Table 1
The primer list of TLRs

Gene	Accession No.		Primers	Bases	Product size (bp)	Annealing
TLR1	NM003263	Sense	5′-TGCCCTGCCTATATGCAA-3′	(381–398)	555	54
		Anti-sense	5′-GAACACATCGCTGACAACT-3′	(918-936)		
TLR2	XM003304	Sense	5′-GCCAAAGTCTTGATTGATTGG-3′	(1783–1803)	346	52
		Anti-sense	5′-TTGAAGTTCTCCAGCTCCTG-3′	(2110–2129)		
TLR3	NM003265	Sense	5′-CGCCAACTTCACAAGGTA-3′	(277–294)	689	54
		Anti-sense	5′-GGAAGCCAAGCAAAGGAA-3′	(949–966)		
TLR4	XM005336	Sense	5′-TGGATACGTTTCCTTATAAG-3′	(1768–1787)	506	52
		Anti-sense	5′-GAAATGGAGGCACCCCTTC-3′	(2256–2274)		
TLR5	NM003268	Sense	5′-ATCTGACTGCATTAAGGGGAC-3′	(2274–2294)	567	52
		Anti-sense	5′-TTGAGCAAAGCATTCTGCAC-3′	(2822–2841)		
TLR6	NM006068	Sense	5′-CCTCAACCACATAGAAACGAC-3′	(832–852)	531	50
		Anti-sense	5′-CACCACTATACTCTCAACCCAA-3′	(1342–1363)		
TLR7	NM016562	Sense	5′-AGTGTCTAAAGAACCTGG-3′	(2222–2239)	544	50
		Anti-sense	5′-CCTGGCCTTACAGAAATG-3′	(2749–2766)		
TLR8	NM016610	Sense	5′-CAGAATAGCAGGCGTAACACATCA-3′	(1909–1932)	639	56
		Anti-sense	5′-AATGTCACAGGTGCATTCAAAGGG-3′	(2522–2545)		
TLR9	NM017442	Sense	5′-GTGCCCCACTTCTCCATG-3′	(791–808)	259	50
		Anti-sense	5′-GGCACAGTCATGATGTTGTTG-3′	(1030–1050)		
TLR10	NM030956	Sense	5′-CTTTGATCTGCCCTGGTATCTC-3′	(2286–2307)	497	52
		Anti-sense	5′-AGCCCACATTTACGCCTATCCT-3′	(2783–2286)		
GAPDH	XM033263	Sense	5′-CCATCACCATCTTCCAGGAG-3′	(293–312)	575	60
		Anti-sense	5′-CCTGCTTCACCACCTTCTTG-3′	(849–868)		

ELISA. To quantify cytokine secretion, culture supernatants were harvested and the level of IL-6 and IL-8 was assayed by human cytokine-specific ELISA (Biosource, Camarillo, CA).

Real-time semi-quantitative PCR. This was performed on an ABI-prism 7700 (Applied Biosystems, Foster City, CA) according to a previously described protocol [27] and the manufacturer's instructions. Total cellular RNA extraction and the first cDNA synthesis were as described above. The primers and probes for human IFN-β, human molecules possessing ankyrin-repeats induced by LPS (MAIL), and human GAPDH were from Perkin-Elmer Applied Biosystems. Previously reported primer and TaqMan probes for human IL-6 and IL-8 were used. The primers for IL-6 were 5′-TGACAAACAAATT CGGTACATCCT-3′ and 5′- AGTGCCTCTTTGCTGCTTTCAC-3′; the TaqMan probe for human IL-6 was 5′-TTACTCTTGTTACA TGTCTCCTTTCTCAGGGCTG-3′ [28]. The primers for human IL-8 were 5′-GCGCCAACACAGAAATTATTGTAA-3′ and 5′-TTATGA ATTCTCAGCCCTCTTCAA-3′; the TaqMan probe for IL-8 was 5′-TTCTCCACAACCCTCTGCACCCAGTT-3′ [27]. The probes were synthesized by Perkin-Elmer Applied Biosystems. To amplify human IL-6, IL-8, IFN-β, MAIL, and GAPDH cDNA, PCR was performed in a 25-μl total volume that contained a 1 μl cDNA template in 2× TaqMan universal PCR master mix (Applied Biosystems) at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The results were analyzed with sequence detection software (Applied Biosystems); the expression level of each mRNA was normalized to the expression of the human housekeeping gene GAPDH.

Data analysis. Data were expressed as means ± SE and evaluated by Student's *t* test using the Excel program.

Results

Expression of TLR3-specific mRNA in human corneal epithelium

We first examined whether human corneal epithelium expresses specific mRNA for TLRs 1–10. TLR-specific RT-PCR showed that mRNA from all but TLR8 was present in normal human corneal epithelium (Fig. 1). Among TLR-specific mRNA tested, TLR3 was expressed most intensely. When, as a positive control, we also subjected mRNA isolated from HPMC to RT-PCR, we found that these cells expressed TLRs 1–10. We then isolated, subcloned, and sequenced the PCR products. The obtained sequences were >95% identical with the known nucleotide sequences of human TLRs. Our findings suggest that while human corneal epithelium harbors messages for most TLRs, TLR3 is the one with the highest expression level. The expression of TLR3 was higher, while that of the other TLRs was lower, in human corneal epithelium than HPMC.

Primary HCEC express TLR3, but not TLR2 and TLR4, on the cell surface

Next we examined the cell-surface expression of TLR2, TLR3, and TLR4 on primary HCEC. While TLR3 was expressed on the surface of primary HCEC, TLR2 and TLR4 were not (Fig. 2). In positive controls, TLR2 and TLR4 were expressed on the cell surface of human peripheral blood monocytes and TLR3 was expressed on the cell surface of MRC-5 [29].

Primary HCEC respond to polyI:C but not LPS

Next we determined whether HCEC respond to polyI:C, a mimic of TLR3 ligand dsRNA. We first examined the production of inflammatory cytokines by primary HCEC stimulated with polyI:C and LPS. As

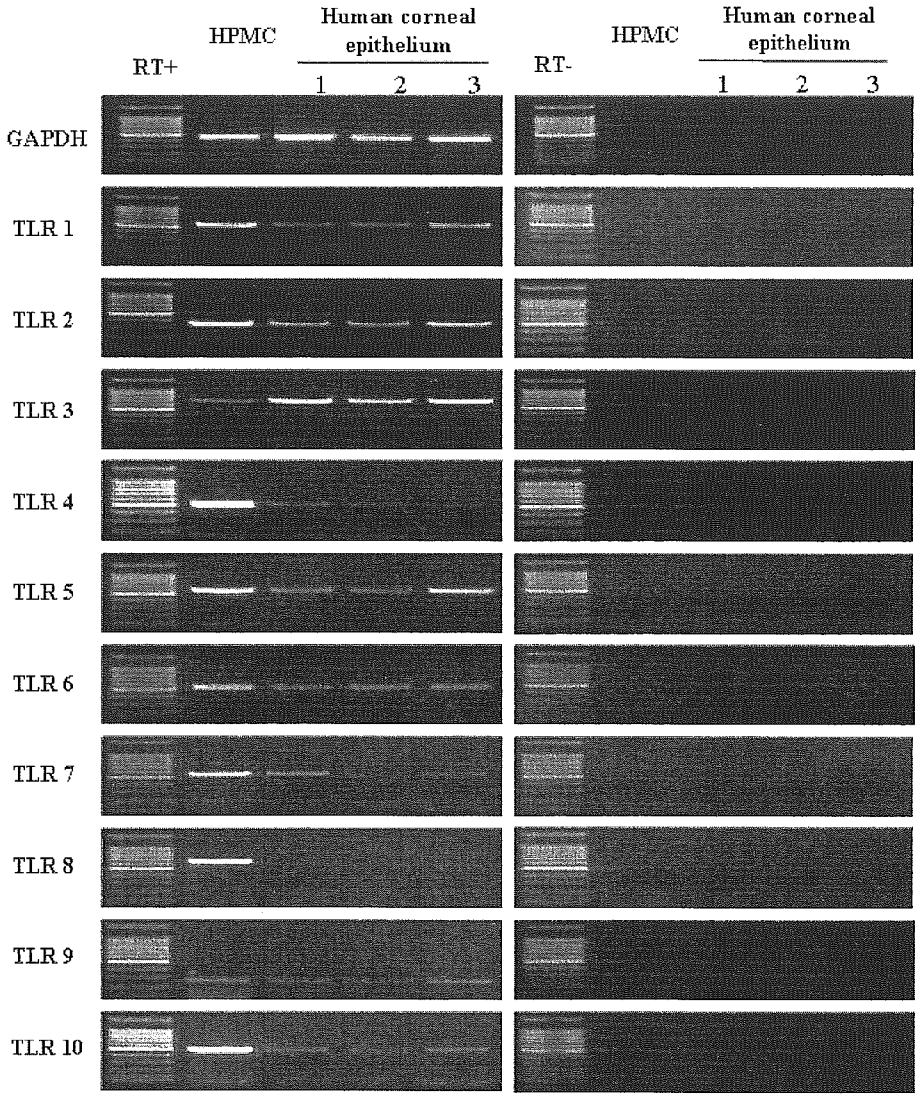


Fig. 1. The level of TLR3 expression is higher in human corneal epithelium than HPMC. Normal human corneal epithelium expresses mRNAs for TLRs 1–7 and 9–10 but not TLR8. As a positive control, mRNA isolated from human peripheral mononuclear cells (HPMC) was subjected to RT-PCR (left column). RT– indicates data were obtained without reverse transcription (controls).

shown in Fig. 3A, polyI:C stimulation induced the secretion of IL-6 and IL-8 while LPS treatment did not; in LPS-treated primary HCEC the level of IL-6 and IL-8 was similar to that seen in unstimulated cells. On the other hand, LPS stimulation significantly increased the production of IL-6 and IL-8 by HPMC and HCFB.

These findings were confirmed at the mRNA expression level. In primary HCEC, stimulation with polyI:C, but not LPS, resulted in the increased expression of IL-6- and IL-8-specific mRNA. Conversely, HPMC responded to LPS- but not to polyI:C stimulation and HCFB responded to both LPS and polyI:C (Fig. 3B).

IFN-β is controlled with TLR3/IRF-3 signaling. Thus, IFN-β-specific mRNA was significantly elevated in polyI:C- but not LPS-stimulated primary HCEC. Similarly, polyI:C but not LPS stimulated the induction of IFN-β in HPMC and HCFB. Surprisingly, IFN-β-specific mRNA expression was markedly higher in primary HCEC than HPMC and HCFB (Figs. 4A and B). Although primary human fibroblasts such as HCFB and MRC-5 expressed TLR3 on their cell surface, the cell-surface expression of TLR3 was more notable in primary HCEC (Fig. 4C).

Induction of IκBα- and MAIL-specific mRNA by polyI:C, but not LPS, in primary HCEC

In epithelial cells, the transcription factor NF-κB plays a central role in regulating genes that govern the onset of mucosal inflammatory responses. The primary consequences of TLR activation are NF-κB activation, cytokine secretion, and the expression of co-stimulatory molecules [9,30]. These responses help to promote and shape the critical immunological processes that facilitate

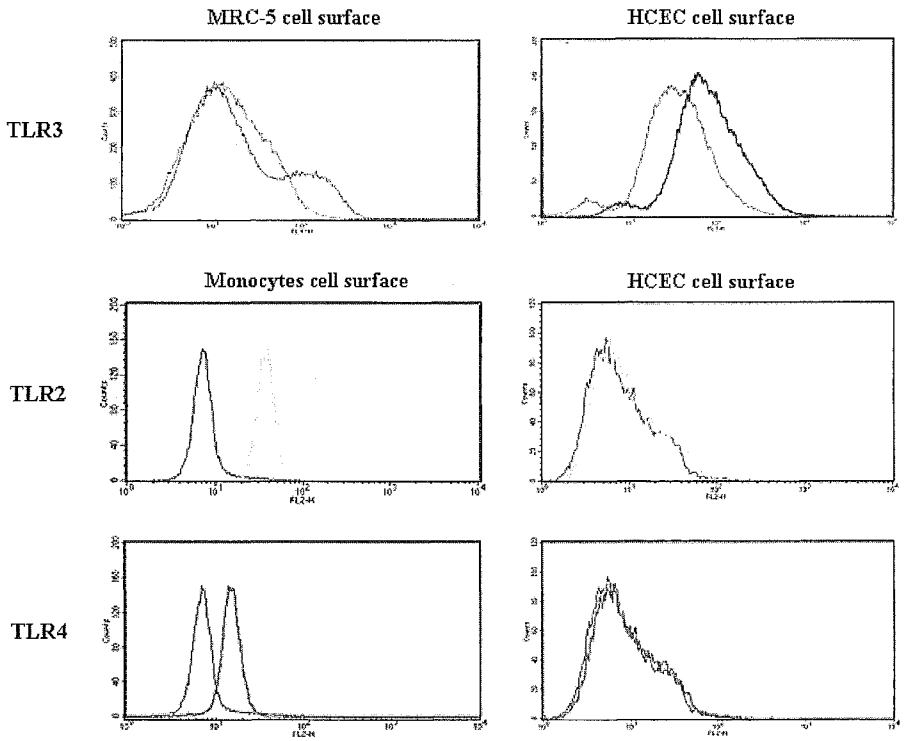


Fig. 2. Primary HCEC express TLR3, but not TLR2 and TLR4, on the cell surface. The cell-surface expression of TLR2, TLR3, and TLR4 was examined by flow cytometry. For TLR3 expression, cells were incubated (30 min, 4 °C) with mouse anti-human TLR3 monoclonal antibody or isotype control mouse IgG1. Alexa Fluor 488 goat anti-mouse IgG (H + L) was the secondary antibody. For TLR2 and TLR4 expression, cells were incubated (30 min, 4 °C) with PE-conjugated mouse anti-human TLR2 (TL2.1), TLR4 (HTA125) monoclonal antibody, or isotype control mouse IgG2a. The histogram data are representative of three separate experiments.

the control and clearance of pathogens. We examined whether polyI:C stimulation of primary HCEC induced mRNA specific for the IκB family, regulators of NF-κB, such as IκBα and MAIL. We found that the expression of IκBα- and MAIL-specific mRNA was in fact elevated by polyI:C but not LPS (Fig. 5). These mRNAs were not up-regulated in polyI:C-stimulated HPMC, but their expression was significantly up-regulated upon stimulation with LPS as described previously [31]. It is of note that MAIL-specific mRNA was elevated by both polyI:C and LPS in HCFB. Taken together, these findings show that polyI:C could up-regulate IκBα and MAIL expression in primary HCEC via TLR3 (Fig. 5).

PolyI:C stimulates the gene expression and surface expression of TLR3 in primary HCEC

In macrophages, high TLR3- but not TLR4 gene expression levels are induced by TLR3 and TLR4 agonists [32]. In the context of this autocrine loop of TLR3 expression, we examined whether TLR-specific mRNA was inducible in primary HCEC by the TLR3 agonist polyI:C. As shown in Fig. 6A, TLR3-specific mRNA was highly elevated in primary HCEC stimulated with polyI:C. Interestingly, increased TLR2 and TLR4 gene expression was also observed in polyI:C- but not LPS-stimulated primary HCEC. Furthermore, as shown in Fig. 6B, the cell-surface expression of TLR3, but not of TLR2 and TLR4, was increased. These observations raise interesting questions regarding the role of TLR3 in the host defense mounted by corneal epithelium.

Discussion

We provide evidence for the gene and surface expression of TLR3 in human corneal epithelium and suggest that expressed TLR3 is functionally active in the secretion of the inflammatory mediators IL-6, IL-8, and IFN-β. We thus documented that polyI:C can induce the secretion of inflammatory mediators by primary HCEC. The ability of IFN-β to prevent the death of anergic cells, in addition to its anti-proliferative effect, may be one way in which the immune system regains a quiescent state after activation [33]. Further studies are necessary to elucidate the pathological role of IFN-β produced by HCEC. It is noteworthy that TLR3 expression was up-regulated by the TLR3 agonist polyI:C. Furthermore, the up-regulation in primary HCEC of IκB-α and MAIL (a human homologue of IκBζ, a NF-κB regulator in the nucleus) by polyI:C suggests a

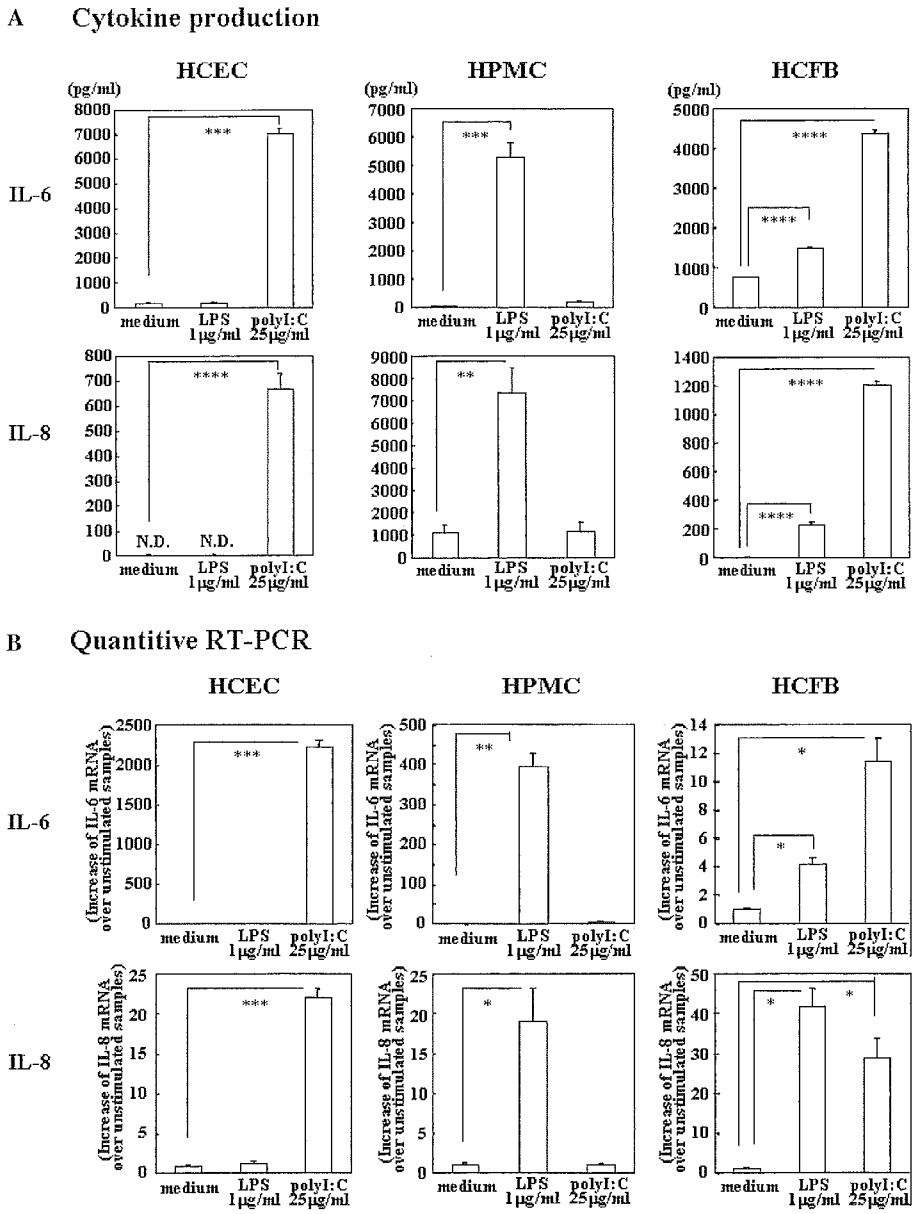
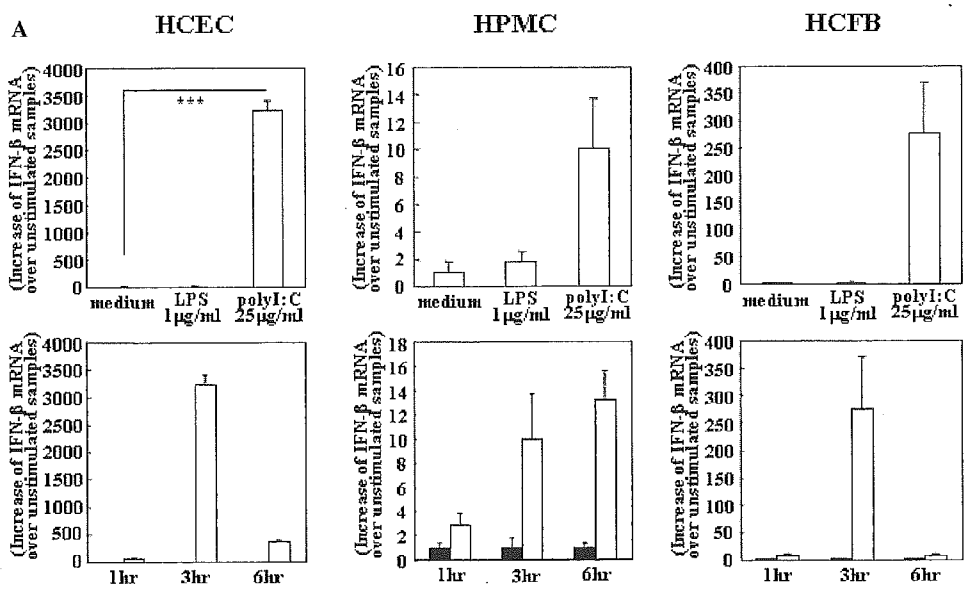


Fig. 3. The production and mRNA expression of IL-6 and IL-8 by primary HCEC. Primary HCEC and HCFB were cultured to sub-confluence and exposed to 1 μg/ml LPS from *P. aeruginosa* or 25 μg/ml polyI:C for 6 h. HPMC were cultured at a density of about 1×10^6 cells/ml and exposed to either 1 μg/ml LPS from *P. aeruginosa* or 25 μg/ml polyI:C for 6 h. (A) The culture supernatants were harvested and assayed by cytokine-specific ELISA for IL-6 and IL-8. (B) Total RNA was isolated from these cells with the Trizol reagent (Life Technologies). The RT reaction was performed with the SuperScript preamplification system (Invitrogen). Real-time semi-quantitative PCR was on an ABI-prism 7700. The *Y* axis shows the increase of specific mRNA over unstimulated samples. Data are representative of three separate experiments and show means ± SEM from an experiment carried out in triplicate wells (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; and ****$p < 0.001$).

novel role for TLRs in ocular surface physiology. The new findings presented here contribute to a better understanding of innate ocular surface immunity.

A novel IκB protein, IκBζ/MAIL, induced by IL-1 and PAMPs regulates NF-κB in the cell nucleus. The induction of IκBζ is controlled by NF-κB, which, in turn, is negatively regulated by IκBζ, thereby forming an autonomous negative-feedback loop [34]. We postulate that IκBζ in ocular surface epithelium negatively regulates the pathological progression of ocular surface inflammation [35].

IκBζ was originally reported as a regulator of NF-κB induced by IL-1 and LPS [36]. LPS stimulation induced IκBζ in macrophages [31,34], but not primary HCEC (Fig. 4C). Furthermore, IκBζ is reportedly indispensable for IL-6 production in response to TLR ligands and is a positive regulator of NF-κB in the two-step process of IL-6 gene activation [37]. The implications of the



B **The ratio of IFN-β/GAPDH mRNA at 3hr**

	HCEC	ratio to HCEC	HPMC	ratio to HCEC	HCFB	ratio to HCEC	MRC-5	ratio to HCEC	HeLa	ratio to HCEC
unstimulated samples	0.00074 ±0.00014	1	0.01821 ±0.01295	24.6	0.00031 ±0.00002	4/10	0.00003 ±0.00002	4/100	0.0004 ±0.00013	5/10
stimulated with polyI:C	2.39136 ±0.13371	1	0.14682 ±0.06039	6/100	0.11038 ±0.01085	5/100	0.0462 ±0.00201	2/100	0.0468 ±0.00338	2/100
increasing ratio	3224	1	8	2/1000	360	1/10	1540	5/10	117	4/100

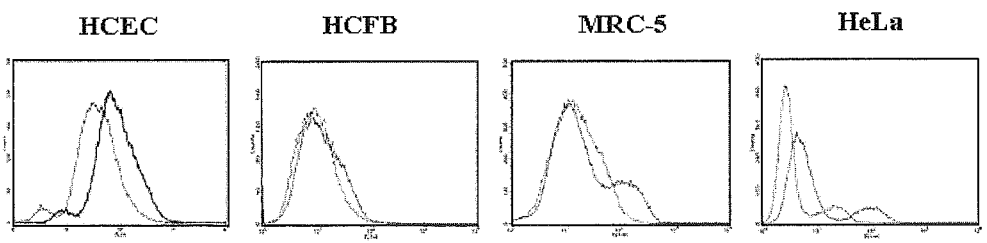


Fig. 4. Comparison of IFN-β mRNA expression and TLR3 cell-surface expression. (A) HCEC, HCFB, and HPMC were cultured as in Fig. 3 and exposed to either 1 μg/ml LPS from *P. aeruginosa* or 25 μg/ml polyI:C for 3 h. Subsequent procedures were as in the assay of mRNA expression. The *Y* axis shows the increase of specific mRNA over unstimulated samples. Data are representative of two separate experiments and show means ± SEM from an experiment carried out in triplicate wells (***$p < 0.005$). (B) The methods were as in (A). The actual ratio of IFN-β/GAPDH mRNA and the relative ratio in HCEC are summarized in the table. (C) Cell-surface expression of TLR3 was examined by flow cytometry. Cells were incubated (30 min, 4 °C) with mouse anti-human TLR3 monoclonal antibody or isotype control mouse IgG1. Alexa Fluor 488 goat anti-mouse IgG (H + L) was the secondary antibody. The histogram data are representative of three separate experiments.

induction by polyI:C of IκBζ/MAIL in primary HCEC remain to be determined.

The epithelial expression of TLRs may be of importance in inflammation and immunity in response to pathogens [38–41]. Unique patterns of TLR expression appear to exist at different host-environment tissue interfaces. Under physiological conditions, the corneal epithelium appears to be hyporesponsive to commensal bacteria to which it is consistently exposed. We previously reported that HCEC failed to respond functionally to LPS or PGN because they lack TLR2 and TLR4 on their cell surface [20]. Despite the presence of TLR2 and TLR4 in their cytoplasm, HCEC did not respond to experimentally translocated LPS [20]. This is indicative of a characteristic difference between HCEC and immune-competent cells such as macrophages. The selective expression of TLR3 in human corneal epithelium (Fig. 1) contrasts with the ubiquitous expression of TLR family members in HPMC and indicates that the regulation and localization of TLR3 are different

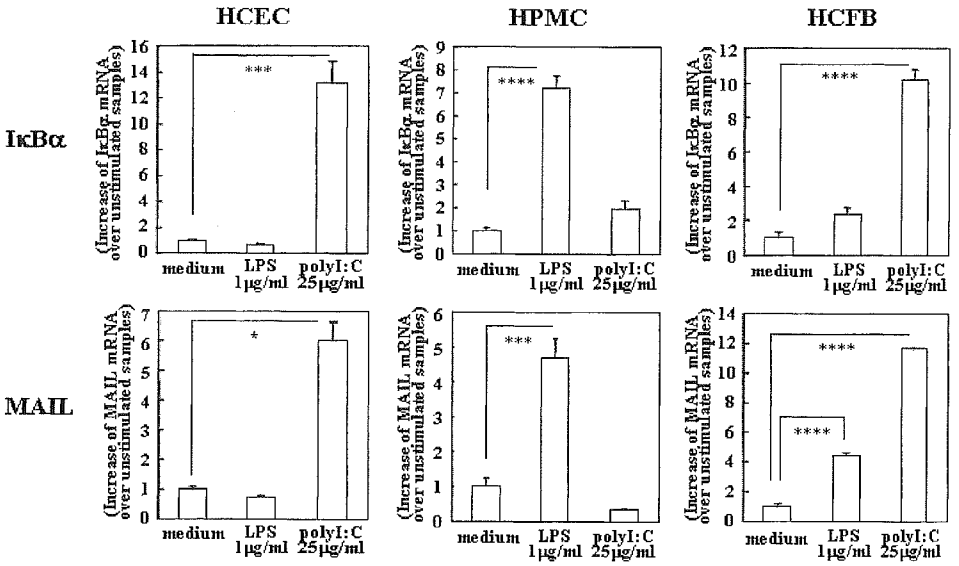


Fig. 5. Augmentation of MAIL and IκB-α gene expression in polyI:C-stimulated primary HCEC. HCEC, HCFB, and HPMC were cultured as in Fig. 3 and exposed to either 1 μg/ml LPS from *P. aeruginosa* or 25 μg/ml polyI:C for 3 h, subsequent procedures were as in the assay of mRNA expression. The *Y* axis shows the increase of specific mRNA over unstimulated samples. Data are representative of two separate experiments and show means ± SEM from an experiment carried out in triplicate wells ($*p < 0.05$; $***p < 0.005$; and $****p < 0.001$).

in these cells. This may reflect the participation of cell type-specific multiple pathways in antiviral IFN induction via TLR3 [42]. However, the previous work does not exclude the role of inflammation-dependent TLRs on HCEC or on antigen-presenting cells infiltrating corneal tissue. IFN-α up-regulated TLR3 mRNA expression in epithelial cells and IFN-γ enhanced TLR3 expression in epithelial- and endothelial cells [43]. In macrophages, TLR3 expression is inducible by both TLR3 and TLR4 ligands, although these stimuli fail to induce TLR4 expression. Furthermore, TLR3 and TLR4 require the IFN-β autocrine/paracrine feedback mechanism to induce TLR3 expression and to activate and/or enhance genes required for antiviral activity [32]. IFN-α/β is critical for the measles virus-mediated up-regulation of TLR3 induction [44]. Given that cell-surface TLR3 expression was up-regulated by an agonist of TLR3, polyI:C (Fig. 6), and that polyI:C was able to induce the gene expression of IFN-β in HCEC (Fig. 4B), it is conceivable that IFN-β is crucial for the innate immune response of the ocular surface to pathogenic and nonpathogenic viruses and bacteria.

LPS up-regulates TLR3 expression in murine phagocytic cells through autocrine IFN-β induction. In humans, however, the IFN-β-induced up-regulation of TLR3 was blocked by pretreatment with LPS [45]. This observation coincides with our present results that TLR3 expression was not up-regulated by an agonist of TLR4, LPS (Fig. 6), and that LPS was incapable of inducing the gene expression of IFN-β in HCEC (Fig. 4A). The species-specific differences between humans and mice in their responses to LPS coincide with the presence of different, evolutionary nonconserved promoter sequences in both species. The intriguing functionality of TLR3 in HCEC and the far more potent induction of IFN-β by a TLR3 agonist in HCEC than fibroblasts require elucidation of the molecular mechanisms that regulate TLR3 expression on HCEC.

Although all TLRs activate NF-κB, not all TLRs activate IRF3 or induce IFN-β expression. TLR3 and TLR4 are the best-characterized TLRs known to activate IRF3 [30]. The TIR domain-containing adapter-inducing IFN-β (TRIF) is also an adapter for TLR3 and TLR4 [46,47]. Unlike the other TLRs including TLR4 that use the common MyD88-dependent pathway, TLR3 seems to employ only MyD88-independent TRIF-dependent pathways. These biochemical findings may account for the distinctly different responses elicited by polyI:C and LPS in the present study.

Type I IFN is induced not only by viral but also by bacterial infection [22,48]. An understanding of the role of the TLR3-IFN-β-link is crucial for understanding the involvement of type I IFN in TLR3-induced biological effects on the ocular surface. O'Conell et al. [49] reported that type I IFNs play a different role in bacterial and viral infections. The sophisticated interplay between bacteria and viruses may culminate in the exacerbation of pathological inflammation on the ocular surface.

In summary, the innate immune responses in mucosal epithelial cells such as HCEC differ from those in immune-competent cells such as macrophages. The elucidation of the unique innate immune response in mucosal epithelium is critical for a better understanding of the symbiotic relationship between mucosal epithelial cells and commensal bacteria inhabiting the mucosal surface.

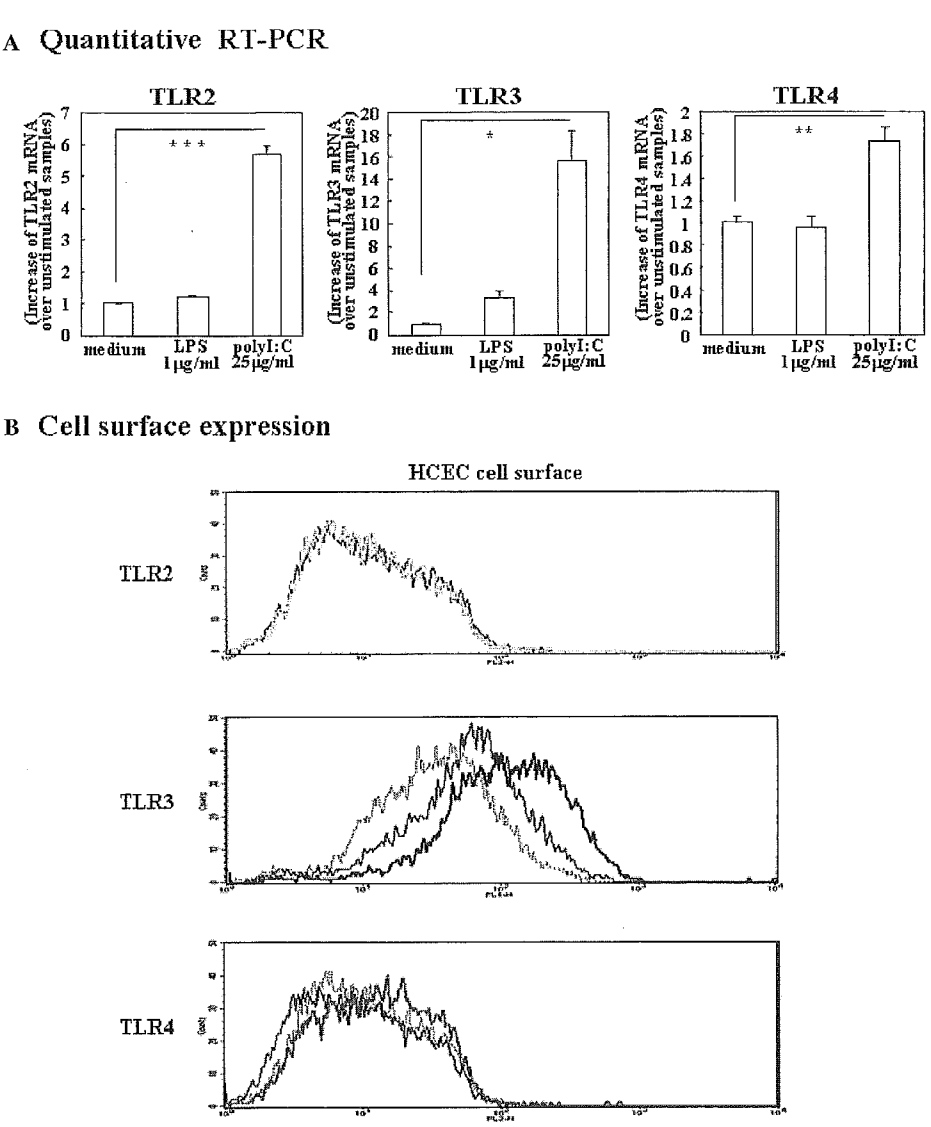


Fig. 6. Augmentation of TLR3 expression on HECE by polyI:C stimulation. (A) Augmentation of TLR3 gene expression in polyI:C-stimulated primary HCEC. Primary HCEC were cultured to sub-confluence in 25 cm^2 flasks (2×10^6 cells/flask) and exposed to 1 μg/ml LPS from *P. aeruginosa* or 25 μg/ml polyI:C for 6 h. RNA extraction, RT reaction, and real-time semi-quantitative PCR were as in Fig. 3. The *Y* axis shows the increase of specific mRNA over unstimulated samples. Data are representative of three separate experiments and show means ± SEM from an experiment carried out in triplicate wells. (B) Augmentation of TLR3 cell-surface expression on polyI:C-stimulated primary HCEC. Primary HCEC were cultured to sub-confluence in 75 cm^2 flasks (6×10^6 cells/flask) and untreated or exposed to 25 μg/ml polyI:C for 6 h. The cell-surface expression of TLR2, TLR3, and TLR4 was examined by flow cytometry. For TLR3 expression, cells were incubated (30 min, 4 °C) with mouse anti-human TLR3 monoclonal antibody or isotype control mouse IgG1. Alexa Fluor 488 goat anti-mouse IgG (H + L) was the secondary antibody. For TLR2 and TLR4 expression, cells were incubated (30 min, 4 °C) with PE-conjugated mouse anti-human TLR2 (TL2.1), TLR4 (HTA125) monoclonal antibody, or isotype control mouse IgG2a. The histogram data are representative of two separate experiments (dotted line, isotype control; thin line, untreated; and bold line, stimulated with 25 μg/ml polyI:C for 6 h).

Acknowledgments

This work was supported in part by Grants-in-Aid for scientific research from the Japanese Ministry of Health, Labour and Welfare, the Japanese Ministry of Education, Culture, Sports, Science and Technology, CREST from JST, a research grant from the Kyoto Foundation for the Promotion of Medical Science, and the Intramural Research Fund of Kyoto Prefectural University of Medicine. We thank Dr. T. Seya and Dr. J. Yamada for the gift of MRC5, HeLa cells, and HCFB, respectively, and Ms. C. Mochida for her technical assistance.

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GASTROENTEROLOGY 2005;128:922–934

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.

0016-5085/05/$30.00
doi:10.1053/j.gastro.2005.01.013

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 antiapoptotic 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′-TGCACCTGACGCCCTTCAC-3′; antisense, 5′-TAGCTGATTCGACCATT TGCCTGA-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense, 5′-TTCACCACCATGGAGAAGGC-3′; antisense, 5′-GGCATGGACTGTGGTCATGA-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

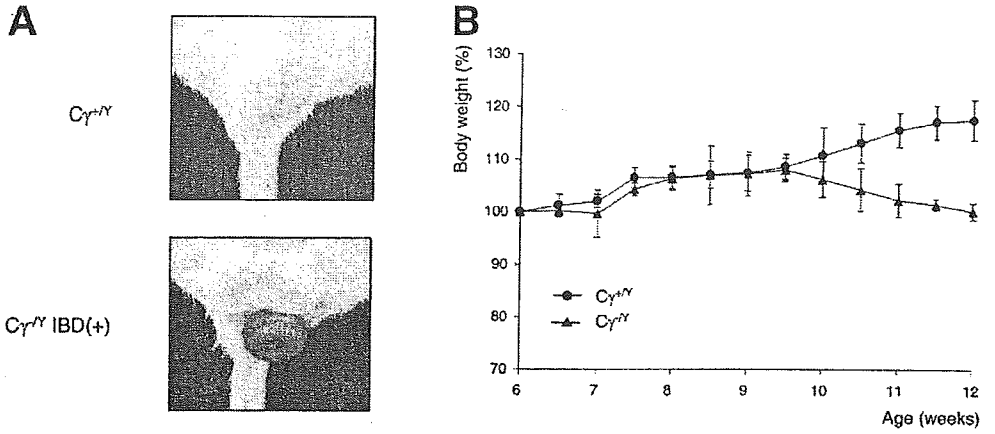


Figure 1. Gross appearance of the (*A*) anorectal prolapse and (*B*) body weight changes in $CR\gamma^{+/Y}$ mice, $CR\gamma^{-/Y}$ mice without IBD, and $CR\gamma^{-/Y}$mice with IBD. $CR\gamma^{-/Y}$ mice with IBD showed anal prolapse beginning at 8 weeks of age and obvious lack of body weight gain 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 $CR\gamma^{-/Y}$ 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

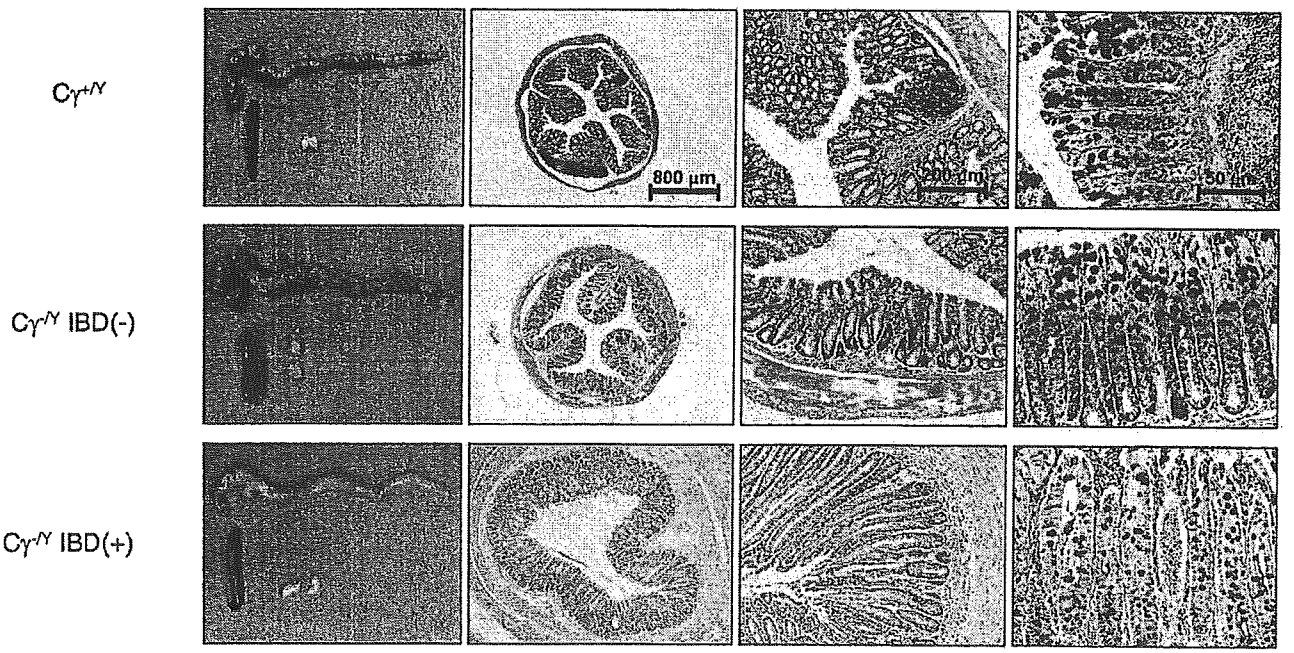


Figure 2. Macroscopic and microscopic appearance of the large intestine in $CR\gamma^{+/Y}$ mice, $CR\gamma^{-/Y}$ mice without IBD, and $CR\gamma^{-/Y}$ mice with IBD. The large intestine of the mice was dissected for routine histologic analysis, including fixing with 4% paraformaldehyde, embedding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

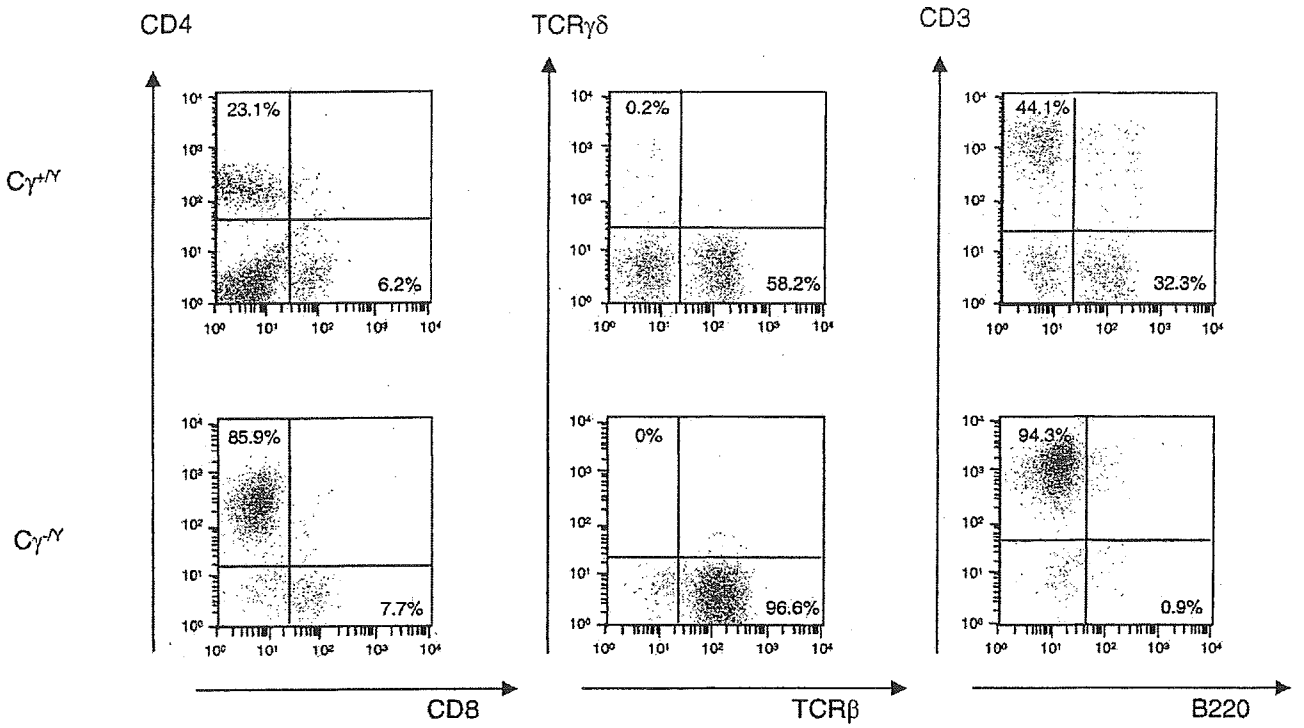


Figure 3. Flow cytometric analysis of colonic LP lymphocytes isolated from CRγ$^{-/Y}$ and CRγ$^{+/Y}$ mice. CRγ$^{-/Y}$ mice did not possess mature B cells or TCR γδ T cells but had increased numbers of CD4$^+$ T cells. The data are representative of results from 2 independent experiments.

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

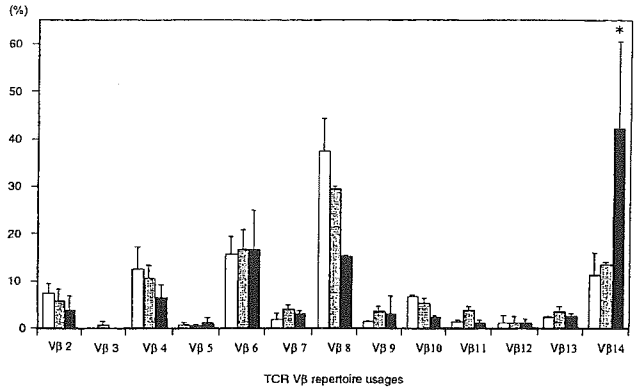


Figure 4. Flow cytometric analysis of the TCR Vβ repertoire of CD4+ T cells isolated from colonic LP of the CRγ$^{-/Y}$ and CRγ$^{+/Y}$ mice. Mononuclear cells isolated from CRγ$^{-/Y}$ mice with or without colitis and control CRγ$^{+/Y}$ mice were costained with mAbs specific for FITC/TCR Vβ and PE/CD4. 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+

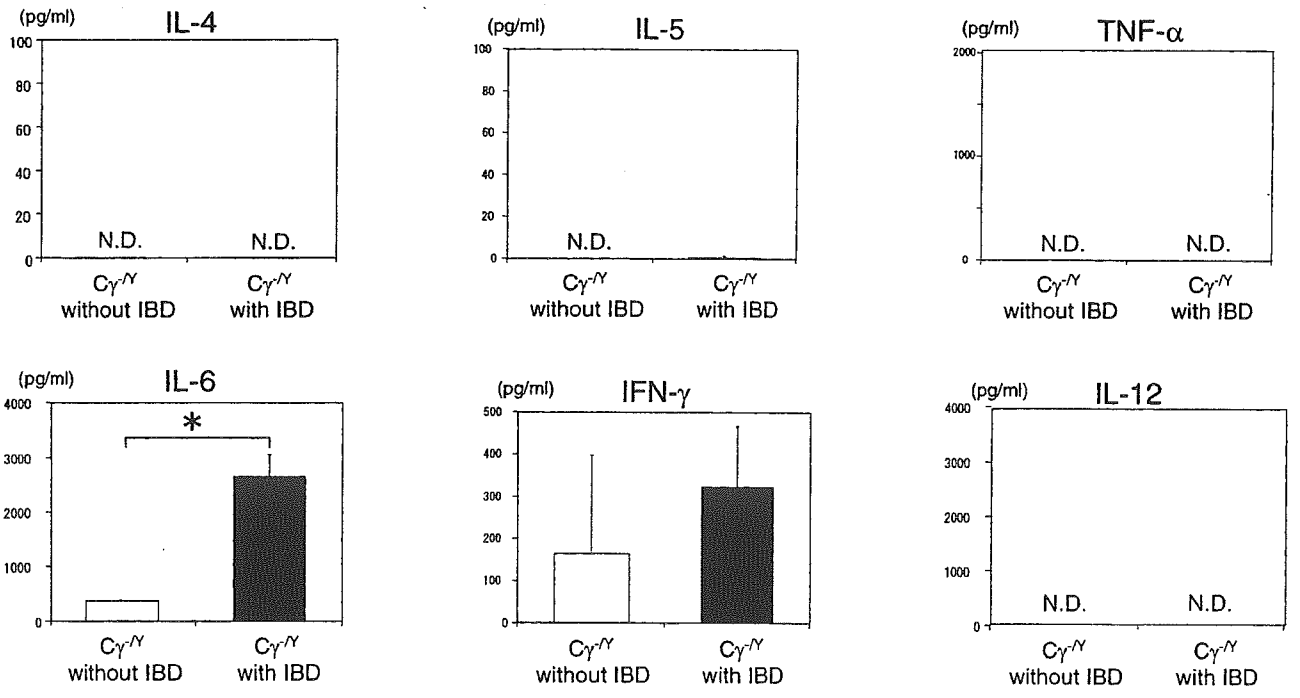


Figure 5. Analysis of cytokine production expression by colonic CD4+ T cells isolated from CRγ$^{-/Y}$ mice with (*closed squares*) and without (*open squares*) IBD. Whole colonic LP mononuclear cells were incubated in complete medium with 10% fetal calf serum for 48 hours without any exogenous stimulation. 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 (*$P < .05$).

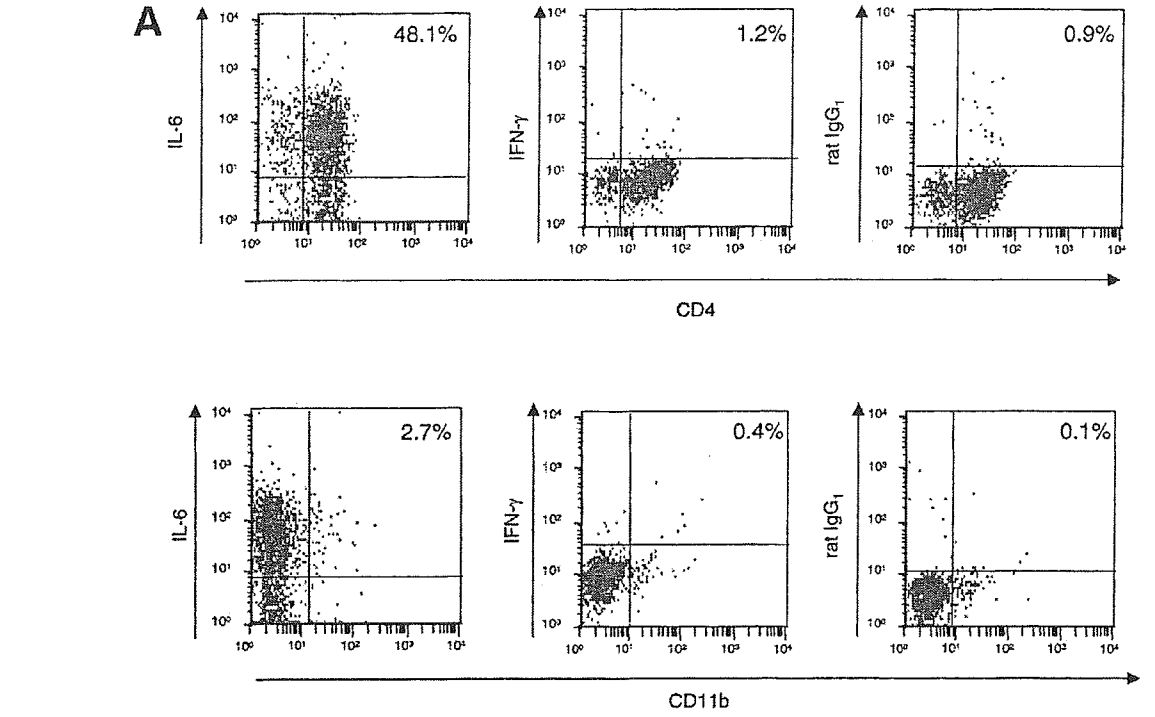


Figure 6. Characterization of colonic IL-6–producing $CD4^+$ T cells from diseased mice. (*A*) Lymphocytes were isolated from the colons of $CR\gamma^{-/Y}$ mice with IBD and then immediately costained with the appropriate combination of CD4, CD11b, IFN-γ, and IL-6 for intracellular cytokine FACS analysis. (*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-

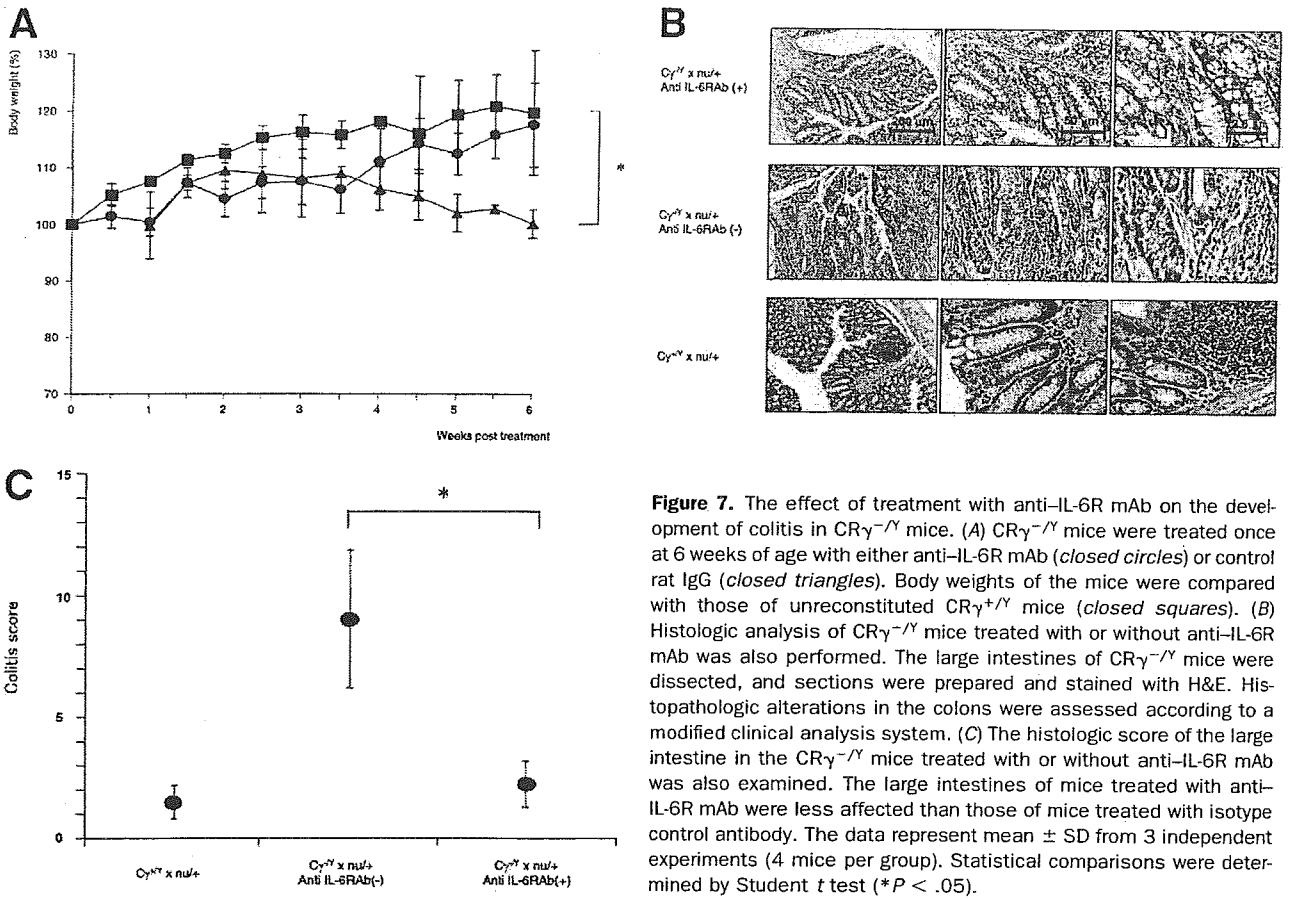
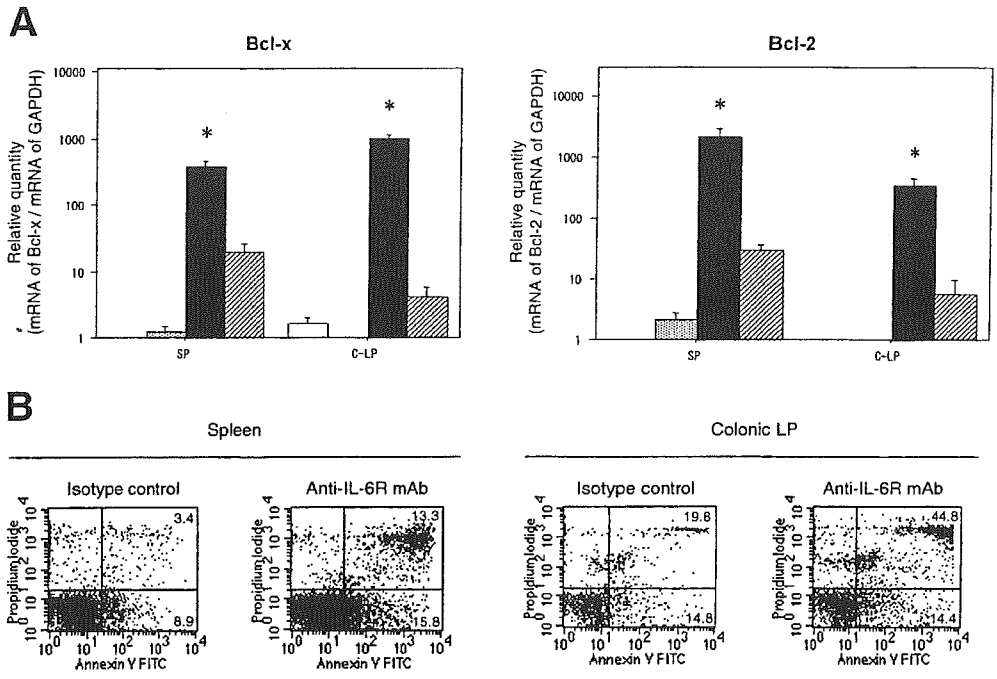


Figure 7. The effect of treatment with anti–IL-6R mAb on the development of colitis in CRγ$^{-/Y}$ mice. (*A*) CRγ$^{-/Y}$ mice were treated once at 6 weeks of age with either anti–IL-6R mAb (*closed circles*) or control rat IgG (*closed triangles*). Body weights of the mice were compared with those 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

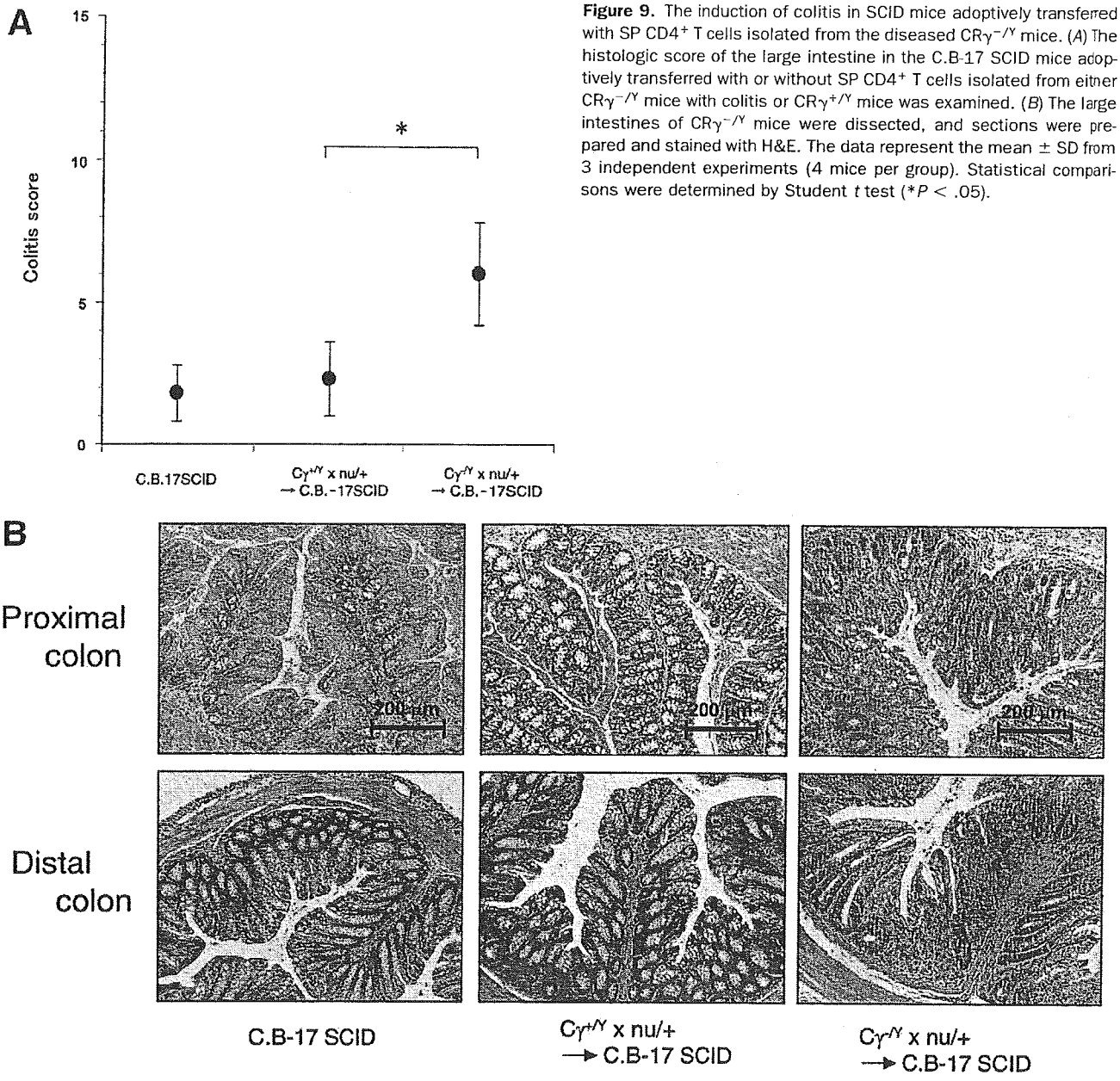


Figure 9. The induction of colitis in SCID mice adoptively transferred with SP CD4+ T cells isolated from the diseased CRγ$^{-/Y}$ mice. (*A*) The histologic score of the large intestine in the C.B-17 SCID mice adoptively transferred with or without SP CD4+ T cells isolated from either CRγ$^{-/Y}$ mice with colitis or CRγ$^{+/Y}$ mice was examined. (*B*) The large intestines of CRγ$^{-/Y}$ 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 CRγ$^{-/Y}$ or CRγ$^{+/Y}$ 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 CRγ$^{+/Y}$ 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 CRγ$^{-/Y}$ 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 CRγ$^{-/Y}$ 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.[52]

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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