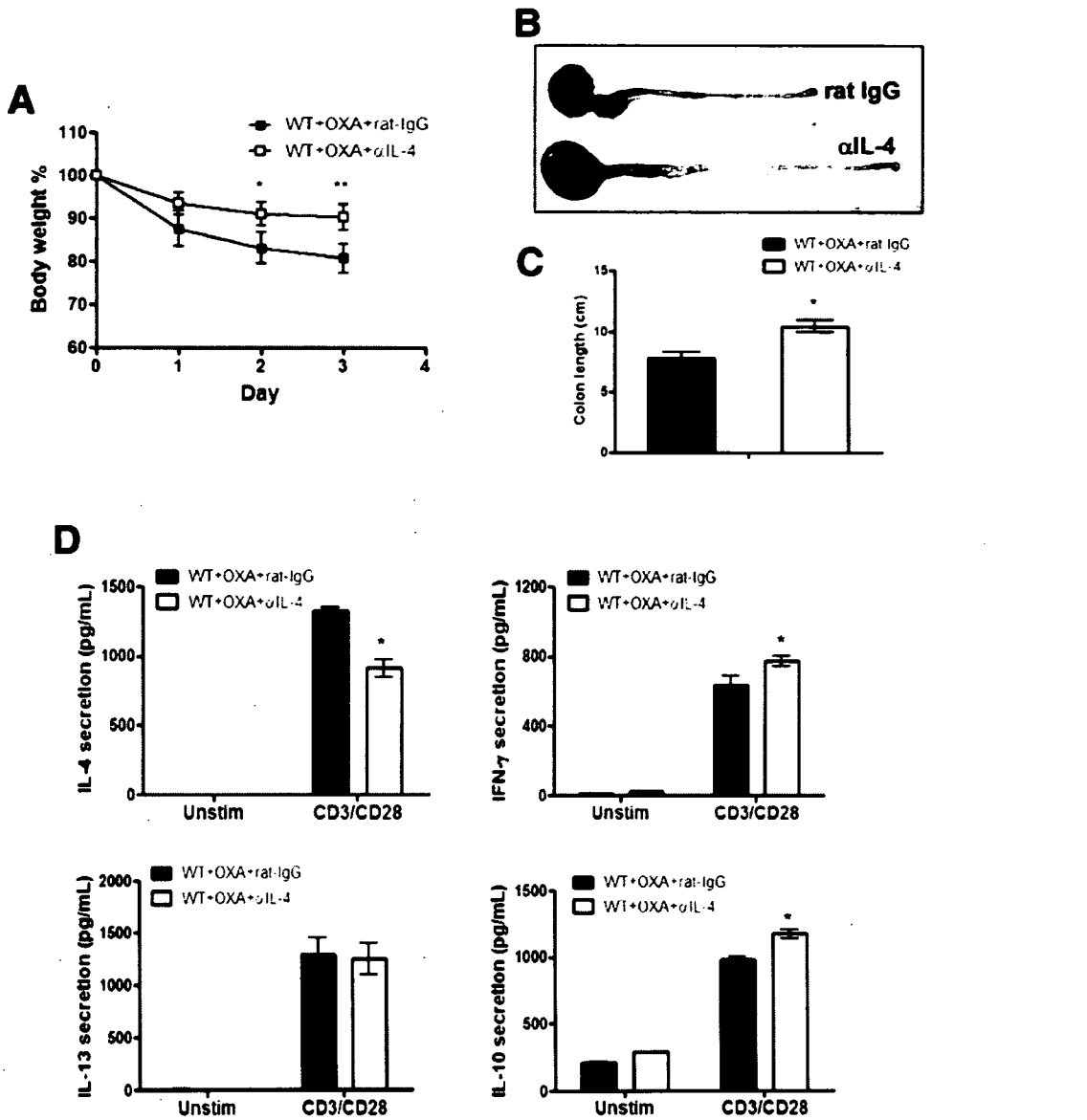

Supplementary Figure 3. Effects of in vivo treatment with anti-IL-4 mAb on OXA-induced colitis. WT mice were treated with anti-IL-4 (0.3 mg per dose) or control rat IgG (0.3 mg per dose) at the time of colitis induction with OXA. The body weight loss (*A*), macroscopic appearance of colons (*B*) and colon length (*C*) were monitored daily. Cytokine production (*D*) on day 3 after OXA treated was assayed by ELISA. Each *column* and *vertical bar* indicates means ± SD for 5 mice of each group. Data of a representative are shown from 3 independent experiments. Statistically significant differences are shown (*$P < .05$ or **$P < .01$).

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Original Article

Blockade of interferon-γ-inducible protein-10 attenuates chronic experimental colitis by blocking cellular trafficking and protecting intestinal epithelial cells

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The role of chemokines, especially CXCL10/interferon-γ-inducible protein 10 kDa (IP-10), a chemokine to attract CXCR3$^+$ T-helper 1-type CD4$^+$ T cells, is largely unknown in the pathophysiology of inflammatory bowel disease; ulcerative colitis and Crohn's disease. The authors have earlier shown that IP-10 neutralization protected mice from acute colitis by protecting crypt epithelial cells of the colon. To investigate the therapeutic effect of neutralization of IP-10 on chronic colitis, an anti-IP-10 antibody was injected into mice with newly established murine AIDS (MAIDS) colitis. Anti-IP-10 antibody treatment reduced the number of colon infiltrating cells when compared to those mice given a control antibody. The treatment made the length of the crypt of the colon greater than control antibody. The number of Ki67$^+$ proliferating epithelial cells was increased by the anti-IP-10 antibody treatment. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)$^+$ apoptotic cells were observed in the epithelial cells of the luminal tops of crypts in control MAIDS colitis, whereas TUNEL$^+$ apoptotic epithelial cells were rarely observed with anti-IP-10 antibody treatment. In conclusion, blockade of IP-10 attenuated MAIDS colitis through blocking cellular trafficking and protecting intestinal epithelial cells, suggesting that IP-10 plays a key role in the development of inflammatory bowel disease as well as in chronic experimental colitis.

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Chemokines, which are chemotactic cytokines, control the essential process of the attraction of leukocytes to tissues in inflammation.[1,2] The chemokine family consists of two major subfamilies, termed CXC and CC according to the arrangement of the first two conserved cysteines that are separated by one amino acid and are adjacent, respectively.[1,2] The interferon (IFN)-γ-inducible protein of 10 kDa (IP-10/CXCL10) is a member of the CXC chemokine family, and a potent chemoattractant for activated T lymphocytes, natural killer (NK) cells, and monocytes.[3,4] It is also considered as a regulator of the T-helper 1 (Th1) inflammatory response.[5] It has been reported that the expression of IP-10 was elevated in several diseases such as ulcerative colitis, hepatitis, multiple sclerosis, and Sjögren's syndrome, suggesting the involvement of IP-10 in the development of these diseases.[6–9] Information on the role of IP-10 in inflammatory bowel disease is limited, and needs further detailed investigation.

We have recently reported that neutralization of IP-10 protected mice from dextran sulfate sodium (DSS)-induced acute colitis by promoting crypt cell survival, without altering the infiltration of immune cells.[10] Furthermore, recombinant IP-10 administration into mice directly inhibited the intestinal epithelial cell proliferation.[10] In contrast, another report showed that IP-10 neutralization dominantly inhibited inflammation, and ameliorated colitis in interleukin-10 (IL-10)-deficient mice.[11] Thus, the therapeutic mechanism of IP-10 neutralization on experimental colitis has yet to be shown clearly.

The LP-BM5 murine leukemia virus (MuLV) is a retrovirus that induces profound immunodeficiency similar to that of human acquired immunodeficiency syndrome (AIDS), therefore it has been studied as a murine model of AIDS, thus termed murine AIDS (MAIDS).[12–15] We have previously reported that systemic exocrinopathy resembling Sjögren's syndrome and pancreatitis-like lesions were induced in mice with MAIDS, and colitis was not induced in MAIDS mice.[16,17] In contrast, nude mice inoculated with lymph node cells from mice with MAIDS developed chronic inflammatory bowel disease-like colitis, which we termed MAIDS colitis.[18] The precise mechanism of pathogenesis of the colitis remains largely unknown, however, regulatory T cells (Treg) deficiency might play a role in its development because there are some reports of colitis modulated by Treg.[19,20] We demonstrated that the pathological lesions of MAIDS colitis resembled ulcerative colitis, and that the major populations of colon-infiltrating cells in MAIDS colitis were $Mac1^+$ macrophages and $CD4^+$ T cells with polarized immune responses toward Th2.[21] Thus, MAIDS colitis could serve as an animal model for ulcerative colitis.

There have been other colitis models developed including DSS colitis, IL-10-deficient mice, and $Rag2^{-/-}$ mice reconstituted with $CD4^+CD45RB^{high}$ T cells, which are characterized as a Th1-dependent disease, mimicking Crohn's disease.[22] The diversity in the etiopathophysiology of these colitis models might induce different effects with IP-10 blockade. Therefore, to confirm the therapeutic mechanism of IP-10 neutralization, it is better to analyze the effect of anti-IP-10 treatment in different type of colitis models. In this report, to address this point, we investigated the effect of IP-10 neutralization on MAIDS colitis.

MATERIALS AND METHODS

Animals

Four-week-old female C57BL/6 (B6) mice were purchased from Charles River Japan (Atsugi, Japan). B6 nude mice were provided by Dr Norimitsu Satoh at the Animal Center of Niigata University School of Medicine. All mice were maintained at the same animal center under specific pathogen-free conditions. All animal experiments were performed according to the Guide for Animal Experimentation of Niigata University School of Medicine.

Induction of MAIDS and MAIDS colitis

Four-week-old B6 female mice were injected i.p. with 0.3 mL LP-BM5 MuLV stock solution. Induction of MAIDS was confirmed when the mice developed generalized lymphadenopathy. Eight weeks after virus inoculation, mice with MAIDS were killed by cervical dislocation under ether anesthesia, and their all lymph nodes were collected. To induce MAIDS colitis, the lymph nodes were pressed and passed through a steel mesh, and the cell suspension was transferred i.v. to 10–13-week-old female B6 nude mice at a dose of $3–5 \times 10^7$ lymph node cells/head. Symptoms of colitis such as diarrhea and anal bleeding were observed 3 weeks after cell transfer, and all the mice died within 6 weeks after cell transfer. LP-BM5 MuLV was prepared from the supernatant of cloned G6 cells infected with the retrovirus as reported previously.[16] For blocking experiments, PBS containing 100 μg/100 μL anti-CXCL10 mAb[10] or antihuman parathyroid-related peptide mAb, which was the IgG1 subclass-matched control mAb, or PBS alone were administered i.p. at the time of cell transfer and once a week thereafter. Three weeks after cell transfer, the mice were killed and their colons were removed for further analysis. Five mice were analyzed for each group and all the experiments were repeated three times.

Histopathological examination

Tissue samples were taken from the sigmoid colon, fixed in 10% buffered formalin, and then embedded in paraffin wax blocks. Four μm-thick sections were made in the usual way and stained with HE. The stained sections were then examined by light microscopy.

The entire colon (5 mice/group) was sampled. We analyzed the distal colon tissue section located approximately 10 mm from the anal verge to calculate the mean number of infiltrating cells of five different points in the lamina propria of the colon in a high-power field (×400) under a microscope. The crypt length of the colon of each mouse was also calculated as a mean value of five different crypts.

Preparation and flow cytometric analysis of cells that infiltrated the colon

Mouse colonic mucosal mononuclear cells were prepared as follows. Lamina propria mononuclear cells were isolated from the colon at 3 weeks after the induction of colitis as described here. Briefly, the entire colon was opened longitudinally, washed with PBS, and cut into small pieces. The pieces were then incubated with Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) containing 2.5% fetal bovine serum (FBS) and 1 mmol dithiothreitol (DTT) (Sigma-Aldrich, St Louis, MO, USA) for 30 min to remove mucus and then serially incubated twice in Ca^{2+}- and Mg^{2+}-free HBSS containing 2.5% FBS and 0.75 mmol EDTA (Sigma-Aldrich) for 1 h each. The supernatants from these incubations were collected, pooled, and treated with 1 mg/mL collagenase

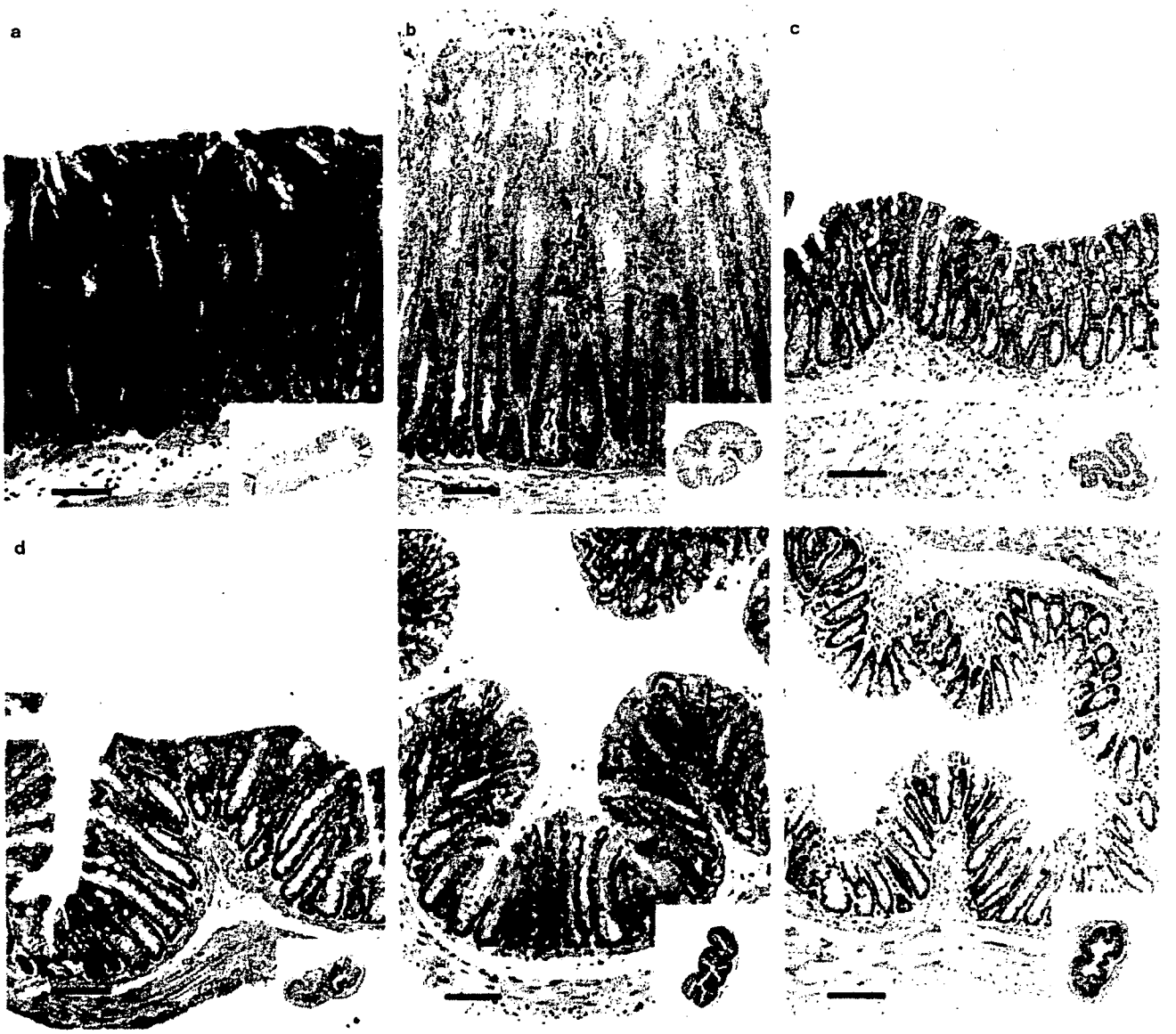

Figure 1 (**a–d**) Neutralization of interferon-γ-inducible protein 10 kDa (IP-10/CXCL10) attenuated murine acquired immunodeficiency syndrome (MAIDS) colitis. Distal colon tissues: (**a**) control antibody-treated mouse at 3 weeks after induction of colitis, (**b**) anti-IP-10 antibody-treated mouse, (**c**) normal B6 nude mouse, (**d**) normal B6 mouse, (**e**) anti-IP-10 antibody-treated B6 nude mouse, (**f**) anti-IP-10 antibody-treated B6 mouse. (**a**) Mild erosions of crypt epithelial cells at the tip of the crypt, and crypt abscess were observed in the MAIDS colitis. HE, original magnification ×100. Bars, 100 μm.

(Worthington Biomedical, Freehold, NJ, USA) and 0.01% Dnase (Worthington Biomedical) in medium for 2 h. The cells were pelleted twice through a 40% isotonic Percoll solution (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and then further purified by Ficoll-Hypaque (Pharmacia, Inc., Pisataway, NJ, USA) density gradient centrifugation (40/75%) at 1300 *g*.

Cell suspensions were prepared in PBS containing 1% fetal calf serum and 0.1% sodium azide. The cells were incubated with anti-Fc receptor mAb (2.4G2) for 10 min at 4°C, and then incubated with fluorescent isothiocyanate (FITC)-conjugated mAb and phycoerythrin-conjugated mAb for 30 min. The stained cells were washed twice, resuspended, and analyzed using FACScan (Becton-Dickinson, Mountain View, CA, USA).

Immunohistochemical staining for Ki67

To evaluate the number of proliferating crypt epithelial cells of the colon, we used rabbit polyclonal anti-Ki67 antibody (YLEM, Rome, Italy). Formalin-fixed and paraffin-embedded

colon sections were deparaffinized with xylene and ethanol, and then incubated with the first antibody. FITC-labeled anti-rabbit sheep antibody was then reacted as a secondary antibody. The number of $Ki67^+$ crypt epithelial cells was evaluated under high-power fields on immunofluorescence microscopy.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

Apoptotic cells were identified using an *in situ* apoptosis-detection kit (Takara Biomedicals, Otsu, Japan) according to the manufacturer's instructions. In brief, paraffin-embedded colon sections were deparaffinized, rehydrated and then incubated with terminal deoxynucleotidyl transferase mixture containing FITC-dUTP for 90 min at 37°C. After mounting, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling $(TUNEL)^+$ cells were counted in a crypt at five different points in high-power fields (×400) on fluorescent microscopy.

Statistical analysis

Data are expressed as mean ± SD. The unpaired Student's *t*-test or the non-parametric Mann–Whitney test was used for statistical analysis. Differences were considered significant at $P < 0.05$.

RESULTS

IP-10 neutralization attenuated MAIDS colitis

In mice treated with control antibody, a marked mononuclear cellular infiltration was observed in the mucosal and submucosal layer at 3 weeks after the induction of colitis (Fig. 1a) in comparison to normal B6 (Fig. 1c) or nude mice (Fig. 1d). Mild erosions of crypt epithelial cells were observed at the tip of crypt, and crypt abscess was also observed in some crypts (Fig. 1a). The number of infiltrating cells in the colon increased gradually with colon weight, and the crypt length significantly increased in MAIDS colitis with control antibody compared to untreated B6 or nude mice (Fig. 2). To evaluate the effect of IP-10 neutralization, we have analyzed the lesions of MAIDS colitis at 3 weeks after the induction of colitis (Fig. 1b). Because a long-term anti-IP-10 antibody treatment induced immunodeficiency and opportunistic infection such as pulmonary abscess at 6 weeks, we therefore carried out the analysis at 3 weeks after the induction of colitis. Neutralization of IP-10 decreased cellular infiltration into the colon of mice with MAIDS colitis (Fig. 2a). In addition, the length of the crypt became significantly greater by IP-10 blockade compared to control MAIDS colitis (Fig. 2b). Neutralization of IP-10 did not produce any significant change either in the number of cells in the lamina propria of the colon or in the length of the crypt of the colon in B6 nude or B6 mice (Figs 1e,f,2).

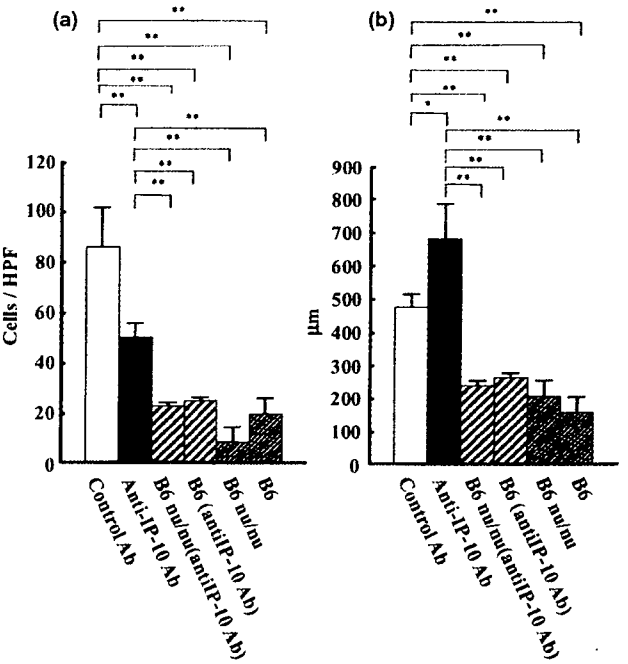

Figure 2 Effect of neutralization of interferon-γ-inducible protein 10 kDa (IP-10/CXCL10) on (**a**) number of infiltrating cells in the lamina propria of the colon at 3 weeks after induction of colitis and (**b**) crypt length of colon of mice at 3 weeks after induction of colitis. HPF, high-power field. $^*P < 0.05$; $^{**}P < 0.01$.

IP-10 neutralization reduced the number of immune cells in the colon of MAIDS colitis

Our previous immunohistochemical analyses showed that $CD4^+$ T cells and $Mac1^+$ macrophages are major populations in the colon of MAIDS colitis, with minor populations of $CD8^+$ T, and B cells.[18,21] In the present study, to determine the effect of IP-10 neutralization on the cellular components of the colon of MAIDS colitis, we analyzed the population of mucosal mononuclear cells that were isolated from the colon using a flow cytometer. In MAIDS colitis, $CD4^+$ and $CD8^+$ T cells with αβ T-cell receptors, and $Mac1^+$ cells were major populations that infiltrated the colon (Fig. 3). NK cells and granulocytes were minor populations (Fig. 3). Neutralization of IP-10 reduced the total number of inflammatory mononuclear cells infiltrating in the colon from 1.4×10^6 cells to 0.4×10^6 cells at 3 weeks after induction of the colitis. Additionally, IP-10 blockade significantly reduced the percentage

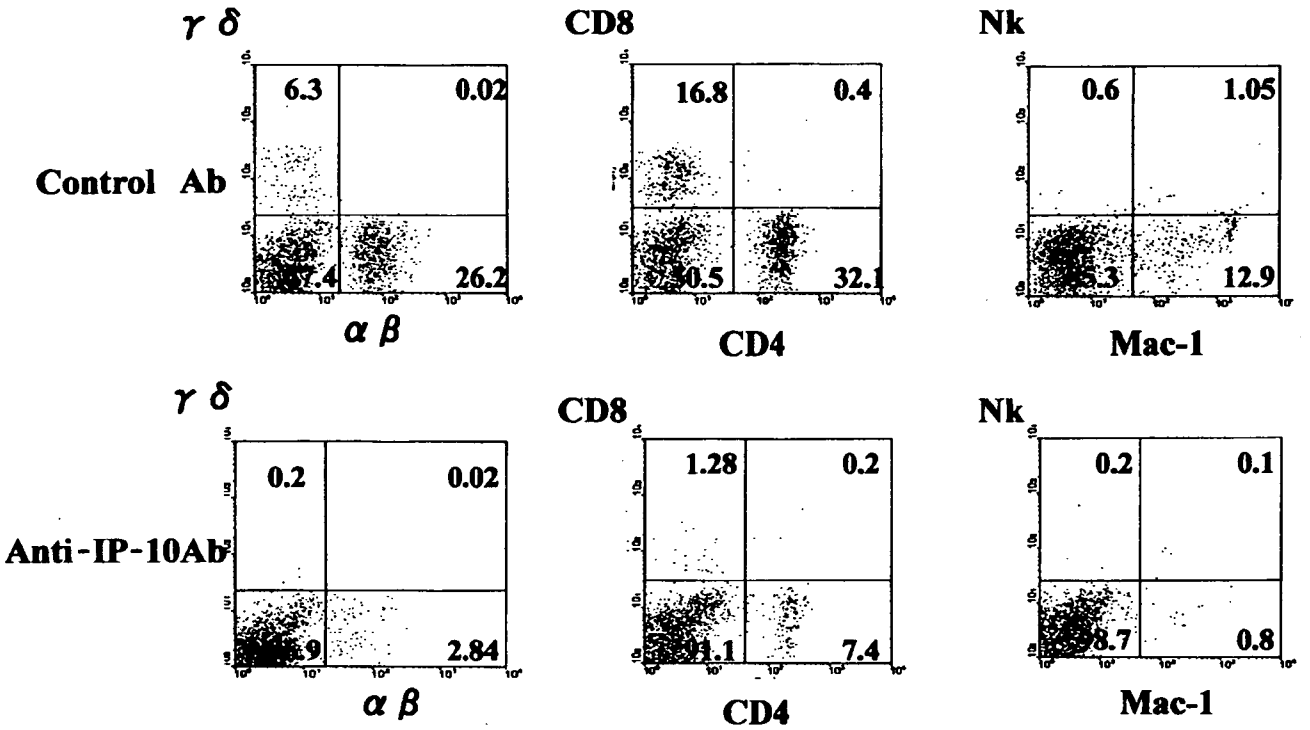

Figure 3 Neutralization of interferon-γ-inducible protein 10 kDa (IP-10/CXCL10) inhibited immune cell trafficking into the colon of murine acquired immunodeficiency syndrome (MAIDS) colitis. Two-color staining for T-cell receptor (TCR)-αβ and TCR-γδ, CD4 and CD8, and Mac1 and NK-1.1. Mononuclear cells in the colon of MAIDS mice at 3 weeks after induction of colitis were analyzed by flow cytometry; cells of mice treated with anti-IP-10 antibody were compared with those of mice treated with control antibody. Numbers in the figure represent the percentages of fluorescence-positive cells in corresponding areas. Representative results of three experiments are depicted.

of each population of $CD4^+$ and $CD8^+$ T cells possessing αβ T-cell receptors, and $Mac1^+$ cells in the colon (Fig. 3).

IP-10 neutralization increased $Ki67^+$ cells and decreased $TUNEL^+$ cells in the crypt epithelia of MAIDS colitis

We assessed the Ki67 staining to evaluate the proliferation effect of anti-IP-10 antibody treatment on colonic epithelial cells in attenuation of the colitis. In untreated B6 or B6 nude mice, $Ki67^+$ crypt epithelial cells were detected at basal one-fourth of a crypt (Fig. 4c–e). As we reported previously, crypt epithelial hyperplasia was observed in MAIDS colitis, and $Ki67^+$ cells were detected at one-third of the basal side of a crypt in the colon of MAIDS colitis with significantly increased number (Fig. 4a,e). Anti-IP-10 antibody treatment remarkably increased the number of $Ki67^+$ cells, and they were detected at a lower half of the crypt in anti-IP-10 mAb-treated mice (Fig. 4b,e).

Our previous report showed anti-apoptotic effect by IP-10 blockade on colonic epithelial cells in DSS colitis, therefore we assessed the apoptotic epithelial cells in the colons of mice with MAIDS colitis using the TUNEL method to detect DNA fragmentation *in situ*. $TUNEL^+$ apoptotic cells were observed in the epithelial cells of luminal tops of crypts in control MAIDS colitis (Fig. 5a). In anti-IP-10 antibody-treated mice, $TUNEL^+$ apoptotic epithelial cells were rarely observed (Fig. 5b).

DISCUSSION

In the present study we demonstrated that blockade of IP-10 attenuated MAIDS colitis not only by blocking cellular trafficking, but also by protecting crypt epithelial cells of the colon.

IP-10 was initially characterized as a chemoattractant for T lymphocytes, and binds to its receptor CXCR3, which is associated with Th1 immune responses.[1–4] The concept of selective mobilization of Th1 lymphocytes by IP-10 has been supported by several types of disease models[5] including multiple sclerosis,[23] rheumatoid arthritis, and inflammatory bowel disease.[11,24] Inflammatory bowel disease consists of two major forms: ulcerative colitis and Crohn's disease. Crohn's disease is suggested to be mediated by Th1-associated cytokines such as IL-23, IL-12, and IFN-γ that are overproduced by macrophages and T cells of lamina propria.

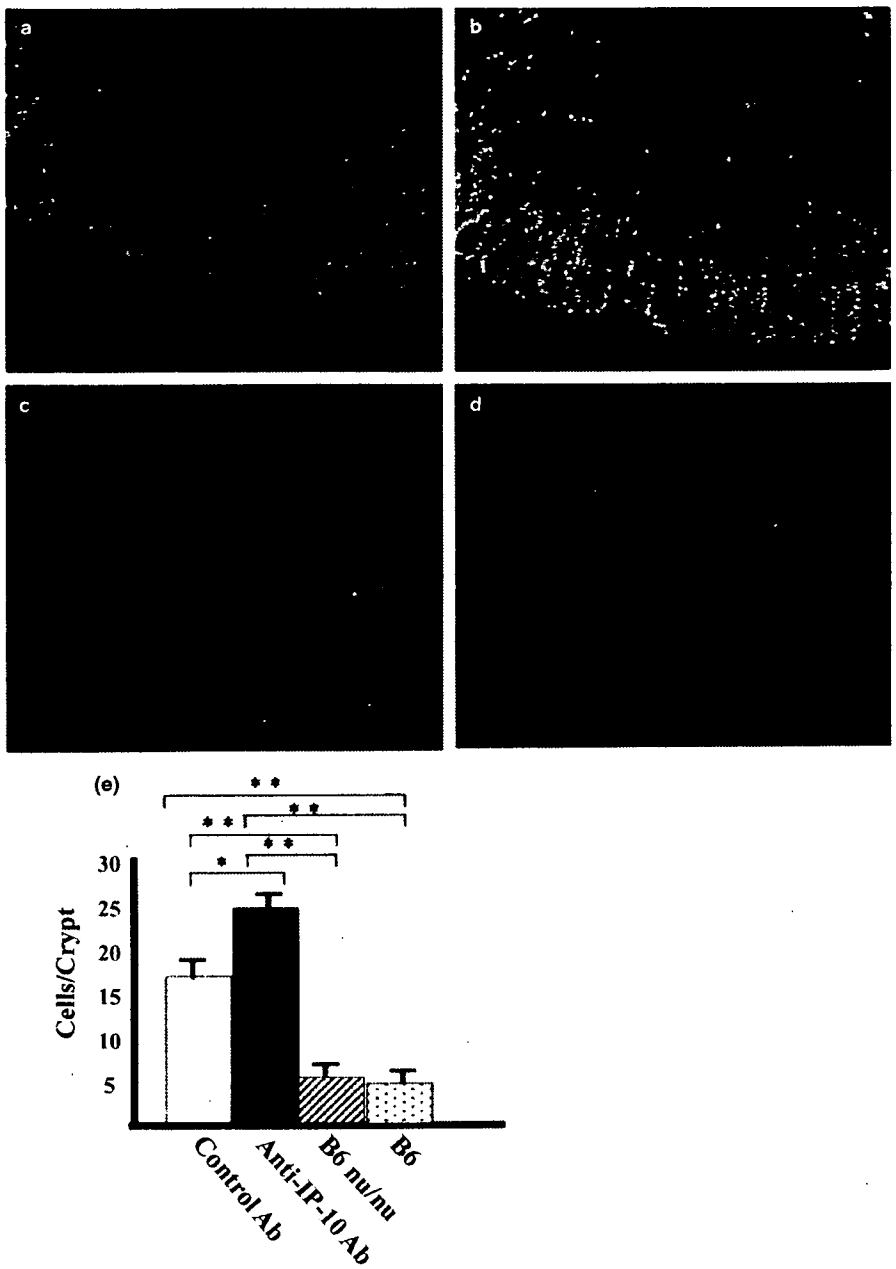

Figure 4 Neutralization of interferon-γ-inducible protein 10 kDa (IP-10/CXCL10) enhanced proliferation of crypt epithelial cells of the colon of murine acquired immunodeficiency syndrome (MAIDS) colitis. Ki67$^+$ crypt epithelial cells were detected in (**a**) control antibody-treated MAIDS colitis mouse at 3 weeks after induction of colitis; (**b**) anti-IP-10 antibody-treated MAIDS colitis mouse; (**c**) normal B6 nude mouse; and (**d**) normal B6 mouse. (**e**) The number of Ki67$^+$ crypt epithelial cells per crypt was increased in MAIDS colitis as compared with B6 and B6 nude mice. The number of cells was significantly increased by anti-IP-10 antibody treatment.

There have been developed many inflammatory bowel disease animal models. Among these, colitis observed in both IL-10-deficient mice and Rag2$^{-/-}$ mice reconstituted with CD4$^+$CD45RB^{high} T cells has been characterized as a Th1-dependent disease, mimicking Crohn's disease.[22] Scheerens *et al.* have reported increased expression of mRNA of IP-10 in Rag2$^{-/-}$ mice reconstituted with CD4$^+$CD45RB^{high} T cells, but not in IL-10-deficient mice.[24] Singh *et al.* showed that inhibition of IP-10 abrogates colitis in IL-10$^{-/-}$ mice supposedly by inhibiting the Th1 immune response.[11] They did not observe the hyperplasia of crypt epithelial cells of the colon induced by the blockade of IP-10 in the model. Thus, IP-10 neutralization is supposed to inhibit Th1 inflammatory reaction resulting in amelioration of colitis. To confirm the efficacy of the aforementioned mechanism, it is better to analyze the effect of anti-IP-10 treatment in different types of colitis models. We have established another experimental colitis model, that is, MAIDS colitis resembling ulcerative colitis.[18,21] In the present study, using this MAIDS colitis model, we also showed that IP-10 blockade significantly inhibited immune cell trafficking, leading to attenuation of the disease. Additionally we have recently reported that anti-IP-10 treatment ameliorates autoimmune-like pancreatic lesions in mice with MAIDS by blocking cellular trafficking.[25] Therefore, it is con-

Figure 5 Anti-apoptotic death of crypt epithelial cells of colon by neutralization of interferon-γ-inducible protein 10 kDa (IP-10/CXCL10). (**a**) Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)$^+$ apoptotic cells were observed in the epithelial cells of luminal tops of crypts in murine acquired immunodeficiency syndrome (MAIDS) colitis; (**c**) TUNEL$^+$ apoptotic cells were rarely observed from B6 mice, (**d**) B6 nude mice and (**b**) mice treated with anti-IP-10 antibody.

ceivable that the main therapeutic effect of IP-10 neutralization in colitis, as well as in the other autoimmune diseases, is induced by inhibition of selective Th1 cells mobilization.

In our previous report we showed that neutralization of IP-10 protected mice from DSS-induced acute colitis by promoting crypt cell survival without altering immune cell infiltration.[10] Furthermore, recombinant IP-10 administration into normal mice directly inhibited intestinal crypt cell proliferation and migration *in vivo*.[10] Thus, we consider IP-10 a negative regulator of crypt cell proliferation and migration in the intestine. Initially DSS colitis was considered to be a T-cell-independent model because of the induction of colitis in T- and B-cell-lacking severe combined immunodeficient mice.[26] Later, however, both macrophage and T-cell responses were shown to play a pivotal role in the disease process, and the pathophysiology of DSS colitis is different from colitis observed in IL-10-deficient mice and $Rag2^{-/-}$ mice reconstituted with $CD4^+CD45RB^{high}$ T cells.[22] This difference in pathophysiology of colitis might explain the different effect of IP-10 blockade on enhanced proliferation and anti-apoptosis of crypt epithelial cells or on inhibition of immune cell trafficking into the colon. As we have reported previously, both Th1 and Th2 immune responses were observed in MAIDS colitis, and hyperplasia of crypt epithelial cells is one of the characteristics of MAIDS colitis.[18,21] In the present study we demonstrated that IP-10 neutralization induced marked proliferation of crypt epithelial cells in MAIDS colitis as well as inhibition of immune cell trafficking. Neutralization of IP-10 did not show any change in the length of the crypt of the colon in B6 nude and B6 mice. In contrast, IP-10 neutralization for MAIDS colitis accelerated intestinal epithelial proliferation more significantly than untreated MAIDS colitis. These results suggest that IP-10 neutralization exerts its cell-proliferating effect more actively on the intestinal epithelial cells in inflammation, rather than on those in normal condition. We should analyze the synergistic relationship between IP-10 neutralization and cytokines and growth factors such as IFN-γ, IL-13, and IL-10, which are secreted by the immune cells recruited by IP-10, for the promotion of proliferation and anti-apoptosis of intestinal epithelial cells.

In conclusion, we have demonstrated that blockade of IP-10 ameliorated MAIDS colitis not only by blocking cellular trafficking, but also by facilitating the proliferation and anti-apoptosis of crypt epithelia of the colon. Neutralization of IP-10 could be a promising adjunctive therapy for inflammatory bowel disease.

ACKNOWLEDGMENTS

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EDITORIAL

Recent advances in basic and clinical aspects of inflammatory bowel disease: Which steps in the mucosal inflammation should we block for the treatment of inflammatory bowel disease?

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Abstract

There are four steps in the interaction between intestinal microbes and mucosal inflammation in genetically predisposed individuals from the viewpoints of basic and clinical aspects of inflammatory bowel disease (IBD). The first step is an interaction between intestinal microbes or their components and intestinal epithelial cells *via* receptors, the second step an interaction between macrophages and dendritic cells and mucosal lymphocytes, the third step an interaction between lymphocytes and vascular endothelial cells, and the fourth step an interaction between lymphocytes and granulocytes producing proinflammatory cytokines or free radicals and mucosal damage and repair. Recent therapeutic approaches for IBD aim to block these four steps in the intestinal inflammation of patients with IBD.

Key words: Ulcerative colitis; Crohn's disease; inflammatory bowel disease; Treatment; Immunology

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INTRODUCTION

Ulcerative colitis and Crohn's disease are the nonspecific inflammatory bowel diseases (IBD) with unknown etiology. Ulcerative colitis was described by Wilks and Moxon in 1859, and Crohn's disease by Crohn, Ginzburg, and Oppenheimer in 1932 with their clinical and pathological analyses[1]. Ulcerative colitis was not a common disease before 1945 with an average annual incidence rate of 8.4 per 100000 population, but after 1945 it showed a gradual rise in average annual incidence rate[2]. In Europe, a steep rise in the incidence of Crohn's disease began after 1945 and a high incidence of Crohn's disease and a low incidence of ulcerative colitis were reported in the 1980s. After 1990 the incidence of Crohn's disease increased from 5.2 per 100000 population in 1988-1990 to 6.4 per 100000 population in 1997-1999, and that of ulcerative colitis decreased from 4.2 to 3.5 per 100000 population, respectively[3]. However, in Asia, Eastern Europe, and South America, the annual incidence rate of ulcerative colitis and Crohn's disease was low before 1990, but it has been steadily increasing over last 10 years[4-7]. These changes in incidence rates may be influenced by various environmental factors, because the etiopathogenesis of IBD is thought to be caused by the mutual reactions among host susceptibility genes (CARD15/NOD2, HLA-class II), environmental factors including enteric flora and food antigens, and abnormal immune balance[8,9].

ETIOPATHOGENESIS OF IBD

Step 1: Interaction between microbes and epithelial cells

Human and murine studies of Crohn's disease have shown an increased expression of T-helper 1 (Th1) cytokines by intestinal *lamina propria* lymphocytes characterized by excessive production of interleukin (IL)-12/IL-23, interferon γ and tumor necrosis factor (TNF) α. These immune responses may be induced by defects in the epithelial barrier, an increased intestinal permeability, adherence of bacteria, and decreased expression of defensins[10]. Intestinal epithelial cells have toll-like receptors (TLR) to exert direct antibacterial effects *via* secretion of antimicrobial peptides and play an important role in the interrelation between the innate and adaptive immunity of the intestine. Moreover, a single nucleotide polymorphism of the nucleotide-binding oligomerization domain 2 (NOD2), which activates nuclear factor κB (NF-κB), is one of candidates for the susceptibility genes of Crohn's disease, because CARD15/NOD2 is expressed

in intestinal epithelial cells and triggers human beta-defensin (HBD)-2 transcription[11]. In Crohn's disease patients with a mutation in the NOD2 gene, which is an intracellular peptidoglycan receptor, the ileal Paneth cell defensins, human defensin (HD)-2 and HD-3, which are antimicrobial peptides are diminished[12,13]. NOD2 mutant macrophages were reported to produce larger amounts of IL-12 in response to stimulation with microbial components than wild-type cells. Therefore, defects in the innate immune response, which is important for immunological protection against intestinal microbes, investigated by Podolsky's group, may contribute to the deve-lopment of Crohn's disease, especially of the ileal type. However, Japanese and Korean patients with Crohn's disease have no mutations in the CARD15/NOD2 gene. Therefore, there be many routes between intestinal microbes and intestinal epithelial cells and *lamina propria* antigen-presenting cells leading to the development of Crohn's disease.

When experimental mice which spontaneously developing spontaneously severe colitis were raised under specific pathogen-free conditions, they developed mild gastrointestinal inflammation[14]. Alteration of the intestinal microflora by antibiotic or probiotic therapy may induce and main-tain remission in colitis mice. Oral administration of Lactobacillus GG induced and maintained remission of some patients with Crohn's disease. In addition, the VSL#3 probiotic-mixture containing 3600 billion bacteria good for the intestine induced remission in patients with active ulcerative colitis[15]. One of the reasons under lying this mechanism is that bacterial flagellin is a dominant antigen in Crohn's disease[16]. When the intestinal epithelial cells were exposed to flagellin, they produced chemokines that induced subsequent migration of immature dendritic cells, probably *via* TLR5. There are many papers stressing that probiotics and prebiotics were effective for the treatment of IBD. Ewaschuk *et al* demonstrated that Bacteroides spp, Enterococcus faecalis, Enterobacter cloacae, intestinal Helicobacter spp, Fusobacterium spp, adherent/invasive *E. coli* strains, Eubacterium, and Peptostreptococcus spp were aggressive intestinal microbes and that the beneficial intestinal microbes were Lactobacillus spp, Bifidobacterium spp, Streptococcus salivarius, Saccharomyces boulardii, Clostridium butyricum, Ruminococci, and *E coli* Nissle 1917[17]. Altering the composition of the intestinal microflora using probiotics and prebiotics is one of the promising therapies for ameliorating chronic intestinal inflammation and may be preventives against IBD in people with disease susceptibility genes.

Step 2: Interaction between macrophages and dendritic cells and T lymphocytes

There are numerous macrophages and dendritic cells in the subepithelial space of mucosal *lamina propria*. Intestinal dendritic cells in active Crohn's disease patients are matured and activated by antigens, and then have an enhanced expression of TLR2 and TLR4. Toll-like receptors in mucosal dendritic cells will recognize invading microbes and their components, because the permeability of interepithelial spaces is increased in the intestinal mucosa in Crohn's disease. Dendritic cells will be activated by Toll-like receptor signaling, *via* MyD88-dependent or independent pathways, and produce interferon γ, TNF-α, IL-6 and IL-12/18. Thus, T cells activated by antigens may undergo distinct developmental pathways, gaining effector functions and properties.

T-helper cells are thought to differentiate into Th1 and Th2, and recently Th17 cells producing IL-17 have been found to induce autoimmunity and regulatory T cells[18]. Intestinal dendritic cells produce IL-12 and IL-6. Antibodies against IL-12 and -6 are effective for the treatment of human Crohn's disease. Intestinal macrophages will be abnormally differentiated in the subsets, producing excessive IL-12 and IL-23 in response to bacteria in IL-10-deficient conditions[19]. On the other hand, human and murine studies of ulcerative colitis have shown an increased expression of atypical Th2 cytokines or natural killer (NK) T cells by *lamina propria* lymphocytes characterized by excessive production of IL-13[20]. Intestinal inflammation may be induced by an imbalance between the memory (effector) T cells inducing inflammation and regulatory T cells suppressing inflammation, probably due to loss of oral tolerance. In the animal model of colitis, IL-7 exacerbated chronic colitis with expansion of memory IL-7R^{high} CD4+ mucosal T cells[21]. Kanai and Watanabe reported that naturally arising CD4+CD25+ regulatory T cells suppressed the expansion of colitogenic CD4+CD44^{high} CD62L-effector memory T cells[22]. It was reported that colonic CD4+CD25 positive regulatory T cells increased with disease activity in patients with active ulcerative colitis[23]. A balance between effector T cells and regulatory T cells seems to be very important for induction of colonic inflammation, because it was also reported that CD8 regulatory T cells were decreased in the colonic *lamina propria* of patients with IBD[24]. The Th1 and Th2 T cell responses that underlie IBD may depend on NF-κB transcriptional activity. NF-κB proteins are regulators of innate and adaptive immunity, inflammatory stress, and proliferative and apoptotic responses of cells to a number of different stimuli. Proinflammatory cytokines such as TNF α and IL-6 are induced by the immuno-competent cells after activation of NF-κB. NF-κB is thought to provide a mechanistic link between inflammation and cancer. In addition, the p38 mitogen-activated protein kinase (MAPK) regulates the expression of proinflammatory cytokines such as IL-8. Inhibitors of intracellular transcriptional factors will be useful for the treatment of IBD in the near future[25]. After NF-κB activation, anti-proinflammatory cytokine antibody therapy such as infliximab, adalimumab and anti-IL-6 monoclonal antibody (MRA) have been used for the treatment of Crohn's disease and ulcerative colitis[26-29].

Step 3: Interaction between lymphocytes and vascular endothelial cells

Intestinal dendritic cells in active Crohn's disease patients were matured and activated upon exposure to intestinal microbes or their components. Memory T cells sensitized by antigens may proliferate and produce proinflammatory cytokines, and then are transported in the mesenteric lymph nodes *via* intestinal lymphatics where they will be modulated. They home again into the intestinal mucosa

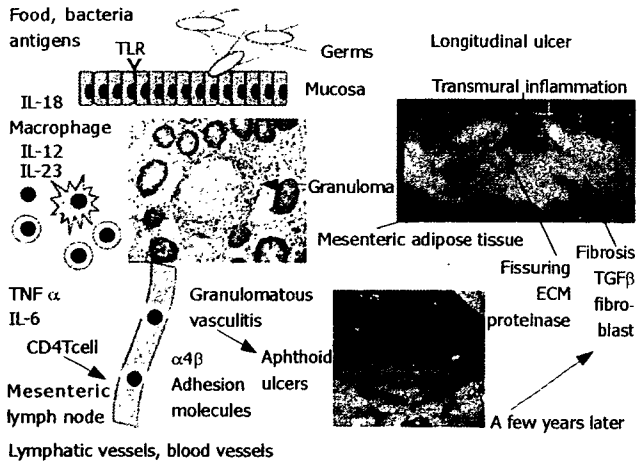

Figure 1 Schema of the pathogenesis of Crohn's disease TLR: toll-like receptors; IL: interleukin; TNF: tumor necrosis factor; ECM: extracellular matrix; TGF: transforming growth factor.

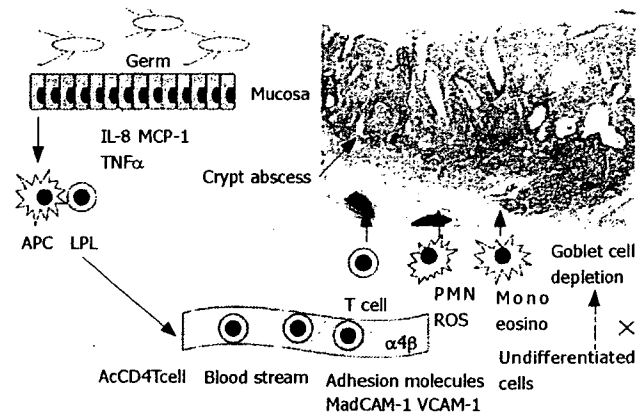

Figure 2 Schema of the pathogenesis of ulcerative colitis. IL: interleukin; MCP: monocyte chemoattractant protein; TNF: tumor necrosis factor; APC: antigen-presenting cells; LPL: lamina propria lymphocytes; AcCD4Tcell: activated CD4Tcell; PMN: polymorphonuclear cell; ROS: reactive oxygen species; mono: monocyte; MadCAM: mucosal addressin cell adhesion molecule; VCAM: vascular cell adhesion molecule.

via the thoracic duct and this homing of lymphocytes is induced by a mutual interaction between the receptors L-selectin and α4β integrin on CD4 T cells and endothelial cell receptors. Anti-adhesion molecule antibody against α4 integrin is effective for the treatment of Crohn's disease with a few complications such as progressive multifocal leukoencephalopathy[30]. Intestinal lesions may occur macroscopically and microscopically in a spotted and regional manner in Crohn's disease and diffusely in ulcerative colitis. The pathologic features of Crohn's disease may occur in association with vascular injury, focal arteritis, fibrin deposition, and arterial occlusion mainly at the level of the *lamina propria*, resulting in multifocal gastrointestinal tissue infarction and lastly longitudinal ulcers in a few years after occurrence of multiple aphthoid ulcers (Figure 1). The majority of granulomas in Crohn's disease may be formed within or near the walls of blood and lymphatic vessels, suggesting granulomatous vasculitis as an early element in the path-ogenesis of Crohn's disease[31,32]. Blockade of intestinal lymphatic flow and mesenteric lymph nodes are also very important factors in the pathogenesis of Crohn's disease, because Crohn's disease reveals dilated lymphatic vessels and edema in the mucosa and submucosa, resulting in intestinal protein loss and albuminemia[33,34].

Step 4: Interaction between cytokines and mucosal damage and repair

Intestinal inflammation occurring in ulcerative colitis is characterized by the mucosal and submucosal infiltration of numerous lymphocytes and granulocytes and the depletion of goblet cells with crypt abscess in most of moderate and severe cases (Figure 2). Goblet cell depletion is thought to be induced by disturbed transformation of undifferentiated cells into goblet cells under an environment exposed to proinflammatory cytokines. Recently, granulocytes including neutrophils have again attracted attention in the pathophysiology of IBD, because leukopheresis is effective for the treatment of active ulcerative colitis and Crohn's disease[35-37]. In experimental colitis induced by dextran sulfate sodium (DSS), deletion of neutrophils by administration of antibody against neutrophils decreased severity of colonic inflammation and production of reactive oxygen species[38]. IL-8 may induce mucosal infiltration of neutrophils at first and then lymphocytes. Mucosal IL-8 is produced by macrophages, colonic epithelial cells and neutrophils when they were activated by antigens and proinflammatory cytokines[39]. The mucosal levels of IL-8 were closely correlated with levels of luminol-dependent chemiluminescence and myeloperoxidase in the mucosa of patients with ulcerative colitis[40]. On the other hand, lower neutrophil accumulation in the intestine and lower production of IL-8 were found in Crohn's disease than in ulcerative colitis[41]. Production of reactive oxygen species by polymor-phonuclear cells was not increased in Crohn's disease when compared with that of controls[42]. Activation of neutrophils was induced in contact with activated platelets *via* P-selectin, resulting in neutrophil-mediated tissue injury *via* excessive production of free radicals[43]. Therefore, active chronic inflammation is a very important factor for the pathogenesis of ulcerative colitis.

Oxidative stress-induced intestinal epithelial cell injury may be induced *via* Rho/ROK/PKC pathway activation[44]. However, precise mechanisms of free radicals-induced cell injury have not been clarified in human ulcerative colitis. There is a clinical trial reporting that lecithinized superoxide dismutase was effective for the treatment of ulcerative colitis[45]. However, in transgenic mice overexpressing human CuZn-SOD, the severity of colitis and the levels of myeloperoxidase in DSS colitis were worsened, implying divergent roles of superoxide and nitric oxide[46]. The mesenchymal cells including fibroblasts, endo-thelial cells involved in angiogenesis, and platelets may play an important role in the pathophysiology of IBD. Fibroblasts and platelets are very important factors for ulcer healing and tissue remodeling[47]. Microvessels having strongly positive staining of αV β3 integrins were increased in density in the colonic mucosa of IBD. Activated platelets released CD40L which was involved in the CD40/CD40L

system and reactive oxygen species from granulocytes when they contacted granulocytes of patients with active IBD[45]. Reconstitution of damaged tissues and epithelial cells is one of targets for wound healing. Watanabe's group found that bone-marrow derived cells could promote the regeneration of damaged epithelia in human gastrointestinal tract[49,50]. In bone -marrow transplantion recipients, epithelial cells of donor origin were distributed throughout the gastrointestinal tract. Bone-marrow transplantation has been tried clinically as one of the treatment for Crohn's disease[51].

CONCLUSION

There are four steps in the interaction between intestinal microbes and mucosal inflammation in individuals genetically pre-disposed to IBD. The first step is an interaction between intestinal microbes or their components and intestinal epithelial cells *via* receptors including Toll-like receptors, the second step an interaction between macrophages and dendritic cells and mucosal lymphocytes, the third step an interaction between lymphocytes and vascular endothelial cells, and the fourth step an interaction between lymphocytes and granulocytes producing proin-flammatory cytokines or free radicals and mucosal damage and repair. Recent therapeutic approaches for IBD are to block these four steps in the intestinal inflammation of patients with IBD.

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Enhanced TLR-mediated NF-IL6–dependent gene expression by Trib1 deficiency

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Toll-like receptors (TLRs) recognize a variety of microbial components and mediate downstream signal transduction pathways that culminate in the activation of nuclear factor κB (NF-κB) and mitogen-activated protein (MAP) kinases. Trib1 is reportedly involved in the regulation of NF-κB and MAP kinases, as well as gene expression in vitro. To clarify the physiological function of Trib1 in TLR-mediated responses, we generated Trib1-deficient mice by gene targeting. Microarray analysis showed that Trib1-deficient macrophages exhibited a dysregulated expression pattern of lipopolysaccharide-inducible genes, whereas TLR-mediated activation of MAP kinases and NF-κB was normal. Trib1 was found to associate with NF-IL6 (also known as CCAAT/enhancer-binding protein β). NF-IL6–deficient cells showed opposite phenotypes to those in Trib1-deficient cells in terms of TLR-mediated responses. Moreover, overexpression of Trib1 inhibited NF-IL6–dependent gene expression by down-regulating NF-IL6 protein expression. In contrast, Trib1-deficient cells exhibited augmented NF-IL6 DNA-binding activities with increased amounts of NF-IL6 proteins. These results demonstrate that Trib1 is a negative regulator of NF-IL6 protein expression and modulates NF-IL6–dependent gene expression in TLR-mediated signaling.

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Abbreviations used: 24p3, lipocalin-2; BLP, bacterial lipoprotein; C/EBP, CCAAT/enhancer-binding protein; Jnk, c-Jun N-terminal kinase; MALP-2, macrophage-activating lipopeptide–2; MAP, mitogen-activated protein; mPGES, prostaglandin E synthase; TLR, Toll-like receptor.

Innate immunity is promptly activated after the invasion of microbes through recognition of pathogen-associated molecular patterns by pattern-recognition receptors, including Toll-like receptors (TLRs) (1). The recognition of microbial components by TLRs effectively stimulates host immune responses such as proinflammatory cytokine production, cellular proliferation, and up-regulation of co-stimulatory molecules, accompanied by the activation of NF-κB and mitogen-activated protein (MAP) kinases (2, 3). Although the inhibitory protein IκB family members sequester NF-κB in the cytoplasm of unstimulated cells, TLR-dependent IκB phosphorylation by the IκB kinase complex and degradation by the ubiquitin–proteasome pathway permit translocation of NF-κB to the nucleus (4). MAP kinases such as c-Jun N-terminal kinase (Jnk) and p38 are also rapidly phosphorylated and activated by upstream kinases in response to TLR stimulation (5). Moreover, TLR-mediated activity of NF-κB and MAP kinases is shown to be regulated at multiple steps regarding the strength and the duration of the activation (6).

Recent extensive experiments have identified a variety of modulators that have positive and negative effects on the activation of NF-κB and MAP kinases, including a family of serine/threonine kinase-like proteins called Trib (7). Trib consists of three family members: Trib1 (also known as c8fw, GIG2, or SKIP1), Trib2 (also known as c5fw), and Trib3 (also known as NIPK, SINK, or SKIP3) (7–12). Trib3 has been shown to interact with the p65 subunit of NF-κB and to inhibit NF-κB–dependent gene expression in vitro (11). In terms of MAP kinases, Trib1, Trib2, and Trib3 reportedly bind to Jnk and p38, and affect the activity of MAP kinases and IL-8 production in response to PMA or

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TLR ligands/IL-1 (12). However, whether Trib family members regulate TLR-mediated signaling pathways under physiological conditions is still unknown.

In this study, we generated Trib1-deficient mice by gene targeting and analyzed TLR-mediated responses. Although the activation of NF-κB and MAP kinases in response to LPS was comparable between wild-type and Trib1-deficient cells, microarray analysis revealed that a subset of LPS-inducible genes was dysregulated in Trib1-deficient cells. Subsequent yeast two-hybrid analysis identified the CCAAT/enhancer-binding protein (C/EBP) family member NF-IL6 (also known as C/EBPβ) as a binding partner of Trib1, and phenotypes found in NF-IL6–deficient cells were opposite to those observed in Trib1-deficient cells. Moreover, overexpression of Trib1 inhibited NF-IL6–mediated gene expression and reduced amounts of NF-IL6 proteins. Inversely, NF-IL6 DNA-binding activity and LPS-inducible NF-IL6–target gene expression were up-regulated in Trib1-deficient cells, in which amounts of NF-IL6 proteins were increased. These results demonstrate that Trib1 plays an important role in NF-IL6–dependent gene expression in the TLR-mediated signaling pathways.

RESULTS

Comprehensive gene expression analysis in Trib1-deficient macrophages

To assess the physiological function of Trib1 in TLR-mediated immune responses, we performed a microarray analysis to compare gene expression profiles between wild-type and Trib1-deficient macrophages in response to LPS (Fig. 1 A and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070183/DC1). Out of 45,102 transcripts, we first defined the genes induced more than twofold after LPS stimulation in wild-type cells as "LPS-inducible genes" and identified 790 of them (Table S1). We next compared the LPS-inducible genes in wild-type and Trib1-deficient macrophages after LPS stimulation and found 59, 703, and 28 genes as up-regulated, similarly expressed, and down-regulated in Trib1-deficient cells, respectively (Table S1).

Among the up-regulated genes, several were subsequently tested by Northern blotting to confirm the accuracy. LPS-induced expression of prostaglandin E synthase (mPGES), lipocalin-2 (24p3), arginase type II, and plasminogen activator inhibitor type II, which were highly up-regulated in the microarray analysis (Table S1), was indeed enhanced in Trib1-deficient macrophages (Fig. 1 B). Furthermore, in contrast to proinflammatory cytokines such as TNF-α and IL-6, which were similarly expressed between wild-type and Trib1-deficient cells in response not only to LPS but also to other TLR ligands, IL-12 p40 was down-regulated in Trib1-deficient cells compared with wild-type cells (Fig. 1 C; Fig. S2, A–C, available at http://www.jem.org/cgi/content/full/jem.20070183/DC1; and Table S1). Thus, the comprehensive microarray analysis revealed that a subset of LPS-inducible genes is dysregulated in Trib1-deficient cells.

Previous in vitro studies demonstrate that human Trib family members modulate activation of MAP kinases and

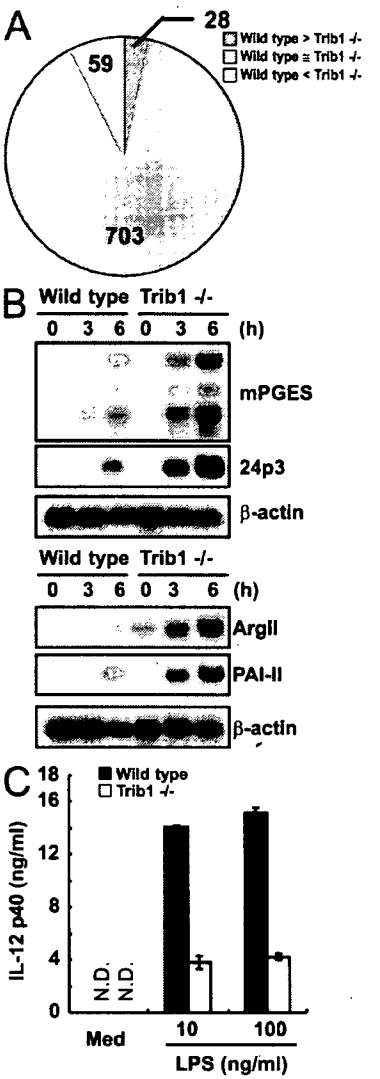

Figure 1. Dysregulation of a subset of LPS-inducible genes in Trib1-deficient cells. (A) Summary of DNA chip microarray analysis. 790 LPS-inducible genes were divided into up-regulated (yellow), similarly expressed (pink), and down-regulated (blue) groups, with the indicated amounts of each. (B) Peritoneal macrophages from wild-type or Trib1-deficient mice were stimulated with 10 ng/ml LPS for the indicated periods. Total RNA (10 μg) was extracted and subjected to Northern blot analysis for the expression of the indicated probes. (C) Peritoneal macrophages from wild-type and Trib1-deficient mice were cultured with the indicated concentrations of LPS in the presence of 30 ng/ml IFN-γ for 24 h. Concentrations of IL-12 p40 in the culture supernatants were measured by ELISA. Indicated values are means ± SD of triplicates. Data are representative of three (B) or two (C) independent experiments. N.D., not detected.

NF-κB (7–12). Both wild-type and Trib1-deficient cells showed similar levels and time courses of phosphorylation of p38, Jnk and extracellular signal-regulated kinase, and IκBα degradation (Fig. S2 D), indicating that the dysregulated

expression of LPS-inducible genes in Trib1-deficient cells might be the independent of activation of NF-κB and MAP kinases.

Interaction of Trib1 with NF-IL6

To explore signaling aspects of Trib1 deficiency other than NF-κB and MAP kinases, we performed a yeast-two-hybrid screen with the full length of human Trib1 as bait to identify a binding partner of Trib1 and identified several clones as being positive. Sequence analysis subsequently revealed that three clones encoded the N-terminal portion of a member of the C/EBP NF-IL6 (unpublished data). We initially tested the interaction of Trib1 and NF-IL6 in yeasts. AH109 cells were transformed with a plasmid encoding the full length of Trib1 together with a plasmid encoding the N-terminal portion of NF-IL6 obtained by the screening (Fig. 2 A). We next examined the interaction in mammalian cells using immunoprecipitation experiments. HEK293 cells were transiently transfected with a plasmid encoding the full length of mouse Trib1 together with a plasmid encoding the full length of mouse NF-IL6. Myc-tagged NF-IL6 was coimmunoprecipitated with Flag-Trib1 (Fig. 2 B), showing the interaction of Trib1 and NF-IL6 in mammalian cells.

TLR-mediated immune responses in NF-IL6–deficient macrophages

An in vitro study showing the interaction of Trib1 and NF-IL6 prompted us to examine the TLR-mediated immune responses in NF-IL6–deficient cells, because LPS-induced expression of mPGES is shown to depend on NF-IL6 (13). We initially analyzed the expression pattern of genes affected by the loss of Trib1 in NF-IL6–deficient macrophages by Northern blotting. LPS-induced expression of 24p3, plasminogen activator inhibitor type II, and arginase type II, as well as mPGES, was profoundly defective in NF-IL6–deficient cells (Fig. 2 C). We next tested IL-12 p40 production by ELISA. As previously reported, IL-12 p40 production by LPS stimulation was increased in a dose-dependent fashion in NF-IL6–deficient cells compared with control cells (Fig. 2 D) (14). In addition, the production in response to bacterial lipoprotein (BLP), macrophage-activating lipopeptide–2 (MALP-2), or CpG DNA was also augmented in

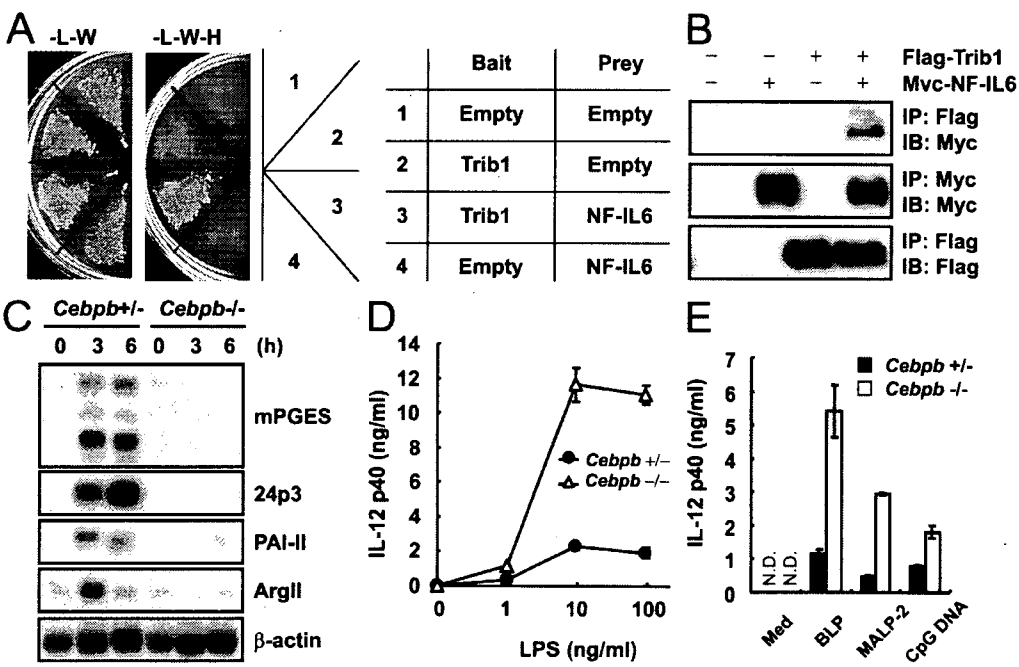

Figure 2. Association of Trib1 with NF-IL6 and TLR-mediated responses in NF-IL6–deficient macrophages. (A) Plasmids expressing human Trib1 fused to the GAL4 DNA-binding domain or an empty vector were cotransfected with a plasmid expressing NF-IL6 fused to GAL4 transactivation domain or an empty vector. Interactions were detected by the ability of cells to grow on medium lacking tryptophan, leucin, and histidine (-L-W-H). The growth of cells on a plate lacking tryptophan and leucine (-L-W) is indicative of the efficiency of the transfection. (B) Lysates of HEK293 cells transiently cotransfected with 2 μg of Flag-tagged Trib1 and/or 2 μg Myc-tagged NF-IL6 expression vectors were immunoprecipitated with the indicated antibodies. (C) Peritoneal macrophages from wild-type or NF-IL6–deficient mice were stimulated with 10 ng/ml LPS for the indicated periods. Total RNA (10 μg) was extracted and subjected to Northern blot analysis for expression of the indicated probes. (D and E) Peritoneal macrophages from wild-type and NF-IL6–deficient mice were cultured with the indicated concentrations of LPS (D) or with 100 ng/ml BLP, 30 ng/ml MALP-2, or 1 μM, CpG DNA (E) in the presence of 30 ng/ml IFN-γ for 24 h. Concentrations of IL-12 p40 in the culture supernatants were measured by ELISA. Indicated values are means ± SD of triplicates. Data are representative of three (B) and two (C–E) separate experiments. N.D., not detected.

NF-IL6–deficient cells (Fig. 2 E). Together, compared with Trib1-deficient cells, converse phenotypes in terms of TLR-mediated immune responses are observed in NF-IL6–deficient cells.

Inhibition of NF-IL6 by Trib1 overexpression

To test whether Trib1 down-regulates NF-IL6–dependent activation, HEK293 cells were transfected with an NF-IL6–dependent luciferase reporter plasmid together with NF-IL6 and various amounts of Trib1 expression vectors (Fig. 3 A). NF-IL6–mediated luciferase activity was diminished by co-expression of Trib1 in a dose-dependent manner. Moreover, RAW264.7 macrophage cells overexpressing Trib1 exhibited reduced expression of mPGES and 24p3 in response to LPS (Fig. S3 A, available at http://www.jem.org/cgi/content/full/jem.20070183/DC1). We next tested NF-IL6 DNA-binding activity by EMSA and observed less NF-IL6 DNA-binding activity in HEK293 cells coexpressing NF-IL6 and Trib1 than in ones transfected with the NF-IL6 vector alone (Fig. 3 B), presumably accounting for the down-regulation of the NF-IL6–dependent gene expression by Trib1. We then examined the effect of Trib1 on the amounts of NF-IL6 proteins by Western blotting. Although the diminution of NF-IL6 by Trib1 was marginal when excess amounts of NF-IL6 were expressed, we found that the transient expression of lower levels of NF-IL6, together with Trib1, resulted in a reduction of NF-IL6 in HEK293 cells (Fig. 3 C). Also, endogenous levels of NF-IL6 proteins in RAW264.7 cells overexpressing Trib1 were markedly less than those in control cells (Fig. 3 D). These results demonstrated that overproduction of Trib1 might negatively regulate NF-IL6 activity in vitro.

Up-regulation of NF-IL6 in Trib1-deficient cells

We next attempted to check the in vivo status of NF-IL6 in Trib1-deficient cells by comparing the NF-IL6 DNA-binding activity in Trib1-deficient macrophages with that in wild-type cells by EMSA. Although LPS-induced NF-κB–DNA complex formation in Trib1-deficient cells was similarly observed, Trib1-deficient cells exhibited elevated levels of C/EBP–DNA complex formation compared with wild-type cells (Fig. 4 A). We further examined whether the C/EBP–DNA complex in Trib1-deficient cells contained NF-IL6 by supershift assay. Addition of anti–NF-IL6 antibody into the C/EBP–DNA complex yielded more supershifted bands in Trib1-deficient cells than in wild-type cells (Fig. 4 B). In addition, the C/EBP–DNA complex was not shifted by the addition of anti-C/EBPδ (also known as NF-IL6β) antibody (Fig. S4 A, available at http://www.jem.org/cgi/content/full/jem.20070183/DC1), suggesting that NF-IL6 DNA-binding activity is augmented in Trib1-deficient cells. We then examined the amounts of NF-IL6 proteins by Western blotting (Fig. 4 C). Compared with wild-type cells, Trib1-deficient cells showed increased levels of NF-IL6 proteins. Finally, we examined NF-IL6 mRNA levels by Northern blotting and observed enhanced expression of NF-IL6 mRNA in Trib1-deficient cells (Fig. 4 D), which is consistent with the autocrine induction of NF-IL6 mRNA

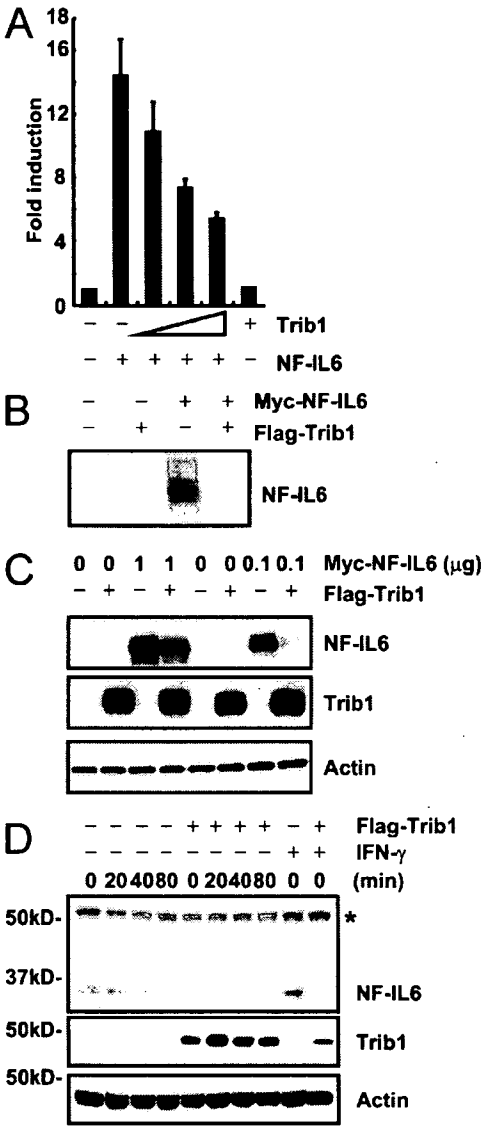

Figure 3. Inhibition of NF-IL6 activity by Trib1 overexpression. (A) HEK293 cells were transfected with an NF-IL6–dependent luciferase reporter together with either Trib1 and/or NF-IL6 expression plasmids. Luciferase activities were expressed as the fold increase over the background shown by lysates prepared from mock-transfected cells. Indicated values are means ± SD of triplicates. (B) HEK293 cells were transfected with 0.1 μg NF-IL6 expression vector together with 4 μg Trib1 expression plasmids. Nuclear extracts were prepared, and C/EBP DNA-binding activity was determined by EMSA using a probe containing the NF-IL6 binding sequence from the mouse 24p3 gene. (C) Lysates of HEK293 cells transiently cotransfected with 2 μg of Flag-tagged Trib1 alone or the indicated amounts of Myc-tagged NF-IL6 expression vectors were immunoblotted with anti-Myc or –Flag for detection of NF-IL6 or Trib1, respectively. (E) RAW 264.7 cells stably transfected with either an empty vector or Flag-Trib1 were stimulated with 10 ng/ml LPS for the indicated periods. The cell lysates were immunoblotted with the indicated antibodies. A protein that cross-reacts with the antibody is indicated (*). Data are representative of three (A and C) and two (B and D), separate experiments.

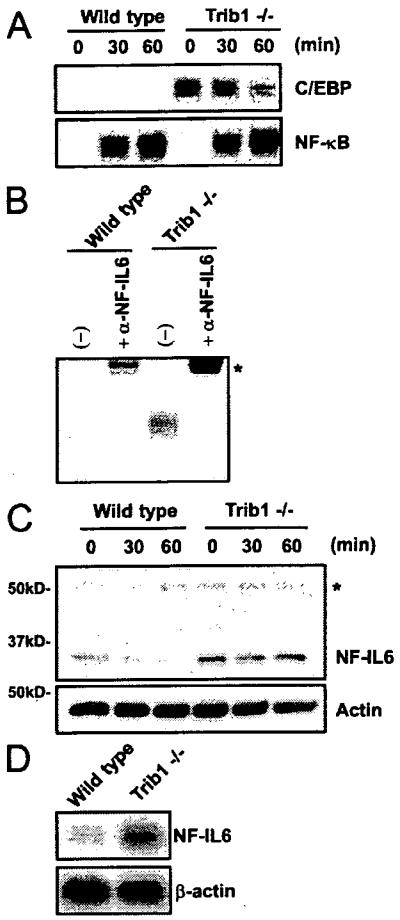

Figure 4. Up-regulation of NF-IL6 activity in Trib1-deficient cells. (A) Peritoneal macrophages from wild-type or Trib1-deficient mice were stimulated with 10 ng/ml LPS for the indicated periods. Nuclear extracts were prepared, and C/EBP DNA-binding activity was determined by EMSA using a C/EBP consensus probe. (B) Nuclear extracts of wild-type and Trib1-deficient unstimulated macrophages were preincubated with anti-NF-IL6, followed by EMSA to determine the C/EBP DNA-binding activity. Supershifted bands are indicated (*). (C) Peritoneal macrophages from wild-type or Trib1-deficient mice were stimulated with 10 ng/ml LPS for the indicated periods and lysed. The cell lysates were immunoblotted with the indicated antibodies. A protein that cross-reacts with the antibody is indicated (*). (D) Total RNA (10 μg) from unstimulated peritoneal macrophages from wild-type or NF-IL6-deficient mice was extracted and subjected to Northern blot analysis for expression of the indicated probes. Data are representative of two (A and B) and three (C and D) separate experiments.

in a previous study (15). Thus, Trib1 may negatively control amounts of NF-IL6 proteins, thereby affecting TLR-mediated NF-IL6–dependent gene induction.

DISCUSSION

In this study, we demonstrate by microarray analysis and biochemical studies that Trib1 is associated with NF-IL6 and negates NF-IL6–dependent gene expression by reducing the amounts of NF-IL6 proteins in the context of TLR-mediated responses. Especially regarding IL-12 p40, although the microarray data showed an almost twofold reduction of the mRNA in Trib1-deficient cells (Table S1), the production was three to four times lower than that in wild-type cells (Fig. 1 C), suggesting translational control of IL-12 p40 by Trib1 in addition to the transcriptional regulation. Moreover, the transcription of the IL-12 p40 gene itself may be affected by not only the amount of NF-IL6 proteins but also the phosphorylation or the isoforms such as liver-enriched activator protein and liver-enriched inhibitory protein (16–18). The molecular mechanisms of how Trib1 deficiency affects IL-12 p40 production on the transcriptional or translational levels through NF-IL6 regulation need to be carefully studied in the future.

The name Trib is originally derived from the *Drosophila* mutant strain *tribbles*, in which the *Drosophila* tribbles protein negatively regulates the level of *Drosophila* C/EBP *slbo* protein and C/EBP-dependent developmental responses such as border cell migration in larvae (19–22). It is also of interest that Trib1-deficient female mice and *Drosophila* in adulthood are both infertile (unpublished data) (18). In mammals, other Trib family members such as Trib2 and Trib3 have recently been shown to be involved in C/EBP-dependent responses (23, 24). Mice transferred with bone marrow cells, in which Trib2 is retrovirally overexpressed, display acute myelogenous leukemia–like disease with reduced activities and amounts of C/EBPα (23). In addition, ectopic expression of Trib3 inhibits C/EBP-homologous protein–induced ER stress–mediated apoptosis (24). Thus, the function of tribbles to inhibit C/EBP activities by controlling the amounts appears to be conserved throughout evolution.

Given the up-regulation of the mRNA in Trib1-deficient cells (Fig. 4 D), the reduction of NF-IL6 in Trib1-overexpressing cells (Fig. 3 C), the auto-regulation of NF-IL6 by itself (15), and the degradation of C/EBPα by Trib2 (23) and slbo by tribbles (22), the loss of Trib1 might primarily result in impaired degradation of NF-IL6 and, subsequently, in excessive accumulation of NF-IL6 via the autoregulation in Trib1-deficient cells.

In this study, we focused on the involvement of Trib1 in TLR-mediated NF-IL6–dependent gene expression. However, given that the levels of NF-IL6 proteins were increased in Trib1-deficient cells, it is reasonable to propose that other non–TLR-related NF-IL6–dependent responses might be enhanced in Trib1-deficient mice. Moreover, Trib3 is also shown to be involved in insulin-mediated Akt/PKB activation in the liver by mechanisms apparently unrelated to C/EBP, suggesting that Trib family members possibly function in a C/EBP-independent fashion (25–27). Future studies using mice lacking other Trib family members, as well as Trib1, may help to unravel the nature of mammalian tribbles in wider points of view.

MATERIALS AND METHODS

Generation of Trib1-deficient mice. A genomic DNA containing the *Trib1* gene was isolated from the 129/SV mouse genomic library and characterized by restriction enzyme mapping and sequencing analysis. The gene encoding mouse Trib1 consists of three exons. The targeting vector was constructed by replacing a 0.4-kb fragment encoding the second exon of the

Trib1 gene with a neomycin resistance gene cassette (*neo*) (Fig. S1 A). The targeting vector was transfected into embryonic stem cells (E14.1). G418 and gancyclovir doubly resistant colonies were selected and screened by PCR and Southern blot analysis (Fig. S1 B). Homologous recombinants were microinjected into C57BL/6 female mice, and heterozygous F1 progenies were intercrossed to obtain *Trib1*$^{+/-}$ mice. We interbred the heterozygous mice to produce offspring carrying a null mutation of the gene encoding Trib1. Trib1-deficient mice were born at the expected Mendelian ratio and showed a slight growth retardation with reduced body weight until 2–3 wk after birth (unpublished data). Trib1-deficient that mice survived for >6 wk were analyzed in this study. To confirm the disruption of the gene encoding Trib1, we analyzed total RNA from wild-type and Trib1-deficient peritoneal macrophages by Northern blotting and found no transcripts for Trib1 in Trib1-deficient cells (Fig. S1 C). All animal experiments were conducted with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases at Osaka University.

Reagents, cells, and mice. LPS (a TLR4 ligand) from *Salmonella minnesota* Re 595 and anti-Flag were purchased from Sigma-Aldrich. BLP (TLR1/TLR2), MALP-2 (TLR2/TLR6), and CpG oligodeoxynucleotides (TLR9) were prepared as previously described (28). Antiphosphorylated extracellular signal-regulated kinase, Jnk, and p38 antibodies were purchased from Cell Signaling. Anti–NF-IL6 (C/EBPβ), C/EBPδ, actin, IκBα, and Myc-probe were obtained from Santa Cruz Biotechnology, Inc. NF-IL6–deficient mice were as previously described (29). Epitope-tagged Trib1 fragments were generated by PCR using cDNA from LPS-stimulated mouse peritoneal macrophages as the template and cloned into pcDNA3 expression vectors, according to the manufacturer's instructions (Invitrogen).

Measurement of proinflammatory cytokine concentrations. Peritoneal macrophages were collected from peritoneal cavities 96 h after thioglycollate injection and cultured in 96-well plates (10^5 cells per well) with the indicated concentrations of the indicated ligands for 24 h, as shown in the figures. Concentrations of TNF-α, IL-6, and IL-12 p40 in the culture supernatant were measured by ELISA, according to manufacturer's instructions (TNF-α and IL-12 p40, Genzyme; IL-6, R&D Systems).

Luciferase reporter assay. The NF-IL6–dependent reporter plasmids were constructed by inserting the promoter regions (−1200 to +53) of the mouse 24p3 gene amplified by PCR into the pGL3 reporter plasmid. The reporter plasmids were transiently cotransfected into HEK293 with the control *Renilla* luciferase expression vectors using a reagent (Lipofectamine 2000; Invitrogen). Luciferase activities of total cell lysates were measured using the Dual-Luciferase Reporter Assay System (Promega), as previously described (28).

Yeast two-hybrid analysis. Yeast two-hybrid screening was performed as described for the Matchmaker two-hybrid system 3 (CLONTECH Laboratories, Inc.). For construction of the bait plasmid, the full length of human Trib1 was cloned in frame into the GAL4 DNA-binding domain of pGBKT7. Yeast strain AH109 was transformed with the bait plasmid plus the human lung Matchmaker cDNA library. After screening of 10^6 clones, positive clones were picked, and the pACT2 library plasmids were recovered from individual clones and expanded in *Escherichia coli*. The insert cDNA was sequenced and characterized with the BLAST program (National Center for Biotechnology Information).

Microarray analysis. Peritoneal macrophages from wild-type or Trib1-deficient mice were left untreated or were treated for 4 h with 10 ng/ml LPS in the presence of 30 ng/ml IFN-γ. The cDNA was synthesized and hybridized to Murine Genome 430 2.0 microarray chips (Affymetrix), according to the manufacturer's instructions. Hybridized chips were stained and washed and were scanned with a scanner (GeneArray; Affymetrix). Microarray Suite software (version 5.0; Affymetrix) was used for data analysis. Microarray data have been deposited in the Gene Expression Omnibus under accession no. GSE8788.

Western blot analysis and immunoprecipitation. Peritoneal macrophages were stimulated with the indicated ligands for the indicated periods, as shown in the figures. The cells were lysed in a lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-Cl [pH 7.5], 5 mM EDTA) and a protease inhibitor cocktail (Roche). The cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. For immunoprecipitation, cell lysates were precleared with protein G–sepharose (GE Healthcare) for 2 h and incubated with protein G–sepharose containing 1 μg of the antibodies indicated in the figures for 12 h, with rotation at 4°C. The immunoprecipitants were washed four times with lysis buffer, eluted by boiling with Laemmli sample buffer, and subjected to Western blot analysis using the indicated antibodies, as previously described (28).

EMSA and supershift assay. 2×10^6 peritoneal macrophages were stimulated with the indicated stimulants for the indicated periods, as shown in the figures. 2×10^6 HEK293 cells were transfected with 0.1 μg Myc–NF-IL6 and/or 4 μg Flag-Trib1 expression vectors. Nuclear extracts were purified from cells and incubated with a probe containing a consensus C/EBP DNA-binding sequence (5′-TGCAGATTGCGCAATCTGCA–3′; Fig. 4, A and B) or mouse 24p3 NF-IL6 binding sequence (sense, 5′-CTTCCTGTTGCTCAACCTTGCA-3′;antisense,5′-TGCAAGGTTGAGCAACAGGAAG–3′; Fig. 3 B), electrophoresed, and visualized by autoradiography, as previously described (28, 30). When the supershift assay was performed, nuclear extracts were mixed with the supershift-grade antibodies indicated in the figures before the incubation with the probes for 1 h on ice.

Online supplemental material. Fig. S1 showed our strategy for the targeted disruption of the mouse *Trib1* gene. Fig. S2 showed the status of proinflammatory cytokine production in response to various TLR ligands and LPS-induced activation of MAP kinases and IκB degradation. Fig. S3 showed decreased expression of NF-IL6–dependent gene in Trib1-overexpressing cells. Fig. S4 showed that the C/EBP-DNA complex in Trib1-deficient cells contained NF-IL6, but not C/EBPδ. Table S1 provides a complete list of the LPS-inducible genes studied. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070183/DC1.

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