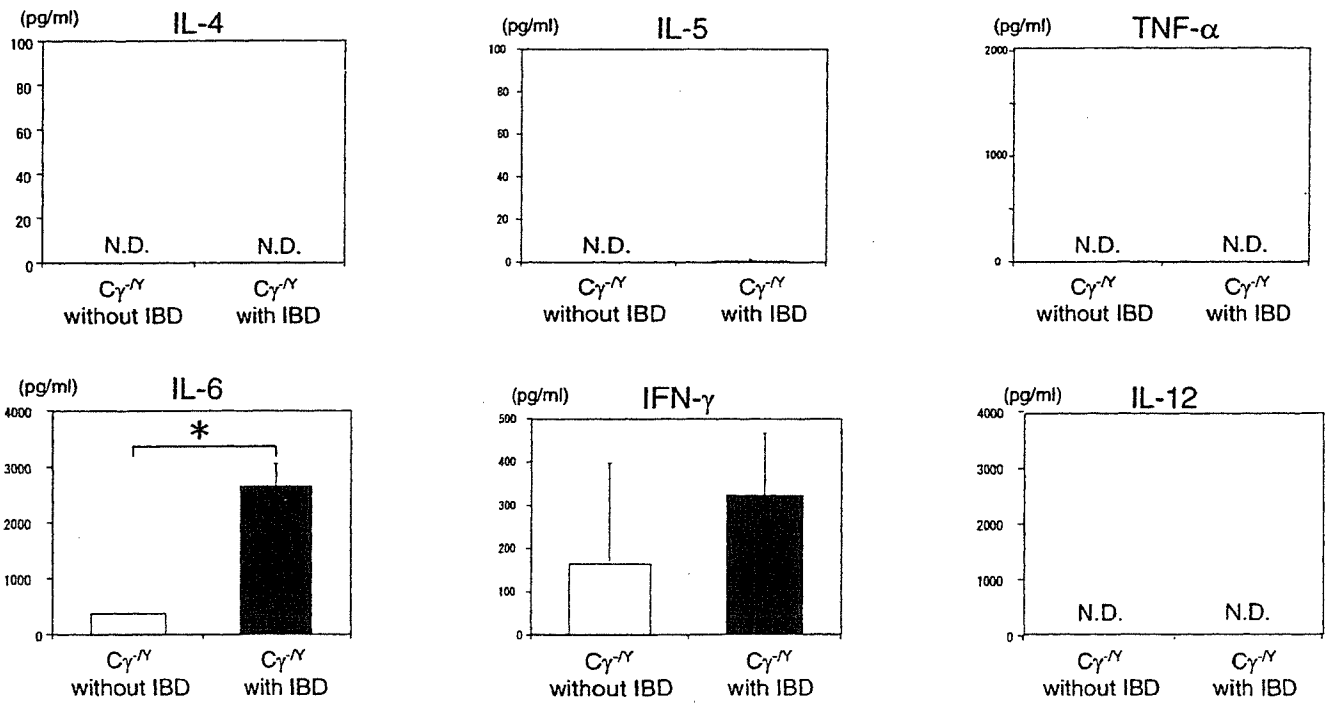


Figure 5. Analysis of cytokine production expression by colonic CD4+ T cells isolated from CRγ$^{-/Y}$ mice with (*closed squares*) and without (*open squares*) IBD. Whole colonic LP mononuclear cells were incubated in complete medium with 10% fetal calf serum for 48 hours without any exogenous stimulation. The culture supernatants were harvested for cytokine-specific ELISA. The data represent the mean ± SD from 3 different experiments. Statistical comparisons were determined by Student *t* test (*$P < .05$).

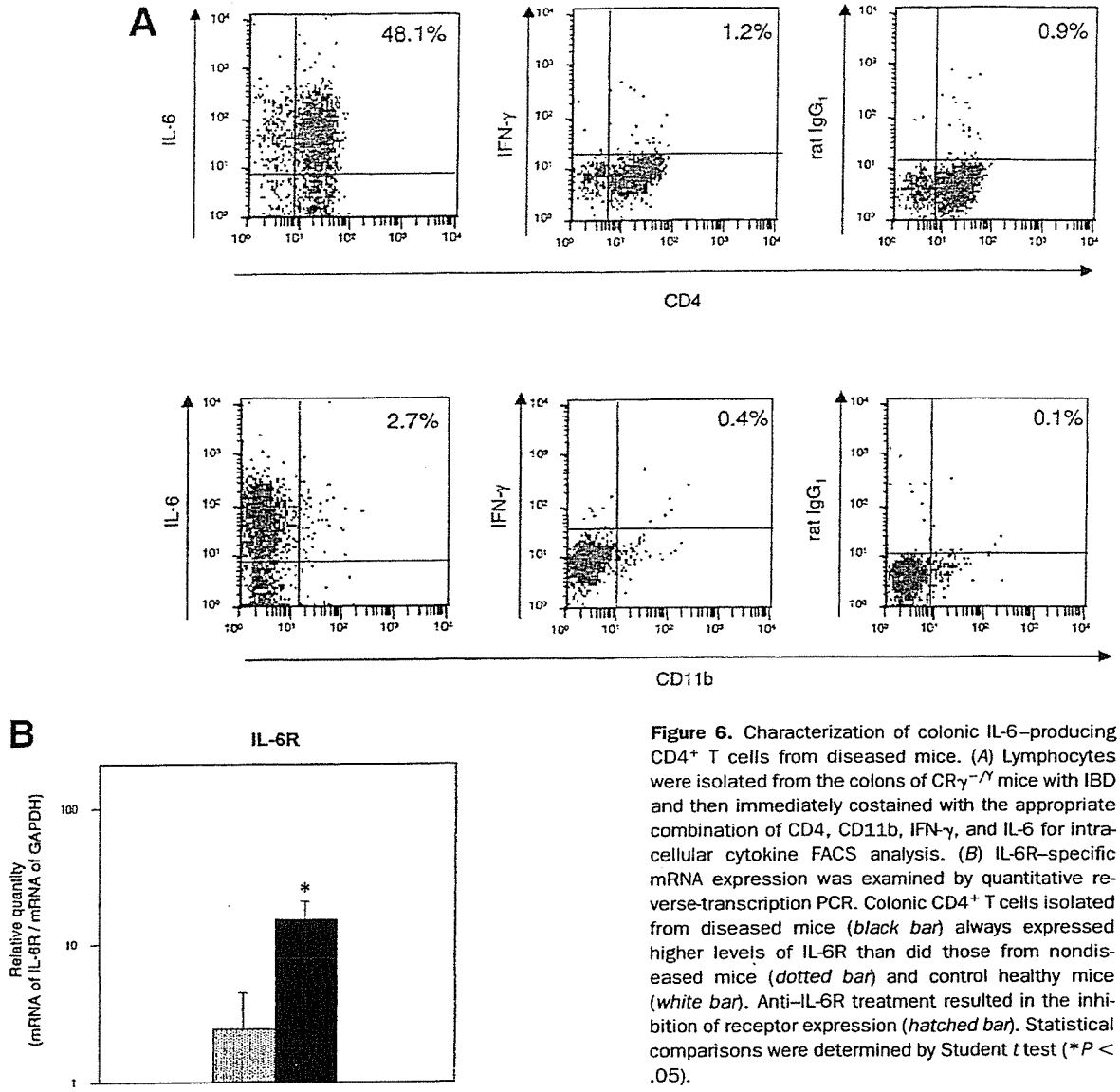


Figure 6. Characterization of colonic IL-6–producing CD4$^+$ T cells from diseased mice. (*A*) Lymphocytes were isolated from the colons of CR$\gamma^{-/Y}$ mice with IBD and then immediately costained with the appropriate combination of CD4, CD11b, IFN-γ, and IL-6 for intracellular cytokine FACS analysis. (*B*) IL-6R–specific mRNA expression was examined by quantitative reverse-transcription PCR. Colonic CD4$^+$ T cells isolated from diseased mice (*black bar*) always expressed higher levels of IL-6R than did those from nondiseased mice (*dotted bar*) and control healthy mice (*white bar*). Anti–IL-6R treatment resulted in the inhibition of receptor expression (*hatched bar*). Statistical comparisons were determined by Student *t* test (*$P <$ .05).

T cells, the level of production was not statistically different from CD4$^+$ T cells isolated from nondiseased mice.

To directly show IL-6 production by colonic LP CD4$^+$ T cells from euthymic (nu/+) mutant CR$\gamma^{-/Y}$ mice with colitis, we next performed intracellular cytokine FACS analysis of such cells. The results obtained by double staining with appropriate fluorescence-conjugated mAb anti-CD4, anti-CD11b, and anti–IL-6 showed that IL-6 was mainly produced by CD4$^+$ T cells but not by CD11b-positive cells (Figure 6*A*). Once we knew that these colonic CD4$^+$ T cells preferentially produce IL-6, we next sought to examine whether these T cells express IL-6R. When IL-6R–specific mRNA expression was examined by quantitative reverse-transcription PCR, colonic CD4$^+$ T cells isolated from the diseased mice always expressed higher levels of the IL-6R message than did those from nondiseased mice (Figure 6*B*). These findings suggest that the increase in pathogenic CD4$^+$ T cells in the large intestine of diseased mice may result from the creation of autocrine-induced antiapoptotic conditions by IL-6 and IL-6R.

Efficacy of In Vivo Treatment With Anti–IL-6R mAb

We next conducted further experiments to confirm our findings that CD4$^+$ Th-cell–derived IL-6 was implicated in the development of colitis. Thus, young adult mice without clinical signs of the disease were treated with anti–IL-6R mAb, mock antibody, or PBS. Like untreated mice, mice treated with the mock antibody or PBS devel-

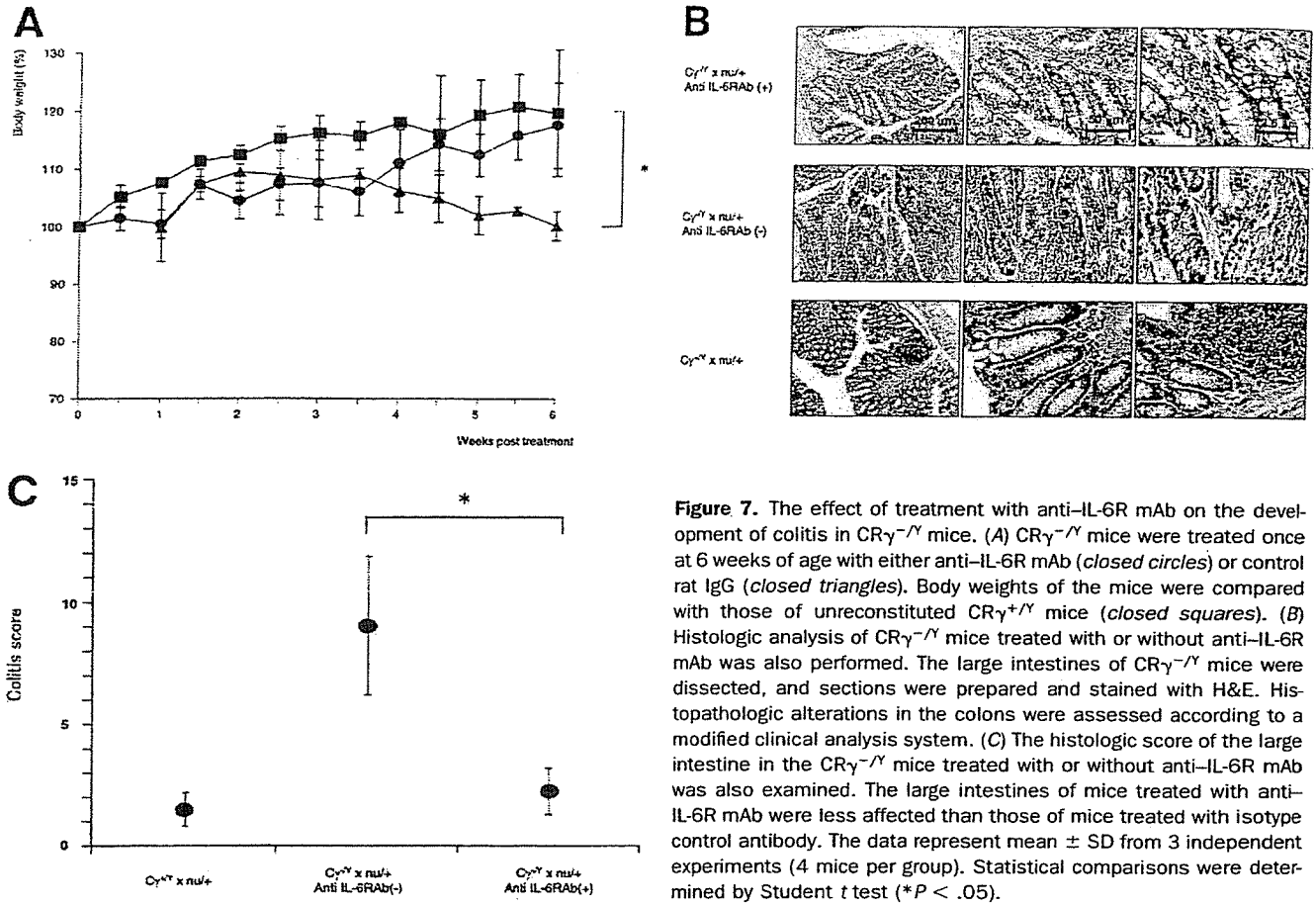
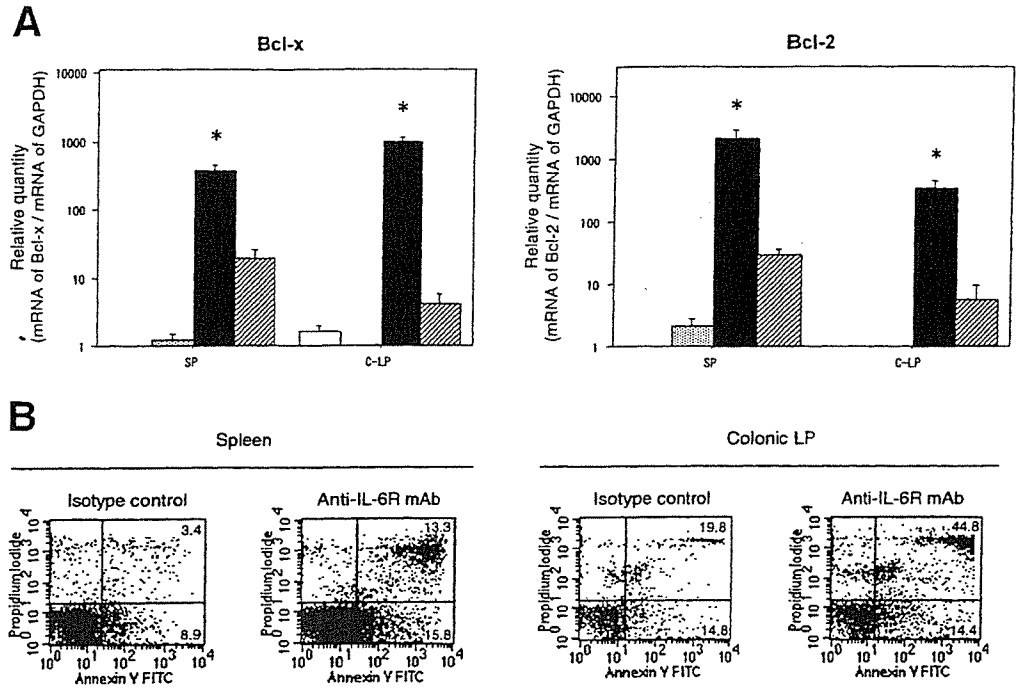


Figure 7. The effect of treatment with anti–IL-6R mAb on the development of colitis in CRγ$^{-/Y}$ mice. (*A*) CRγ$^{-/Y}$ mice were treated once at 6 weeks of age with either anti–IL-6R mAb (*closed circles*) or control rat IgG (*closed triangles*). Body weights of the mice were compared with those of unreconstituted CRγ$^{+/Y}$ mice (*closed squares*). (*B*) Histologic analysis of CRγ$^{-/Y}$ mice treated with or without anti–IL-6R mAb was also performed. The large intestines of CRγ$^{-/Y}$ mice were dissected, and sections were prepared and stained with H&E. Histopathologic alterations in the colons were assessed according to a modified clinical analysis system. (*C*) The histologic score of the large intestine in the CRγ$^{-/Y}$ mice treated with or without anti–IL-6R mAb was also examined. The large intestines of mice treated with anti–IL-6R mAb were less affected than those of mice treated with isotype control antibody. The data represent mean ± SD from 3 independent experiments (4 mice per group). Statistical comparisons were determined by Student *t* test (*$P < .05$).

oped the disease, the symptoms of which include lack of weight gain (Figure 7*A*), anorectal prolapse, and a hunched posture. In contrast, mice treated with anti–IL-6R mAb did not exhibit these pathologic characteristics (Figure 7*A*). Histologically, the intestinal LP of mice treated with anti–IL-6R mAb had less elongation of epithelial villi and much less infiltration of inflammatory cells than did those of mice treated with mock antibody (Figure 7*B*). Also, the number of goblet cells was almost normal in the colons of mice treated with anti–IL-6R mAb but reduced in mock antibody–treated mice (Figure 7*B*). Finally, the clinical scores of anti–IL-6R mAb-treated mice were comparable to those of healthy, control CRγ$^{+/Y}$ mice (Figure 7*C*). Thus, the administration of anti–IL-6R mAb inhibited the pathologic effects induced by CD4$^+$ T-cell–derived IL-6 and thereby seemed to prevent the development of colonic inflammation in the euthymic (nu/+) mutant CRγ$^{-/Y}$ mice.

Anti–IL-6R mAb Treatment Induced Apoptosis in IL-6–Producing Pathogenic CD4$^+$ T Cells

To elucidate the mechanisms by which anti–IL-6R mAb treatment inhibits development of colitis, we next examined the possibility that the mAb could have induced apoptosis because IL-6–producing pathogenic T cells expressed IL-6R (Figure 6*B*). Because IL-6 has been shown to possess antiapoptotic activity,[36] the autocrine manner between IL-6 and IL-6R may lead to the creation of an antiapoptotic environment for the pathologic IL-6-producing colonic IL-6R$^+$ CD4$^+$ T cells. To investigate this possibility, colonic CD4$^+$ T cells were isolated from CRγ$^{-/Y}$ mice with and without large intestinal inflammation and then subjected to Bcl-x– and Bcl-2–specific reverse-transcription PCR analysis. The levels of antiapoptotic gene expression were consistently up-regulated in CD4$^+$ T cells isolated from colonic LP of the diseased mice (Figure 8*A*). Thus, the levels of Bcl-x and Bcl-2 expression by pathogenic CD4$^+$ T cells were higher than those seen in CD4$^+$ T cells isolated from nondiseased mice. It should be noted that anti–IL-6R mAb treatment resulted in the significant reduction of Bcl-x and Bcl-2 expression by colonic pathologic CD4$^+$ T cells (Figure 8*A*). Identical results were obtained when SP CD4$^+$ T cells isolated from the same mice were examined. Thus, the reduction of Bcl-x and Bcl-2 expression by colonic CD4$^+$ T cells suggested that anti–



Stage of Apoptosis		Spleen		Colonic LP	
Annexin V	Propidium iodide	Isotype control	Anti-IL-6R mAb	Isotype control	Anti-IL-6R mAb
+	−	8.6 ± 0.2	14.6 ± 2.4 *	12.2 ± 2.8	16.2 ± 2.0 *
+	+	3.5 ± 0.8	10.9 ± 3.8 *	18.1 ± 1.4	35.2 ± 13.9 *

Figure 8. Characterization of antiapoptotic and apoptotic conditions of CRγ$^{-/Y}$ CD4$^+$ T cells before and after anti–IL-6R mAb treatment. (*A*) Analysis of antiapoptotic Bcl-x and Bcl-2 expressions by colonic and splenic CD4$^+$ T cells isolated from CRγ$^{-/Y}$ mice with IBD. Colonic and splenic CD4$^+$ T cells were isolated from the diseased (*black bars*) and nondiseased (*dotted bars*) CRγ$^{-/Y}$ mice, healthy controls (*white bars*), and anti–IL-6R mAb-treated (*hatched bars*) CRγ$^{-/Y}$ mice and then subjected to Bcl-x– and Bcl-2–specific quantitative reverse-transcription PCR. Pathogenic CD4$^+$ T cells showed higher levels of antiapoptotic gene expression than did CD4$^+$ T cells isolated from nondiseased mice. The levels of antiapoptotic gene expression were reduced in anti–IL-6R mAb-treated mice (*hatched bars*). (*B*) In the second experiment, the apoptosis-inducing effect of anti–IL-6R mAb treatment on CD4$^+$ T cells isolated from colonic LP and SP cells of CRγ$^{-/Y}$ mice was examined. Colonic LP and SP CD4$^+$ T cells from CRγ$^{-/Y}$ mice were cultured with 1 mg/mL of anti–IL-6R mAb (MR-16) or isotype IgG control. After 6 hours of incubation, cells were harvested for FACS analysis using the Annexin V FITC Apoptosis Detection Kit I (BD PharMingen). The data represent the mean ± SD from 3 mice per group. Statistical comparisons were determined by Student *t* test (*$P < .05$).

IL-6R mAb treatment induced apoptosis. To directly elucidate this point, CD4$^+$ T cells were isolated from SP cells and colonic LP of CRγ$^{-/Y}$ mice and then incubated with anti–IL-6R mAb or isotype control. Following 6 hours of incubation, the numbers of annexin V–positive and annexin V and propidium iodide double-positive cells were significantly increased in anti–IL-6R mAb-treated CD4$^+$ T cells when compared with the control (Figure 8*B*). Taken together, these findings suggest that anti–IL-6R mAb treatment interblocks the autocrine antiapoptotic molecular interaction of IL-6 and IL-6R and thus results in the induction of apoptosis in IL-6–producing pathogenic IL-6R$^+$ CD4$^+$ T cells.

Adoptive Transfer of SP CD4$^+$ T Cells From CRγ$^{-/Y}$ Mice With Disease Into SCID Mice

In a final series of experiments, we attempted to directly show the role of IL-6–producing CD4$^+$ T cells in the development of colitis. Thus, we isolated and

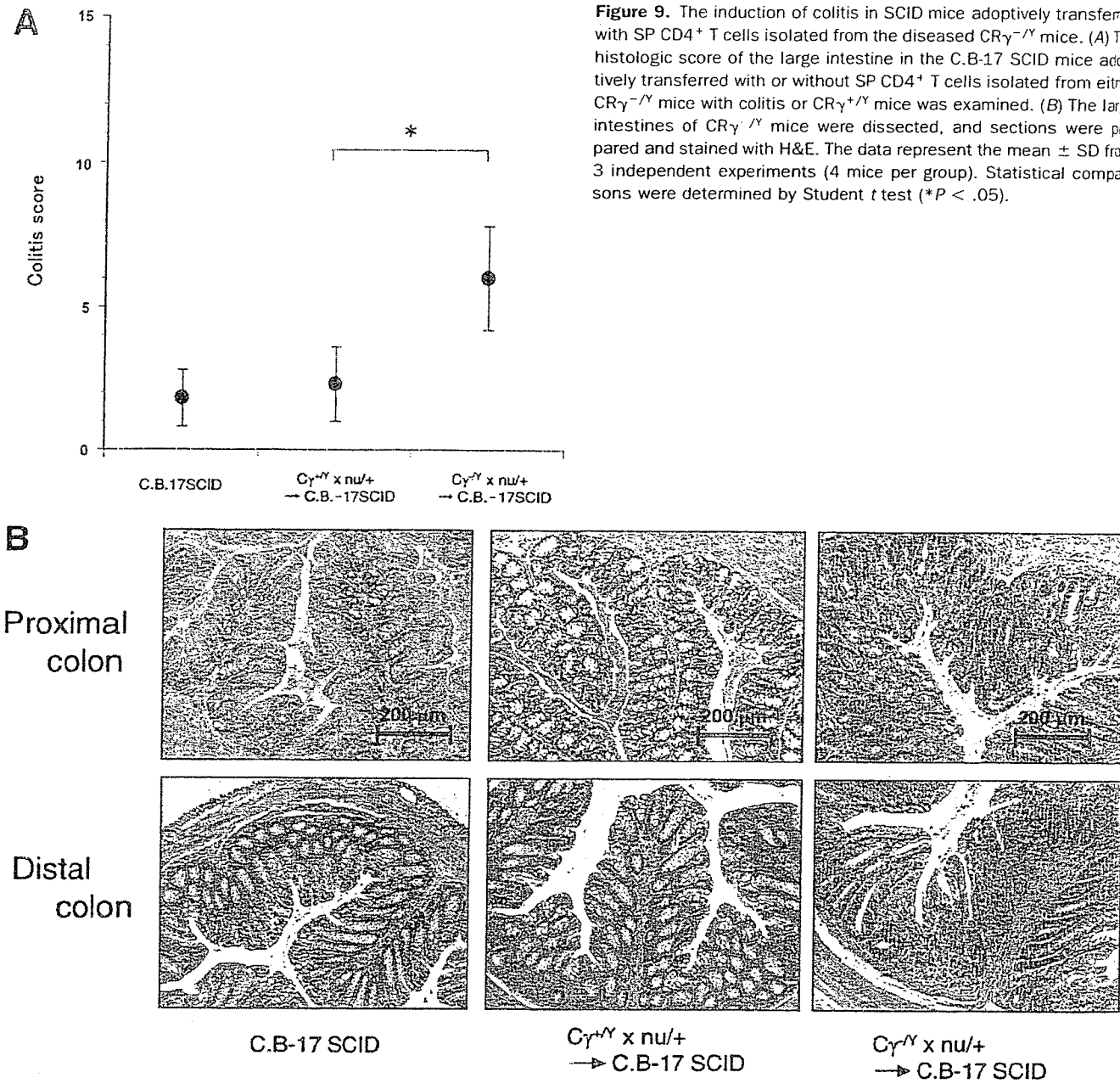


Figure 9. The induction of colitis in SCID mice adoptively transferred with SP CD4+ T cells isolated from the diseased $CR\gamma^{-/Y}$ mice. (*A*) The histologic score of the large intestine in the C.B-17 SCID mice adoptively transferred with or without SP CD4+ T cells isolated from either $CR\gamma^{-/Y}$ mice with colitis or $CR\gamma^{+/Y}$ mice was examined. (*B*) The large intestines of $CR\gamma^{-/Y}$ mice were dissected, and sections were prepared and stained with H&E. The data represent the mean ± SD from 3 independent experiments (4 mice per group). Statistical comparisons were determined by Student *t* test (*$P < .05$).

transferred SP CD4+ T lymphocytes from euthymic (nu/+) mutant $CR\gamma^{-/Y}$ or $CR\gamma^{+/Y}$ mice into age- and sex-matched C.B-17 SCID recipient mice that lacked both T and B cells. The SCID hosts that had been given CD4+ T cells from the $CR\gamma^{+/Y}$ mice showed no clinical or histologic evidence of disease (Figure 9*A* and *B*). In contrast, SCID mice adoptively transferred with SP CD4+ T cells from mutant $CR\gamma^{-/Y}$ mice with the disease developed evidence of colitis, that is, elevated colitis scores (Figure 9*A*) and inflammatory and hyperplastic lesions in the large intestine (Figure 9*B*). When we tested the SP CD4+ T cells for the spontaneous production of IL-6 before the adoptive transfer experiments, we found the cells capable of producing IL-6 (data not shown). The results of these adoptive transfer experiments further incriminated IL-6–producing CD4+ T cells in euthymic (nu/+) mutant $CR\gamma^{-/Y}$ mice in the development of colitis.

Discussion

In patients with IBD, the mucosal immune system, especially the T-cell–dependent regulatory system, is disturbed.[37,38] Results from several studies suggest that immunoregulatory cells, particularly dysregulated CD4+ T cells in intestinal mucosa–associated tissues, are important in the pathogenesis of Crohn's disease and

ulcerative colitis.[10] Our CRγ$^{-/Y}$ IBD model, the mice of which lack Peyer's patches and cryptopatches, provides a unique opportunity to investigate the potential systemic origin of pathologic CD4$^+$ T cells in mucosa-associated tissues and their potential role in the development of the inflammatory disease. Naturally occurring mutations in CRγ are responsible for the X-linked severe combined immunodeficiency disease in humans, characterized by the absence of T and natural killer cells but the presence of B cells.[21] Targeted deletion of CRγ in mice provokes a wide variety of defects in lymphoid development, including absence of natural killer cells, γδ T cells, and gut-associated lymphoid tissue.[25,26] Because these organized lymphoid tissues are necessary for the induction and regulation of the gut mucosal immune system, we believed it was important to examine the possible mechanisms involved in the pathogenesis of colitis in the unique immunologic environment provided by CRγ$^{-/Y}$ mice in this study.

Dysregulation of the delicate balance between Th1- and Th2-type CD4$^+$ T cells results in the development of IBD in various animal models.[10,39] In all murine models initially used, including specific gene-manipulated and hapten-induced mice and adoptive transfer models, the intestinal disease was associated with enhanced Th1-type activity.[10,40,41] However, more recent studies have posited that Th2-like responses are also involved in the colonic inflammation in murine IBD models[12,15,28,42] and in human ulcerative colitis.[38,43,44] In previous studies, the Th2 responses associated with colitis, such as those seen in TCR-α$^{-/-}$, hapten-induced, and oxazolone colitis, were mainly IL-4 dependent.[28,42] Our present findings show that the development of colitis is mediated by IL-6, most likely produced by a selected population of CD4$^+$ αβ T cells using Vβ14 TCR (Figures 3 and 4). The antiapoptotic behavior of these pathogenic CD4$^+$ T cells expressing high levels of Bcl-x and Bcl-2 seemed to be responsible for their increased numbers in the diseased region (Figure 8). Indeed, Bcl-x and Bcl-2 have been shown to be associated with antiapoptotic activity.[45] Because these IL-6–producing pathologic CD4$^+$ T cells simultaneously expressed high levels of IL-6R specific mRNA (Figure 6*B*), it is possible that the antiapoptotic conditions could be created by the autocrine interactions between IL-6 and IL-6R. Lending further support to this view is the finding that IL-6 induces antiapoptotic conditions.[36] Thus, the present study provides the first evidence that IL-6–producing CD4$^+$ T cells can behave as a pathogenic subset via the creation of autocrine antiapoptotic conditions conducive to the development of colitis in the CRγ-deficient condition.

Taken together, these findings provide compelling evidence that Vβ14-expressing CD4$^+$ αβ T cells and IL-6 are implicated in the pathogenesis of colitis in CRγ$^{-/Y}$ mice. First, among colonic lymphocytes isolated from CRγ$^{-/Y}$ mice, γδ T cells were absent, whereas αβ T cells were markedly increased; among such αβ T cells, CD4$^+$ but not CD8$^+$ T cells were increased (Figure 3). Second, among the different cytokines, IL-6, but not IL-4, IL-5, IL-12, or TNF-α, was preferentially produced by colonic CD4$^+$ T cells isolated from CRγ$^{-/Y}$ mice with inflammation. The selectivity of the IL-6 production was further documented by the finding that production of the Th1 cytokine IFN-γ by the colonic CD4$^+$ T cells was not increased in the diseased mice (Figure 5). Third, anti–IL-6R mAb treatment (Figure 7*A–C*), which presumably blocked the autocrine IL-6/IL-6R signaling, prevented the development of colitis, because pathogenic IL-6–producing CD4$^+$ T cells expressed IL-6R for the creation of an antiapoptotic nature (Figure 8*A*). Further, anti–IL-6R mAb treatment resulted in the induction of apoptosis in CD4$^+$ T cells isolated from the colon and spleen of CRγ$^{-/Y}$ mice (Figure 8*B*). This finding corroborates previous reports[31,46] that anti–IL-6R mAb treatment prevented the development of IBD caused by adoptive transfer of CD4$^+$CD45RB^{high} cells. Fourth, on transfer to immunodeficient C.B-17 SCID recipients, SP CD4$^+$ T lymphocytes isolated from CRγ$^{-/Y}$ mice with colitis induced disease that was as severe as that seen in the donors, while transfer of a similar population of T cells from wild-type mice had no such pathologic effect (Figure 9*A* and *B*).

In previous studies, the transfer of CD4$^+$CD45RB^{high} T-cell populations from the spleens of healthy donor mice into SCID mice resulted in the development of colitis in the recipient mice, whereas transfer of CD4$^+$CD45RB^{low} T cells did not.[47–52] Cotransfer of the CD4$^+$CD45RB^{low} T-cell population together with the CD4$^+$CD45RB^{high} T-cell population prevented the colitis.[47] In contrast, in this study, the adoptive transfer of whole CD4$^+$ T-lymphocyte populations from the diseased CRγ$^{-/Y}$ mice, which certainly contained both CD4$^+$CD45RB^{high} and CD45RB^{low} fractions, resulted in colonic inflammation in the SCID mice. This finding suggests that IL-6–producing pathologic CD4$^+$ T cells were resistant to the inhibitory effects of the CD4$^+$CD45RB^{low} T cells.

Previous studies on murine colitis focusing on the transfer of CD4$^+$CD45RB^{high} T cells had also shown IFN-γ to be an important pathogenic cytokine.[52] Because we have found IL-6 produced by CD4$^+$ cells to be the key cytokine in the CRγ$^{-/Y}$ IBD model, an opportunity now is afforded to investigate 2 distinct pathogenic pathways (ie, IFN-γ– or IL-6–producing pathogenic CD4$^+$ T cells) for the development of colitis in cell-transfer models. Moreover, these

findings point to the possible treatment of experimental IBD by the blockage of the IL-6/IL-6R signaling pathway. To this end, it has been shown that the anti–IL-6R treatment is effective for the control of murine colitis induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells.[46] Further, humanized mAb anti–IL-6R is currently in a clinical trial.[32]

In summary, this study showed that unwanted $CD4^+$ T cells, selectively producing the inflammatory cytokine IL-6, can induce colitis in $CR\gamma^{-/Y}$ mice. Also, the colitis can be prevented by inhibiting IL-6/IL-6R interaction with anti–IL-6R mAb. The euthymic (nu/+) mutant $CR\gamma^{-/Y}$ mouse model is yet another example of how a disturbed mucosal immunologic environment can lead to pathologic colonic inflammation. To our knowledge, it is the first colitis model in which $CD4^+$ Th-cell–originated IL-6 is pathogenetically implicated, raising the possibility that certain experimental intestinal inflammatory conditions could be prevented by interrupting the IL-6/IL-6R signaling pathway.

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Nasal IL-12p70 DNA Prevents and Treats Intestinal Allergic Diarrhea[1]

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OVA-induced allergic diarrhea occurs as a consequence of over-expression of Th1 inhibitory IL-12p40 monomers and homodimers in the large intestine, establishing a dominant Th2-type environment. In this study, we demonstrate that intranasally administered murine IL-12p70 naked DNA expression plasmids resulted in the synthesis of corresponding cytokine in the large intestinal CD11c$^+$ dendritic cells, leading to the inhibition of Ag-specific Th2-type response for the prevention of allergic diarrhea and the suppression of clinical symptoms including OVA-specific IgE Ab synthesis. The nasal IL-12p70 DNA treatment proved effective even after the establishment of allergic diarrhea. These results suggest that the mucosal administration of naked IL-12p70 DNA plasmid should be considered as a possible preventive and therapeutic treatment for Th2 cell-mediated food allergic diseases in the intestinal tract. *The Journal of Immunology,* **2005, 174: 7423–7432.**

Allergic diseases of the respiratory and gastrointestinal tracts, including pollinosis, asthma, and allergic diarrhea, respectively, are generally characterized by hyperresponsiveness stemming from the accumulation of eosinophils, mast cells, and mononuclear cells in mucosal tissues along with an elevation of Ag-specific IgE. This hyperreaction is caused by a defect in the immunological balance between Th1 and Th2 lymphocytes, leading to an increase in Th2 cells. The Th2 cytokines IL-4, IL-5, and IL-13 have been shown to be key mediators of the pathology of these allergic diseases (1–3). In our previous study, large intestinal IL-4-producing Th2 cells induced by systemic priming followed by oral challenge with OVA were shown to play a major pathologic role in the development of allergic diarrhea (4). Furthermore, mast cells, IgE and FcεR were also shown to contribute to the induction of this experimental Th2 intestinal allergy (5). Intestinal allergic inflammation was also induced in the small intestine by the eosinophils, eotaxin, and IL-5 associated with a pathologic Th2 environment in mice treated with OVA in enteric-coated beads (6). Our most recent study elucidated the molecular and cellular pathologic mechanisms underlying Th2 cell-mediated intestinal allergic disease by showing that locally produced IL-12p40 contributes to the generation of a dominant Th2-type environment in the large intestine of mice with allergic diarrhea (7).

Dendritic cell (DC)[3]-derived heterodimeric structure, a p35 and a p40 subunit of IL-12 (IL-12p70), is known to promote Th1 cell differentiation and to stimulate the production of IFN-γ from Th1 cells and NK cells (8–10). Production of IL-12 by activated macrophages and DCs also results in the secretion of monomeric and homodimeric IL-12p40 that antagonize IL-12p70 bioactivity via binding to the β1 subunit of the IL-12R (11–13). IL-12p40 transgenic mice have been shown to be more susceptible to the malaria infection due to the reduced Th1 responses (14). These findings, together with our previous observation that large intestinal IL-12p40 contributes to the generation of Th2 cell-mediated allergic disease, suggest that the mucosal IL-12 balance between p70 and p40 might be a key regulator for the Th1 and Th2 networks associated with the mucosal immune compartment (7). Because it is located on chromosome 5q with its allergy-associated cytokine gene cluster of IL-4, IL-5, and IL-13 (15), the IL-12p40 gene in particular can be considered as one of the potent regulators of Th2 cell induction.

Although IL-12p40 can shift the delicate balance existing between the Th1 and Th2 networks more toward the Th2 environment, IL-12p70 is a well-known Th1 inducer with potential for use in treating Th2-dominant diseases such as asthma (16). IL-12 has also been shown to inhibit IL-4 production in bulk cultures of peripheral blood leukocytes from allergic patients and thus to markedly suppress IL-4-mediated IgE production (17, 18). In addition, IL-12 prevents the differentiation of bone marrow cells into eosinophils in a murine model of asthma (19). The i.p. delivery of IL-12 has been shown to inhibit murine Ag-induced eosinophilic inflammation airway hyperresponsiveness and IgE production (20). In a clinical study of allergic asthma, s.c. administration of rIL-12 resulted in a significant decrease in the number of circulating eosinophils, however only minor effects were seen on histamine-associated airway hyperresponsiveness (16). These studies collectively suggest that IL-12 may be an effective suppressor of

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[3] Abbreviations used in this paper: DC, dendritic cell; Flt3, Fms-like tyrosine kinase-3; AFC, Ab-forming cell; DAPI, 4′,6′-diamidino-2-phenylindole; NALT, nasopharynx-associated lymphoid tissue; CLN, cervical lymph node; MLN, mesenteric lymph node; IVIS, in vivo imaging system.

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allergen-induced airway hyperreaction and of atopic asthma-associated inflammation.

Gene therapy using IL-12 may also have a role to play in the development of vaccines administered by mucosal or systemic routes and in disease modification (21, 22). Gene therapy has recently been used both experimentally and clinically as a tool for developing new vaccines or for halting the progression of immunological diseases. Nasal TGF-β DNA plasmid has been shown to be effective in mitigating the severity of ocular and intestinal inflammatory diseases (23, 24) The administration of Fms-like tyrosine kinase-3 (Flt3) ligand DNA has also been shown to increase the number of activated lymphoid DCs and to thereby induce Ag-specific mucosal and systemic Ab responses (25). Although the mucosal delivery of naked DNA specific for regulatory molecules has been shown to be effective for the modulation of immune responses, the fate of the mucosally delivered naked DNA and its efficacy in inducing and regulating immune responses at distant sites remains unknown. In the current study, we demonstrate that nasal IL-12p70 DNA administration results in the expression of the corresponding protein in large intestinal DCs, which promote the shift to a Th1-type cell response at the disease site for the prevention and treatment of Th2-mediated allergic diarrhea.

Materials and Methods

Mice

BALB/c mice were purchased from CLEA Japan. All mice were 6–7 wk of age at the beginning of the individual experiments.

Reagents

The plasmid pORF9-IL-12p70 consists of the pORF9 multiple cloning site (pORF9-mcs) vector plus the full-length recombinant murine IL-12p40 and IL-12p35 cDNA gene (InvivoGen). The pORF is an expression vector containing the hybrid elongation factor 1α/human T cell leukemia virus promoter and the ampicillin-resistant gene. The plasmid DNA was purified using the EndoFree Plasmid Mega kit (Qiagen). pORF-mcs empty plasmid was used as control vector. For use in a time kinetics study, pIRES2-EGFP plasmid was purchased from BD Biosciences. Allophycocyanin anti-CD11b (M1/70, rat IgG2b), allophycocyanin anti-CD11c (HL3, hamster IgG), and allophycocyanin anti-B220 (RA3-6B2, rat IgG2a) were purchased from BD Pharmingen. Anti-IL-12p70 Ab was obtained from R&D Systems and was biotinylated for immunohistochemical analysis. Streptavidin-PE and Alexa Fluor 660-donkey anti-goat IgG were obtained from Molecular Probes. Anti-LYVE (lymphatic vessel endothelial hyaluronan receptor 1) (26) was obtained from Santa Cruz Biotechnology.

Induction of allergic diarrhea

For the induction of allergic diarrhea, we used a previously established protocol (4, 7). Briefly, on the first day of the experiment (day 0), mice were primed by s.c. injection of 1 mg of OVA in CFA (Difco). One week after the systemic priming (day 7), mice were repeatedly challenged with 50 mg of OVA by oral route three times per week for several weeks (4). Within 1–2 h after the tenth administration with OVA, the mice were sacrificed and analyzed.

Nasal IL-12p70 DNA treatment

In vivo nasal treatment was performed as previously described (25). Briefly, BALB/c mice were nasally administered with 50 μg of purified IL-12p70 plasmid or pORF vector (with empty plasmid used as a control) twice a week for the duration of the experiment. Nasal DNA treatment was started at the time of oral challenge to examine preventive effects. In experiments designed to elucidate the therapeutic potential of nasal IL-12 DNA, mice with ongoing allergic diarrhea were treated with nasal IL-12 DNA.

ELISA for OVA-specific IgE Abs in serum

To assess OVA-specific IgE Ab levels in serum, a sandwich ELISA system was adopted as described earlier (4). End-point titers of OVA-specific IgE Abs were expressed as the reciprocal $\log_2$ of the last dilution that showed a level of absorbance 0.1 higher than that of the sera of nonimmune background mice. Total IgE was also analyzed by a sandwich ELISA system that used anti-mouse IgE (R35-72; BD Pharmingen) to capture mAb and biotin anti-mouse IgE (R35-92; BD Pharmingen) for use as the detection mAb (4).

Isolation of mononuclear cells and the Ag-specific Ig production assay

To isolate mononuclear cells from small and large intestines, we used an enzymatic dissociation method (4, 7). Mononuclear cells were incubated in 96-well nitrocellulose plates (Millititer HA; Millipore) precoated with OVA (1 mg/ml) for 4 h at 37°C with 5% CO_2 in air. OVA-specific Ab-forming cells (AFCs) were detected by addition of peroxidase-labeled anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b, and IgA Abs (Southern Biotechnology Associates) and visualized by the reaction of 3-amino-9-ethylcarbozole (Moss). OVA-specific AFCs were automatically counted by using the KS ELISPOT compact (Carl Zeiss).

Ag-induced cytokine analysis

For the detection of IL-4- and IFN-γ-producing $CD4^+$ T cells, intracellular FACS analysis was performed (27). Briefly, mononuclear cells isolated from large intestine or spleen of empty vector-treated allergic mice, IL-12p70 DNA-treated mice, or healthy control mice were incubated with OVA (1 mg/ml) at 37°C for 4 days and were then reacted with 10 μg/ml monensin for the last 5 h of culture. The cells were washed with PBS (pH 7.2) after removal of dead cells using Ficoll gradient separation and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were washed, suspended in staining buffer (PBS containing 2% FCS and 0.01% NaN_3), and incubated with 1 μg/ml PE anti-IL-4 mAb and FITC anti-IFN-γ (BD Pharmingen) in staining buffer containing 0.1% saponin (Sigma-Aldrich) in the presence of 5 μg/ml anti-FcγRII/III (2.4G2; BD Pharmingen) for 30 min at 4°C. After washing three times with staining buffer containing saponin, the cells were stained with 1 μg/ml allophycocyanin anti-CD4 mAb (BD Pharmingen) in staining buffer without saponin for 20 min at 4°C. Following a final washing, the cells were analyzed with a FACSCalibur (BD Biosciences) using the CellQuest software.

Immunoprecipitation and Western blot analyses

For the detection of IL-12p70 heterodimers comprised of IL-12p40 and IL-12p35, large intestinal tissue extracts were prepared as previously described with minor modifications (7). Large intestines were removed, minced in cold PBS with protease inhibitor (Complete Mini; Roche Diagnostics), homogenized and incubated to allow cytokine release from the tissue. After centrifugation and the measurement of their protein concentrations, intestinal tissue extracts were precleared with protein G-Sepharose beads (Pharmacia Biotech), incubated with anti-IL-12p40, and mixed with protein G-Sepharose beads. The beads were washed and then subjected to SDS-PAGE under nonreducing conditions. After electrophoresis, proteins were transferred to a polyvinylidene difluoride microporous membrane (Immobilon; Millipore) and the membrane was reacted with biotinylated anti-IL-12p70 (48110.11, rat IgG1; R&D Systems) and then incubated with a biotin-streptavidin complex (ABC-AP kit; Vector Laboratories). The signal was visualized using a nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate kit (Bio-Rad). The anti-IL-12p70 mAb has no cross-reactivity with recombinant murine IL-12p40.

Immunohistochemical analysis

Following extensive washing, large intestines were fixed in 4% paraformaldehyde-PBS and treated with sucrose gradient, before freezing in OCT embedding medium as previously described with minor modifications (7). For IL-12p70 immunostaining, cryosections were subjected to Ag retrieval using 10 mM citric buffer (pH 6.0) for 5 min at 98°C. Slides were then blocked with anti-FcγRII/III (2.4G2; BD Pharmingen) and incubated with biotin anti-IL-12p70 and goat anti-LYVE for 16 h at 4°C. The sections were then treated with Alexa Fluor 660 anti-goat IgG (Molecular Probes) and Streptavidin-PE (Molecular Probes). For surface marker staining, serial sections were incubated with allophycocyanin anti-CD11b (M1/70; BD Pharmingen) or allophycocyanin anti-CD11c (HL3; BD Pharmingen). Counter staining was performed using 4′,6′-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

In vivo monitoring of protein expression following nasal GFP-DNA administration

For assessing the time kinetics of the protein expression induced by nasal DNA, 50 μg of GFP plasmid was nasally administered to normal BALB/c mice. Then, mice were subjected to in vivo imaging system (IVIS) analysis for 20 s using the Xenogen IVIS CCD camera system at 0, 3, 6, 9, 12, 18,

and 24 h after nasal administration (28). After ventral images were taken, the mice were sacrificed and various tissues removed for ex vivo image analysis using the IVIS. The image data of GFP expression was analyzed by LivingImage software (Xenogen) and the GFP intensity from selected areas was quantified.

Nasal GFP-DNA administration and sample analysis

For the time kinetics study of corresponding protein expression following nasal GFP DNA treatment, mice were sacrificed at the indicated time and then analyzed for GFP expression by FACS or subjected to immunohistochemical analysis. Mononuclear cells from nasal passages, spleen, cervical lymph node (CLN), submandibular glands, and mesenteric lymph node (MLN) were isolated, fixed in 4% paraformaldehyde, stained for cell surface markers such as B220, CD4, CD11b, and CD11c and then analyzed for GFP expression using the FACSCalibur. Nasopharynx-associated lymphoid tissue (NALT) and large intestine were fixed in 4% paraformaldehyde-PBS and treated with sucrose gradient, before being frozen in OCT-embedding medium. For lymphoid vessel staining using anti-LYVE (Santa Cruz Biotechnology) (26), cryosections were subjected to Ag retrieval using 10 mM citric buffer at pH 6.0 for 8 min at 98°C. Slides were then blocked with anti-FcγRII/III (2.4G2; BD Pharmingen), incubated with goat anti-LYVE or control goat IgG, and then treated with Alexa Fluor 660 anti-goat IgG. Counter staining was performed using DAPI (Sigma-Aldrich), and immunohistochemical analysis was performed using confocal laser scanning microscopy (Leica).

Analysis of large intestinal cells

As soon as mice developed diarrhea after the final oral OVA challenge, they were sacrificed and cells were isolated from the large intestine as described earlier. Cytospin slides were prepared and then stained with Diff-Quik (Sysmex) for the morphological analysis of differential cell populations including mononuclear cells, eosinophils, basophils, and neutrophils.

Statistical analysis

Statistical analyses were performed by the two-sample nonparametric Welch test with a significance level of $p < 0.01$ or $p < 0.05$ for IgE levels. Values for cytokine synthesis and IgA, IgG, and IgG1 AFCs in the samples between IL-12p70 and control plasmid-treated mice were analyzed by using Student's t test at values of $p < 0.01$.

A

Immunization	Nasal treatment	Incidence of OVA-induced diarrhea (%)	
SC/PO	None	22/22	100
	Vector	14/14	100
	IL-12p70 plasmid	0/14	0
SC only	None	0/12	0

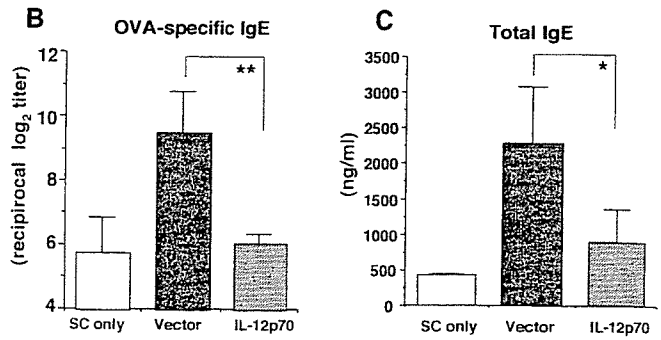

FIGURE 1. Inhibition of allergic diarrhea disease by nasal treatment with IL-12p70 naked DNA plasmid. *A*, The incidence of allergic diarrhea was reduced in mice treated nasally with IL-12p70 DNA when compared with mice treated with empty vector plasmid. Allergic disease was induced in these mice by s.c. immunization and then repeated oral challenge (SC/PO) with OVA. *B*, OVA-specific IgE Abs are reduced in the serum of allergic diarrhea-afflicted mice treated nasally with IL-12p70 DNA. *C*, Total IgE Abs are reduced in the serum of allergic diarrhea-afflicted mice treated with naked IL-12p70 DNA. The data are expressed as the mean ± SD and are representative of five independent experiments. Statistical differences between IL-12p70 DNA- and empty vector-treated mice (**, $p < 0.01$ and *, $p < 0.05$) are indicated.

Results

Nasal IL-12p70 DNA prevents allergic diarrhea

In a previous study, we showed that large intestinal IL-12p40 led to a pathologic Th2-dominant environment conducive to the development of OVA-induced allergic diarrhea (7). Because IL-12p70 is one of the most effective Th1-inducing cytokines (8), we sought to examine whether artificially introduced IL-12p70 DNA could prevent the development of allergic diarrhea by up-regulation of IL-12p70 expression of the disease site. Mice nasally treated with the IL-12p70 plasmid did not develop allergic diarrhea, whereas mice given the empty vector developed severe disease as that observed in OVA-induced diarrhea mice (Fig. 1*A*). Thus, mice receiving the empty vector served as a control for the remainder of the investigation. In addition, elevated OVA-specific IgE Abs were detected in the serum of diarrhea-afflicted mice treated with the control vector DNA, whereas the mice nasally treated with IL-12p70 DNA showed only low OVA-specific IgE Abs (Fig. 1*B*). Furthermore, nasal treatment of IL-12p70 DNA reduced the level of total serum IgE Abs (Fig. 1*C*). These results demonstrate that nasal IL-12p70 plasmid prevented the development of OVA-induced allergic diarrhea and reduced both Ag-specific and total IgE responses. Based on these findings, we hypothesized that nasal treatment with IL-12p70 DNA down-regulates the Th2 environment and thus inhibits the development of allergic diarrhea.

Reduction of large intestinal IgA and IgG production by nasal administration of IL-12p70 DNA

To assess nasal IL-12p70 DNA effects on Th1/Th2 responses, we next examined OVA-specific IgM, IgG, IgG1, IgG2a, IgG2b, and IgA Ab responses in large intestinal lamina propria mononuclear cells using the ELISPOT assay. We found that the numbers of Th2-associated OVA-specific IgA and IgG1 AFCs in large intestinal lamina propria mononuclear cells were significantly reduced in the mice treated nasally with IL-12p70 DNA when compared with those of mice treated with the empty vector DNA (Fig. 2*A*, *left*). As we reported earlier (4), OVA-specific IgG2a, IgG2b (data not shown), and IgM AFCs were not detected in the large intestine of all groups of mice examined. In spleen, no significant differences were detected in the numbers of OVA-specific IgA, IgG, and IgG1 AFCs between IL-12p70-treated and control mice (Fig. 2*A*, *right*). As we have shown previously (4), Ag-specific Ab responses are not induced in the small intestine of mice suffering from allergic diarrhea. Thus, OVA-specific IgA, IgG, and IgG1 Ab responses were not detected in the small intestine of either IL-12p70 DNA-treated or control vector-treated mice (data not shown). Although the exact role of OVA-specific IgA and IgG Ab responses in the large intestine of mice with allergic diarrhea still needs to be elucidated, these findings further support the notion that nasal administration of IL-12p70 DNA can inhibit the generation of locally enhanced Th2 cell-mediated OVA-specific Ab responses in mice suffering from allergic diarrhea.

Suppression of large intestinal Th2 cytokine by nasal IL-12p70 DNA

To directly confirm decreased Th2-type responses in the large intestine after nasal IL-12p70 treatment, we next examined Ag-induced cytokine production of the large intestinal $CD4^+$ T cells using intracellular FACS analysis. Nasal IL-12p70 DNA treatment decreased the number of IL-4-producing cells in large intestinal

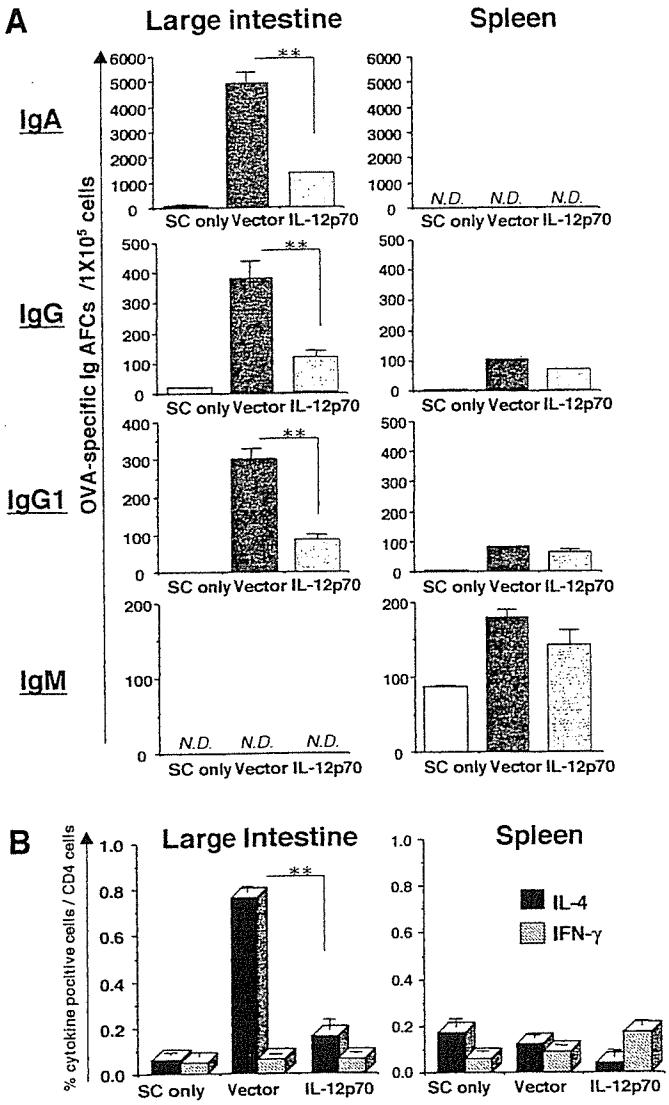

FIGURE 2. Reduction of IgA and IgG hyperresponsiveness in large intestinal B cells and of Th2 cells responses by nasal administration of IL-12p70 DNA. *A*, The frequency of OVA-specific IgA, IgG, IgG1, and IgM AFCs in the large intestine of mice treated with IL-12p70 DNA or empty vector plasmid. *B*, OVA-specific IL-4 and IFN-γ production were analyzed by intracellular staining using FACS analysis. In vivo treatment with IL-12p70 DNA reduced the predominant Th2-type Ag-specific responses by large intestinal mononuclear cells isolated from diarrhea-afflicted mice. The mononuclear cells isolated from the large intestine (1.0×10^6 cells/well) or spleen were cultured with OVA (1 mg/ml) for 4 days. After incubation, cells were harvested and subjected to intracellular staining with anti-IL-4 and IFN-γ. The data are expressed as the percentage of cytokine-positive cells in large intestinal $CD4^+$ T cells. The data are expressed as the mean ± SD and are representative of four independent experiments. Statistical differences between IL-12p70 DNA- and empty vector-treated mice (**, $p < 0.01$) are indicated. N.D., Not detected.

$CD4^+$ T cells when compared with the empty vector-treated mice (Fig. 2*B*, *left*). No difference in the frequency of IFN-γ-producing $CD4^+$ Th cells was seen between the mice treated with nasal IL-12p70 DNA and control vector DNA (Fig. 2*B*, *left*). In contrast, when splenic $CD4^+$ Th cells were examined, no major changes were observed between IL-12p70 DNA-treated and control vector-treated mice (Fig. 2*B*, *right*). However, nasal IL-12p70 treatment did reduce the number of IL-4-producing cells and increase the number of IFN-γ-producing cells in splenic $CD4^+$ Th cells (Fig. 2*B*, *right*). A similar pattern of changes was observed when culture supernatants from the various groups of IL-12p70 DNA- and empty vector-treated mice were examined (data not shown).

Nasal GFP DNA resulted in protein expression in NALT, spleen, and intestine

To directly confirm that the nasal administration of DNA resulted in protein expression in the large intestine, we initially used GFP plasmid DNA for the visualization of corresponding protein expression in vivo. When we used the IVIS to analyze the GFP distribution in vivo following nasal administration of GFP plasmid DNA, the intense fluorescence expression was observed in areas associated with the nasopharynx and CLN as of 3 h after nasal administration (Fig. 3*A*). A high intensity of GFP expression was observed in the intestinal region 9 h after nasal administration (Fig. 3*A*). In addition, we analyzed ex vivo levels of GFP expression in various tissues isolated from mice that had been given nasal GFP plasmid. The spleen and lymph nodes expressed significant GFP beginning at 3 h after administration and continuing up to 18 h (Fig. 3*B*). In mice administered with the GFP gene, GFP expression was particularly strong in CLN. We also analyzed GFP expression levels in the lung, liver, and nasal cavity of mice given nasal IL-12p70 DNA; however, levels of GFP expression were below levels of detection (data not shown). Our effort to analyze GFP expression in the small and large intestine was hampered by autofluorescence, making it difficult to obtain reproducible data.

Next, we assessed whether nasal GFP plasmid would lead to the induction of corresponding protein expression in NALT because the tissue has been shown to be a primary site for the initial uptake of nasal Ags (29). GFP^+ cells were detected under the epithelium of NALT, but not until 12 h after nasal administration (Fig. 4*A*). These GFP^+ cells were examined by costaining them with red fluorescence-conjugated anti-CD11c Ab (Fig. 4*A*, *right*). These data suggest that nasal administration with GFP plasmid resulted in the expression of the corresponding protein in NALT DCs. Because GFP expression had already been noted in other tissues distant from NALT, it can be assumed that some of the administered gene might rapidly pass through NALT without protein expression in the lymphoid tissue.

To chronologically assess the GFP-expressed DCs located in various tissues after nasal GFP plasmid, mice were sacrificed at predetermined time points (0, 1, 3, 6, and 12 h and 1, 2, 3, 4, 5, and 7 days) and mononuclear cells from various lymphoid tissues were analyzed for the presence of GFP^+ DCs at the single cell level. When mononuclear cells from nasal passages, spleen, CLN, MLN, and submandibular glands were examined, we detected some GFP^+ cells in the DC fraction. However, the frequency was not high enough to meet the minimum limits of reliable FACS analysis. For example, we were able to detect GFP^+ $CD11c^+$ cells in MLN at the frequency of ~0.1%. In contrast, it was possible to detect sufficient numbers of GFP-expressing DCs in spleen due to the quantity of naturally occurring $CD11c^+$ cells. Thus, the levels of $CD11c^+$ cells expressing GFP were detected in spleen as early as 3 h, peaked at 12–24 h, and gradually decreased beginning 96 h after nasal administration (Fig. 4*B*). In contrast, CD11b, B220, and $CD4^+$ cells did not express GFP in spleen (data not shown).

To directly confirm GFP expression in intestinal tissues, we next analyzed large intestines for fluorescence expression using immunochemical analysis. GFP^+ cells appeared in large intestines from 6 h to 4 days after nasal administration of GFP plasmid (Fig. 4*C*). High magnification analysis revealed that GFP^+ cells were located in nearby lymphoid vessels (Fig. 4*C*, *right*). Most GFP^+ cells in the large intestines were $CD11c^+$ (data not shown). These findings directly demonstrate that nasal delivery of naked DNA resulted in

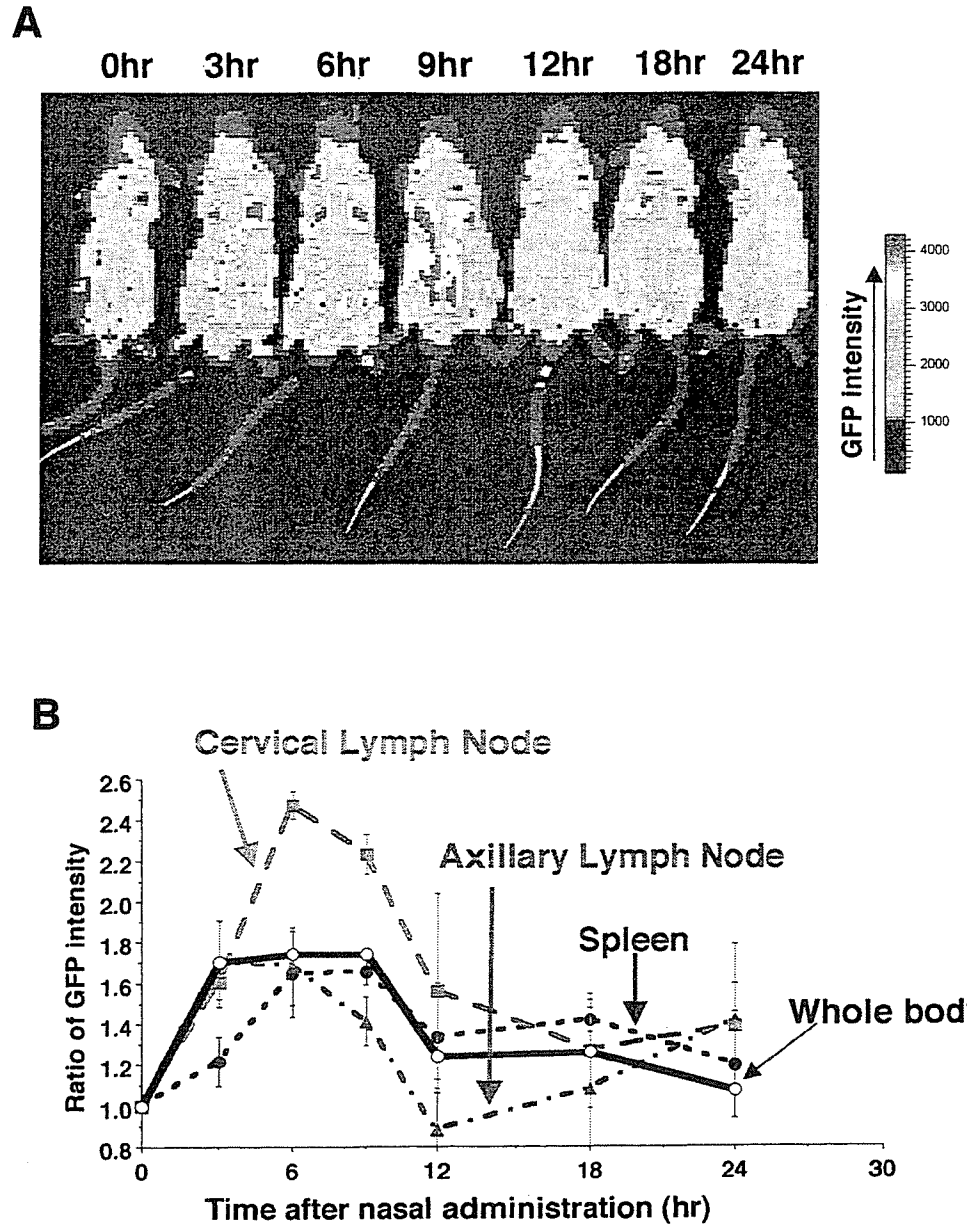

FIGURE 3. Analysis of green fluorescence expression in different tissues following nasal administration of GFP DNA in vivo using IVIS. GFP DNA plasmids were administered nasally for the purpose of analyzing the tissue distribution of GFP expression. GFP expression was detectable in vivo beginning 3 h after nasal administration (*A*). Tissues from sacrificed mice were examined ex vivo for GFP levels using IVIS. The GFP expression levels were quantified by Living Image software (*B*).

the expression of the corresponding protein in a distant mucosal compartment (e.g., large intestine).

Nasal IL-12p70 DNA led to the protein expression in large intestine

To directly demonstrate that nasal IL-12p70 DNA administration induces IL-12p70 expression at the site of disease development, we used immunohistochemical analysis to assess the cytokine expression in the large intestine of mice nasally treated with IL-12p70 DNA. When large intestines were examined, IL-12p70-producing cells were located in nearby lymph vessels (Fig. 5*A*). In contrast, large intestinal cells of mice treated with vector DNA only did not contain any cells expressing IL-12p70 (Fig. 5*A*). Further analysis of the IL-12p70 expressed by large intestinal mononuclear cells revealed that these cells were costained with green fluorescence-coupled anti-CD11c (Fig. 5*C*). These data suggest that nasal administration of IL-12p70 DNA resulted in IL-12p70 protein expression in large intestinal DCs. An identical finding was also observed using nasal GFP DNA treatment (Fig. 4). Furthermore, to finally confirm that IL-12p70 is expressed in mice treated nasally with IL-12p70 DNA, we examined IL-12p70 protein expression by Western blot analysis. Extracts of large intestine from mice nasally treated with IL-12p70 DNA expressed a high intensity band corresponding to IL-12 (Fig. 5*B*). However, only a faint band corresponding to residual IL-12p70 was detected in large intestinal extracts from mice treated with the empty vector. These results indicate that nasal IL-12p70 DNA treatment leads to IL-12p70 expression in large intestinal cells including DCs, accounting for the inhibition of pathologic Th2 reactions and thus for the prevention of allergic diarrhea.

Nasal IL-12p70 DNA administration cures ongoing OVA-induced allergic diarrhea

Once we had established that nasal IL-12p70 DNA prevented allergic diarrhea, we examined whether nasal IL-12p70 DNA could alter the disease condition in mice with existing allergic diarrhea. Severe allergic diarrhea was first induced in systemically primed mice by 10 oral challenges with OVA. When mice were given nasal IL-12p70 DNA, the diarrhea was completely cured after three doses (Fig. 6, *A* and *B*). As one might expect based on our

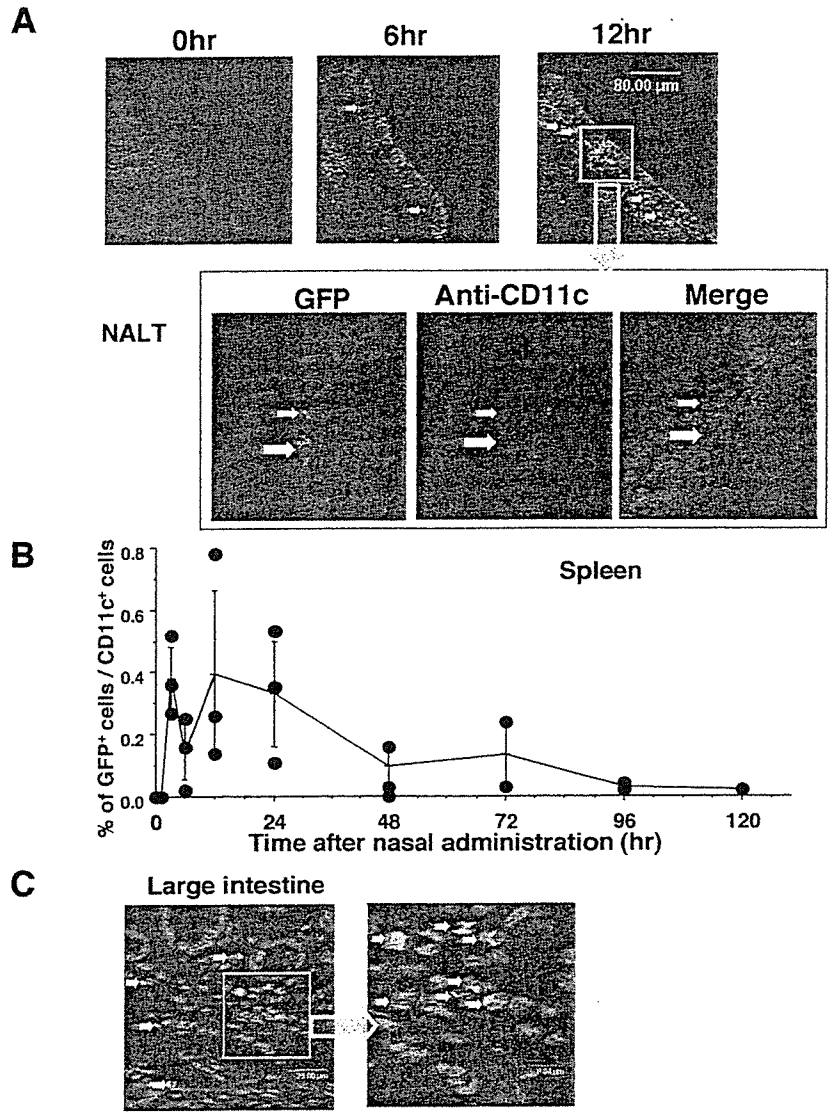

FIGURE 4. Expression fo GFP by $CD11c^+$ cells in systemic (spleen) and mucosal (NALT and large intestine) tissues following nasal administration of GFP DNA. *A*, At 6 and 12 h after nasal administration of GFP DNA, GFP and CD11c double-positive cells were observed in NALT. The arrows point to double-positive cells. *B*, Time kinetic studies using FACS analysis determined the frequency of GFP^+ cells in the spleen of mice nasally treated with GFP DNA. GFP^+ cells were preferentially detected in splenic $CD11c^+$ cells from 3 h to 3 days after nasal DNA administration. GFP^+ cells were not found in B220-positive and $CD11b^+$ populations. *C*, Nasal administration of GFP DNA resulted in the expression of the corresponding protein in the large intestines. They appeared from 6 h to 3 days after nasal DNA administration. GFP^+ cells were located near lymphatic vessels, which are indicated in red. Enlargement (*right*) of the *inset* in the white square (*left*).

previous findings (4), a large number of basophils and eosinophils were seen in the large intestine of mice suffering from allergic diarrhea (Fig. 6*C*, *top*). The nasal treatment with IL-12p70 DNA removed these basophils and eosinophils from the large intestine (Fig. 6*C*, *bottom*). Furthermore, the large intestinal hyperresponse of Th2-associated IgA, IgG, and IgG1 AFCs were also significantly decreased by the nasal IL-12p70 DNA treatment (Fig. 6*D*). Thus, nasal administration with IL-12p70 DNA proved to be effective at inhibiting ongoing large intestinal allergic reaction.

Discussion

Food allergies in humans are caused by hypersensitivity to food allergens and can result in severe diarrhea (30). The OVA-induced Th2 cell-mediated allergic diarrhea model, which involves systemic priming followed by oral challenge, is a useful and adequate model for the investigation of mechanisms that result in food allergies (4, 7). Our most recent study showed that local accumulations of IL-12p40 are a major triggering factor for the creation of an aberrant Th2 environment in the large intestine, one which is conducive to the induction of allergic diarrhea (7). In the current study, our findings demonstrate that nasal IL-12p70 DNA treatment prevents the development of IL-12p40-mediated OVA-induced Th2-dominant allergic disease by inducing IL-12p70 production in the large intestinal tract. Not surprisingly, the inhibition of pathologic Th2 cell responses was seen in the large intestine of mice nasally treated with IL-12p70 DNA. Based on the results obtained by in vivo image and immunohistochemical analyses, we suggested that nasal deposition of naked IL-12p70 DNA resulted in the expression of the corresponding protein at distant sites including large intestine. This finding was further substantiated by results showing that nasal administration of naked GFP plasmid DNA resulted in the expression of GFP-positive DCs but not of macrophages and B cells in the intestinal tract, NALT, and spleen. Although the efficacy of the nasal administration of naked DNA was previously reported using Flt3 ligand and TGF-β DNA plasmid (23, 25), our study is the first to show that expression of nasal DNA occurs in distant mucosal compartments at the single cell level (e.g., IL-12p70-producing large intestinal DCs) and to link that expression to the prevention and treatment of intestinal allergic diseases. Taken together, these data show that noninvasive nasal administration with naked DNA plasmid results in the expression of the corresponding protein in both the mucosal and systemic lymphoid tissues and so may offer a new avenue of therapy for mucosa-associated diseases. Specifically, nasal administration of naked IL-12p70 DNA should be given consideration as a new tool in preventing and treating allergic diseases associated with the distant mucosal tissues.