Pathological Role of Large Intestinal IL-12p40 for the Induction of Th2-Type Allergic Diarrhea

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IL-12 consists of two disulfide-linked subunits, p40 and p35, that form functionally active heterodimers for the induction of Th1 cells. In contrast to IL-12 heterodimers, p40 monomers and homodimers possess inhibitory effects on Th1 cells leading to the creation of a Th2 environment. Although it has been shown that IL-12p40 acts as antagonist of IL-12p70 *in vitro*, no evidence is currently available whether IL-12p40 is functional *in vivo*. We now report that IL-12p40 plays an important pathological role in an intestinal allergic disease. A high expression of IL-12p40 protein was demonstrated in epithelial cells, dendritic cells, and macrophages in large but not small intestine of allergic diarrhea-induced mice. Interestingly, neutralization with anti-IL-12p40 mAbs reduced the incidence and delayed the onset of disease development. Lower levels of ovalbumin (OVA)-specific IgE Abs in serum were detected in anti-IL-12p40 mAb-treated mice than in control Ab-treated mice. The secretion of Th2 cytokines and eotaxin by the mononuclear cells isolated from the large intestine of anti-IL-12p40 mAb-treated mice was significantly decreased. Finally, the removal of the IL-12p40 gene resulted in complete inhibition of disease development. These results show that over-expression of IL-12p40 is an important contributing factor for the generation of the dominant Th2-type environment in the large intestine of mice with allergic diarrhea. (*Am J Pathol* 2004, 164:1327–1335)

In general, intestinal allergic reactions are provoked by the activation of allergen-specific Th2-type cells, excessive eosinophil and mast cell recruitment, and IgE Ab production.^{1,2} We previously reported that systematically

primed BALB/c mice developed severe diarrhea after repeated oral challenge with ovalbumin (OVA).¹ Diarrhea-induced mice revealed a Th2-type allergic response characterized by high levels of Ag-specific IgE Abs in serum, increased numbers of IgG1, IgA and IgE Abs in the large intestine, and high numbers of mast cells and eosinophils in the large intestine. Furthermore, large intestinal CD4⁺ T cells isolated from mice with allergic diarrhea secreted IL-4, IL-5, and IL-13, but not IFN- γ . On the other hand, a murine model of eosinophilic gastrointestinal hypersensitivity induced by challenge with oral allergen, in the form of enteric-coated beads, resulted in marked allergen-induced IL-4 and IL-5 production and eosinophil accumulation in the small intestine. Although several interesting intestinal allergic models were recently reported,^{2,3} the exact underlying molecular and cellular mechanisms remain to be elucidated.

In allergic asthma, allergen-specific T cells have been shown to also acquire the Th2 phenotype and to avoid from the Th1-type pathway.^{4,5} A recent study has demonstrated that Th1/Th2 imbalance induced allergic disease at the level of transcription factors. Interestingly, a high expression of GATA-3 and/or a lack of T-bet signaling markedly influenced the development of allergic asthma.^{6,7} In addition, the expression of not only Th2-type cytokine but also Th1-type cytokine (ie, IFN- γ or IL-12) played a critical role in murine dermatitis and asthma models.^{8,9} It has also been demonstrated that natural killer (NK) cells, like Th2 cells, play an important role in the development of allergen-induced asthma.⁹

It has been suggested that antigen-presenting cells (APCs) play a crucial role in the skewing of Th1 and Th2 differentiation.^{10,11} IL-12 is a heterodimeric cytokine composed of p40 and p35 which strongly promotes the differentiation of naive CD4⁺ T cells to the Th1 phenotype and suppresses the synthesis of Th2-type cytokines.¹² IL-12 is produced primarily by APCs and the production is regulated by IL-10 and IFN- γ .^{13,14} In addition, biological effects of IL-12 are counter-balanced by IL-12p40 itself, which binds to the receptor complex without induc-

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ing intracellular signals.¹⁵ Thus, murine IL-12p40 inhibits IL-12-mediated responses by means of the competitive binding to IL-12 receptor with an affinity similar to that of IL-12p70.^{16,17} Further, IL-12p40 can behave as an IL-12p70 antagonist *in vivo*, delaying the allograft rejection of cardiac myoblast.¹⁸ IL-12p40 transgenic mice also showed increased susceptibility to the malaria infection.¹⁹

With regard to allergic responses, a potential contribution of IL-12 has been suggested for the development of allergic asthma.^{9,20} Mixed Th1- and Th2-associated cytokines, including IFN- γ , IL-2, IL-5, GM-CSF, and IL-12, were secreted by smooth muscle cells located in the sensitized airways of atopic asthma-induced mice.²¹ A previous study demonstrated that IL-12p40 mRNA expression was detected in the lung tissue of mice with asthma.²¹ However, the role of IL-12 in intestinal allergic disease has not yet been carefully examined. To investigate the potential roles of IL-12 in intestinal hypersensitivity, we have assessed the expression pattern of IL-12p40 or IL-12p35 in the intestinal tract of diarrhea-induced mice and examined the therapeutic effects of modulating IL-12 involvement in allergic diarrhea.

Materials and Methods

Mice

BALB/c mice were purchased from Japan Clea Company (Tokyo, Japan). Breeding pairs of IL-12p40-deficient [IL-12p40 knockout (KO)] mice were purchased from The Jackson Laboratories (Bar Harbor, ME), and colonies were established and maintained in the experimental animal facility at the University of Tokyo and the University of Alabama at Birmingham. All mice were 6 to 7 weeks of age at the beginning of individual experiments.

Antibodies

Recombinant murine IL-12 p40 and p70 were obtained from BD PharMingen (San Diego, CA). Biotin-anti-CD11b (M1/70, rat IgG2b) and biotin-anti-CD11c (HL3, hamster IgG) were also purchased from BD PharMingen. Biotin-SP-conjugated, affinity-purified anti-rat IgG (H+L) mouse F(ab')₂ and anti-hamster IgG(H+L) goat F(ab')₂ were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Anti-IL-12p40 (C17.8.20, rat IgG2a) was a generous gift from Dr. G. Trinchieri, Wistar Institute, Philadelphia, PA²² and was purified from ascites on a protein G column (Pharmacia Biotech, Uppsala, Sweden). Purified rat IgG was purchased from Sigma Chemical, Inc. (St. Louis, MO).

Induction of Allergic Diarrhea

For the induction of allergic diarrhea, our well-established protocol was used as described previously.¹ Briefly, on the first day of the experiment (day 0), mice were primed by subcutaneous (SC) injection of 1 mg of OVA in Complete Freund Adjuvant (CFA) (Difco Laboratories, Detroit,

MI). One week after the systemic priming (day 7), mice were repeatedly challenged with 50 mg of OVA by oral administration (PO) three times per week for several weeks.¹ These mice were sacrificed and analyzed within 1 and 2 hours after a total of 10 times of oral administration with OVA. In a timed kinetics study, mice were sacrificed at indicated intervals, ie, 0, 30 minutes, 1 hour, and 2 hours following the last oral administration of OVA. As controls, mice were repeatedly given oral OVA in phosphate-buffered saline (PBS) without systemic priming or were injected SC with 1 mg OVA in CFA without repeated oral challenge.

Treatment of Mice with Anti-IL-12p40

In vivo Ab treatment was performed as described previously.²³ Anti-IL-12p40 (C17.8.20, rat IgG2a) was a generous gift from Dr. G. Trinchieri, Schering-Plough Research Institute, Dardilly, France.²² BALB/c mice were intraperitoneally administered with 0.5 mg to 2.0 mg of purified anti-IL-12p40 (C17.8) or control rat IgG (Sigma Chemicals, Inc.) per week for the duration of the experiment. Ab treatment was started 1 week before or at the time systemic priming with OVA in CFA. Among different concentrations tested, the protocol of 1 mg/mouse of per week was the most optimal condition. Further, when the mAb treatment schedule was compared between the start at 1 week before or at the same time as OVA systemic priming, an identical effect was noted in this study. Thus, the mAb anti-IL-12 (1 mg/mouse) was started on 1 week before the OVA systemic priming.

ELISA for OVA-Specific IgE Abs in Serum

To assess OVA-specific IgE Ab levels in serum, a sandwich ELISA system was adopted.¹ End-point titers of OVA-specific IgE Abs were expressed as the reciprocal log₂ of the last dilution that showed a level of 0.1 higher absorbance than that of sera of non-immune mice as background.

Isolation of Mononuclear Cells and Cytokine-Specific ELISA Assay

To isolate mononuclear cells from small and large intestines, we used a enzymatic dissociation method.²⁴ Briefly, mononuclear cells were dissociated by collagenase from small and large intestines after the removal of Peyer's patches and were then subjected to a discontinuous Percoll gradient.²⁵ Mononuclear cells from small and large intestines were then co-cultured in the presence of 1 mg OVA. After 3 days of culture, the supernatant was collected and assayed for cytokines by using an ELISA Kit specific for IL-4 (Endogen, Woburn, MA), IL-5 (Amersham Pharmacia Biotech, Piscataway, NJ), IL-13 (R&D Systems, Minneapolis, MN) and eotaxin (Techn Corporation, Minneapolis, MN).

Table 1. Primers and Probes Used for Qualitative and Quantitative RT-PCR

Conventional PCR*			
HPRT	primer	F	GTT GGA TAC AGG CCA GAC TTT GTT G
		R	GAG GGT AGG CTG GCC TAT AGG CT
IL-12p40	primer	F	ATG GCC ATG TGG GAG CTG GAG
		R	TTT GGT GCT TCA CAC TTC AGG
IL-12p35	primer	F	ACC CAG TTG GCC AGG GTC
		R	CAA GGC ACA GGG TCA TCA TC
Real-time PCR†			
HPRT	primer	F	AAC TTT GCT TTC CCT GGT
		R	AGT CAA GGG CAT ATC CAA CA
	probe	FITC	CAG TAC AGC CCC AAA ATG GTT AAG GTTGC
		LCRed640	AGC TTG CTG GTG AAA AGG ACG TCT CG
IL-12p40	primer	F	AGA GGA GGG GTG TAA CCA G
		R	GGG AAC ACA TGC CCA CTT G
	probe	FITC	ACC GAA GTC CAA TGC AAA GGC GG
		LCRed640	AAT GTC TGC GTG CAA GCT CAG G
IL-12p35	primer	F	CCT GTG CCT TGG TAG CAT CT
		R	AGA CTG CAT CAG CTC ATC G
	probe	FITC	ACC AGA CAG AGT TCC AGG CCA TCA
		LCRed640	TGA TGG CCT GGA ACT CTG TCT GGT

*, Primers were designed based on the published sequence.¹³

†, Primers and probes were designed and produced by Nihon Gene Research Laboratories (Sendai, Japan).

Immunoprecipitation and Western Blot Analyses

For the detection of different forms of IL-12, intestinal tissue extracts were prepared as previously described with minor modifications.²⁶ Small and large intestines were removed, minced in cold PBS with protease inhibitor, homogenized, and incubated to allow cytokine release from the tissue. After centrifugation intestinal tissue extracts were subjected to the measurement of protein concentration and then pre-cleared with protein G Sepharose beads (Pharmacia Biotech, Uppsala, Sweden), subsequently incubated with anti-IL-12p40, mixed with protein G Sepharose beads. The beads were washed, subjected to SDS-PAGE under non-reducing condition. After electrophoresis, proteins were transferred to a polyvinylidene difluoride microporous membrane (PVDF Immobilon; Millipore, Bedford, MA) and the membrane was reacted with biotinylated anti-IL-12 (C17.8) followed by incubation with biotin-streptavidin complex (ABC-AP Kit; Vector Laboratories, Inc.). Visualization of the signal was performed by NBT/BCIP Substrate Kit (BioRad, Hercules, CA).

Analysis for IL-12 mRNA Accumulation

The expression of IL-12p40 or p35 in small and large intestines of mice was examined using conventional RT-PCR as previously described¹³ and quantitative real-time PCR method using a Lightcycler (Roche Diagnostics, GmbH Mannheim, Germany), with some modifications.^{27,28} The sequences of primers and probes for real-time PCR were designed by Nihon Gene Research Laboratories (Sendai, Japan) (Table 1). Total RNA was extracted by using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and 2 μ g of extracted RNA was subjected to RT reaction using Superscript II Reverse Transcriptase (Life Technologies).¹⁴ Hypoxanthine phos-

phoribosyl transferase (HPRT) cDNA was used to standardize the total RNA content. The cDNA from each experimental sample was then subjected to the Lightcycler FastStart DNA Master Hybridization Probes Kit (Roche Diagnostics). The external standards of HPRT, IL-12p40, and IL-12p35 DNA prepared by PCR between 20 pg to 0.02 femtograms were used for the quantification of specific cDNA in each sample. The ratio of the p40 and p35 increase in experimental mice was calculated and compared with non-treated mice as follows; the ratio = (p40 or p35 mRNA amounts from experimental mice/HPRT mRNA amounts from experimental mice)/(p40 or p35 mRNA amounts from non-treated mice/HPRT mRNA amounts from non-treated mice).

Immunohistochemical Analysis

Following extensive washing, small and large intestines were fixed in 4% paraformaldehyde-PBS and treated with sucrose-gradient, frozen in OCT-embedding medium as previously described with minor modifications.²⁹ For IL-12p40 immunostaining, cryosections were subjected to antigen retrieval using 10 mmol/L citric buffer pH 6.0 for 5 minutes at 98°C. Slides were then blocked with normal mouse IgG and incubated with rat anti-IL-12p40 or control rat IgG for 16 hours at 4°C. The section were then treated with biotinylated goat anti-rat IgG F(ab')₂ (Jackson ImmunoResearch Laboratories, Inc.), ABC-AP Kit, and red chromogen (Vector Red; Vector Laboratories, Inc.). For IL-12p35 immunostaining, we used goat anti-IL-12p35 (Santa Cruz, Inc., Santa Cruz, CA) or control goat IgG. The sections were then treated with biotinylated donkey anti-goat IgG F(ab')₂ and ABC-AP Kit. In the case of surface marker staining, serial sections were incubated with anti-CD11b (M1/70, BD PharMingen) or anti-CD11c (HL3, BD PharMingen), biotinylated second anti-

Figure 1. Selective production of IL-12p40 by the large intestine of diarrhea-induced mice. In **A**, large intestinal tissues from diarrhea-induced mice were immunostained with anti-IL-12p40 mAb, anti-IL-12p35 mAb, or control IgG. Control non-disease mice section gave no signal above background (data not shown). In **B-1**, IL-12p40-specific mRNA was expressed selectively in the large intestine of mice with allergic diarrhea. In **B-2**, quantitative real-time PCR analysis of IL-12p40- and p35-specific mRNA expression was performed. The ratio was obtained as the level of IL-12p40 or p35 expression in non-treated mice as a scale of one. The detailed information for the expression of this ratio is described in the Materials and Methods section. In **C-E**, IL-12p40 was detected in MØ and DC and epithelial cells in the large intestine. The serial sections of the large intestine from diarrhea-induced mice were stained with anti-IL-12p40 mAb and anti-CD11b mAb (**C**), with anti-IL-12p40 mAb and anti-CD11c mAb (**D**). The **arrows** point to double-positive cells. Large intestinal epithelial cells were stained with anti-IL-12p40 mAb (**E**).

body and ABC-AP. The color reaction was developed using Vector Red Substrate Kit I.

Statistical Analysis

Statistical analyses were performed by the two sample non-parametric Welch test with a significance level of 0.01 (**) for body weight and Ig levels, respectively. Mouse disease rates were determined using the Wilcoxon rank-sum test with a significance level of 0.01 (**). Values for cytokine-synthesis in the samples between anti-IL-12 p40-treated and control antibody-treated mice were analyzed by using Student's *t*-test at *P* values of <0.01(**).

Results

Detection of IL-12p40 Protein in the Large Intestine of Allergic Diarrhea Mice

To examine whether IL-12p40 was expressed in the large intestine of OVA-induced diarrhea mice, we analyzed IL-12 expression using a variety of available detection methods. First, we performed immunohistochemical analysis to directly demonstrate the enhanced IL-12p40 expression in the large intestine of mice with allergic diarrhea. As shown in Figure 1A, IL-12p40, but not IL-12p35, was expressed in the large intestine of diarrhea-induced mice. To further confirm enhanced expression of IL-

12p40 in the large intestine of mice with diarrhea, we next performed IL-12-specific RT-PCR analysis. Interestingly, IL-12p40 mRNA was only detected in the large intestine of diarrhea-induced mice, not in control mice without the disease [eg, SC only or per oral challenge (PO) only; Figure 1B]. In contrast, IL-12p35 mRNA expression was detected in both groups of mice (Figure 1B-1). When IL-12-specific mRNA quantitative real-time PCR analysis was performed, high levels of IL-12p40-specific mRNA were noted in the large intestine of OVA-induced allergic diarrhea mice (Figure 1B-2). In contrast, the level of p35 did not vary among the four different groups including experimental diseased (SC/PO) and control non-diseased mice (non-treated, SC only, and PO only). Taken together, these results clearly indicate that IL-12p40, but not p35, was selectively enhanced at the levels of both mRNA and protein in the large intestine of allergic diarrhea mice.

Inasmuch as the induction of IL-12p40 selectively occurred in the large intestine of OVA-induced allergic diarrhea mice, it was important to determine which cell types produced IL-12p40 in the large intestine. Immunohistochemical analysis demonstrated that IL-12p40-producing cells were co-stained with anti-CD11b mAb [ie, macrophages (MØ)]. Further, CD11c⁺ cells [ie, dendritic cells (DC)] were also positively stained for IL-12p40 (Figure 1, C and D). Further, some epithelial cells were also positive for IL-12p40 expression (Figure 1E). Taken together, these findings show that large intestinal macrophages, dendritic cells, and epithelial cells are responsi-

Figure 2. Induction of IL-12p40 homodimer in the large but not small intestine of diarrhea-induced mice. Large and small intestinal tissue extracts were subjected to immunoprecipitation and Western blotting analysis using anti-IL-12p40 (C17.8) mAb under non-reducing conditions (**A**). The captions above the figure indicate the experimental mouse group receiving different *in vivo* treatments. Thus, the samples were obtained from SC/PO mice treated with C17.8 or control antibodies. Further, the samples were isolated from mice treated with PO only, SC only, or non-treated mice. The **arrow** points to IL-12p40 homodimer expression in the large intestine of diarrhea-induced mice. The data represent four independent experiments. In **B**, at the indicated times after oral administration of OVA, large intestinal tissue extracts isolated from diarrhea-induced mice were assayed for IL-12p40 by the same method as in **A**. In **C**, the large intestinal tissue extracts of diarrhea-induced mice were subjected to Western blotting with anti-IL-12p35 Ab as well as anti-IL-12p40. IL-12p70 protein was used as a positive control for the IL-12p35 detection system. As negative control, immunoprecipitation was performed without the tissue specimens (Ab only). The data represent three different experiments.

ble for the production of IL-12p40 at the disease site of OVA-induced allergic diarrhea.

The Western blotting method was adopted for the examination of IL-12 p40 expression in the small and large intestinal tissue extracts from OVA-induced allergic diarrhea mice within 1 to 2 hours after the last oral challenge. In the large intestine of diarrhea-induced mice, the 80kD form of IL-12 predominated clearly demonstrating the presence of IL-12p40 homodimer but not 70kD IL-12 heterodimer, in contrast to the environment observed in the large intestine of control mice or the small intestine of mice with/without diarrhea (Figure 2A). The multiple bands of p40 and p80 are the result of glycosylation heterogeneity.¹⁵ We thus analyzed three bands of p40 and three bands of p80 as specific bands. In the case of spleen, IL-12p40 was detected in control, healthy mice. The levels of IL-12p40 did not change after development of allergic diarrhea (data not shown). To examine the kinetics of the response, we next assessed the time course of IL-12p40 expression in the large intestine of the diarrhea-induced mice. The expression of IL-12p40 or p80 in the large intestine peaked between 1 and 2 hours after the last oral challenge, at the same time that severe symptoms of OVA-induced allergic diarrhea were ob-

Figure 3. Inhibition of allergic diarrhea disease by the treatment with anti-IL-12p40 mAb. In **A**, anti-IL-12p40 mAb (C17.8) treatment (**thin dashed line**) delayed the development of allergic diarrhea when compared with the rat IgG-treated group (**solid line**). Statistical differences were determined by Wilcoxon rank-sum test and are indicated by **, $P < 0.01$. Mice with SC only were used as controls (**thick dashed line**). In **B**, left, body weight was recovered in allergic diarrhea mice treated with anti-IL-12p40 mAb (C17.8). In **B**, right, OVA-specific IgE Abs were reduced in the serum of allergic diarrhea mice treated with anti-IL-12p40 mAb (C17.8). The data are expressed as the mean of $\pm$ SE and are representative of five independent experiments. Statistical differences between anti-IL-12p40 mAb and control rat IgG-treated mice are indicated as **, $P < 0.01$.

served (Figure 2B). These data suggest that there is an intimate relationship between the development of diarrhea and the expression of IL-12p40 in the large intestine.

To further confirm the expression of IL-12p80 or p40 instead of the p70 form, the protein extracts from the large intestine of the diarrhea-induced mice were immunoprecipitated with anti-IL-12p40 mAb and then Western blotting was performed using anti-IL-12p35 mAb. No molecular bands corresponding to IL-12p70 proteins were detected in the large intestine of diarrhea-induced mice, while predominant IL-12p40 protein was detected (Figure 2C). The large molecular weight band above the p70 and p80 bands was non-specific and was caused by the nature of antibody used in the immunoprecipitation, since the large molecular weight band was also seen following immunoprecipitation in the absence of tissue specimens (Ab only in Figure 2C). These results indicate that the secretion of IL-12p40, but not IL-12p70, in the large intestine is critically important in the development of OVA-induced allergic diarrhea.

Anti-IL-12p40 Treatment Reduced the Symptoms of Allergic Diarrhea

Inasmuch as the preferential localization of IL-12p40 was observed in mice with allergic diarrhea, we next performed a neutralization experiment using anti-IL-12p40 mAb (C17.8). We observed a significant delay in the onset of diarrhea and reduced the frequency of diarrhea to 40% by treatment with anti-IL-12p40 mAb (Figure 3A). Obvious body weight loss was seen in control Ig-treated

Figure 4. *In vivo* treatment with anti-IL-12p40 (C17.8) reduced the predominant antigen-specific Th2 type responses by large intestinal mononuclear cells isolated from diarrhea-induced mice. The mononuclear cells isolated from the large intestine (1.5×10^5 cells/well) were cultured with OVA (1 mg/ml) for 3 days. Culture supernatants were harvested and then assayed for IL-4, IL-13, IL-5, and eotaxin by ELISA assay. These data are expressed as the mean $\pm$ SE and are representative of three independent experiments. The statistical differences between anti-IL-12p40 mAb and control antibody treated mice are indicated as **, $P < 0.01$.

diarrhea mice, while treatment with anti-IL-12p40 mAb resulted in partial recovery from body weight loss (Figure 3B, left). In addition, high levels of OVA-specific IgE Abs were detected in the serum of diarrhea-induced mice treated with control Ab, whereas the mice treated with anti-IL-12p40 mAb showed low levels of OVA-specific IgE Abs (Figure 3B, right). These results indicate that treatment with anti-IL-12p40 mAb alters the environment from a disease-inducing one to one fastening recovery in OVA-induced allergic diarrhea.

Suppression of Intestinal Th2-Type Cytokine by Anti-IL-12p40 Treatment

To confirm decreased Th2-type responses in the large intestine after anti-IL-12p40 mAb treatment, we next examined antigen-induced cytokine production by the large intestinal mononuclear cells. Interestingly, the anti-IL-12p40 treatment resulted in decreased levels of OVA-induced Th2 cytokine synthesis including those of IL-4, IL-5, and IL-13 (Figure 4). Production levels of the Th2 cytokines were comparable to those of control mice without allergic diarrhea (SC only). In contrast to the alterations observed in OVA-induced Th2 cytokine synthesis, there was no difference in the level of IFN- γ production between the mice treated with anti-IL-12p40 mAb and control IgG (data not shown). We further confirmed that IL-4 producing cells were CD4⁺ Th2 cells by intracellular staining (data not shown).

Finally, the level of eotaxin, a well-known chemokine for eosinophil recruitment in allergic disease,³⁰ was also examined, since our previous study demonstrated that the frequency of eosinophils was increased in the large intestine of allergic diarrhea mice.¹ Likewise, the level of eotaxin could also be presumed to be increased in the large intestine of allergic diarrhea mice (Figure 4). Interestingly however, the level of eotaxin synthesis was sig-

Figure 5. Suppression of allergic diarrhea development in IL-12p40 KO mice. In **A**, the incidence of allergic diarrhea was reduced in the IL-12p40 KO mice when compared with wild-type mice immunized subcutaneously and then given OVA repeatedly by the oral route (SC/PO). In **B**, the large intestinal LP mononuclear cells from IL-12p40 KO mice did not produce IL-4. Mononuclear cells isolated from the large intestine were restimulated with OVA for the assessment of IL-4 synthesis as described in Figure 4A. The data are expressed as the mean $\pm$ SE and represent three different experiments.

nificantly decreased by the treatment with anti-IL-12p40 mAb (Figure 4). These results indicate that anti-IL-12p40 mAb inhibited the immunopathological Th2 cytokine environment of the large intestine in allergic diarrhea mice. Thus, an interesting scenario could be the presence of high levels of IL-12p40 monomer and/or homodimers instead of IL-12p70 in the disease site of OVA-induced allergic diarrhea mice. Therefore, treatment with anti-IL-12p40 mAb might result in the inhibition of Th2-type responses in the large intestine of allergic disease mice.

IL-12p40-Deficient Mice Do Not Develop Allergic Diarrhea

To directly confirm the pathological role of IL-12p40 in the development of allergic diarrhea, IL-12p40 knockout (KO) mice were used. IL-12p40 KO mice did not develop the allergic diarrhea completely (Figure 5A). OVA-induced IL-4 production by large intestinal LP mononuclear cells was not detected in IL-12p40 mice (Figure 5B). The levels of other Th2-type cytokines (IL-5 and IL-10) were also reduced in IL-12p40 KO mice (data not shown). Taken together, these results clearly show that IL-12p40 plays an important role in the development of this large intestinal allergic disease.

Discussion

Our present findings provide new, strong evidence for an immunopathological role for locally produced IL-12p40 in the development of OVA-induced allergic diarrhea. Here we demonstrate the high expression of IL-12p40, without IL-12p35, in the large intestine but not in the small intestine of mice with allergic diarrhea. These IL-12p40 were locally produced by large intestinal M ϕ , DC, and epithelial cells. Based on our knowledge, this is the first demonstration of the presence of IL-12p40 in the selected part of the intestinal tract (eg, large intestine) in mice with allergic diarrhea. Although IL-12 is thought to drive the Th1-dominant environment,³¹ our present findings provide additional supportive evidence that IL-12p40 contributes to the generation of a Th2-dominant environ-

ment.^{18,19} It should be noted that our results directly demonstrate the *in vivo* immunopathological contribution of locally produced mucosal IL-12p40 to the development of OVA-induced diarrhea. Thus, the anti-IL-12p40 treatment reduced the incidence of OVA-induced allergic diarrhea. An attractive explanation would be that large intestinal MØs and DCs as well as epithelial cells contribute to the development of pathological Th2-dominant responses by the production of IL-12p40 in OVA-induced allergic diarrhea. Thus, the administration of anti-IL-12p40 resulted in the inhibition of the locally produced, mucosal IL-12p40-created, pathological Th2 condition, leading to the reduction of disease development.

Our present and previous results clearly show that large intestinal antigen-specific Th cells produce high levels of Th2 cytokine in OVA-induced allergic diarrhea.¹ The presence of monomeric or dimeric forms of IL-12p40, behaving as an antagonist to IL-12p70, is an additional contributing factor for the creation of a dominant pathological Th2 environment. Thus, the severe symptoms of allergic diarrhea were reduced by treatment with anti-IL-12p40 mAb, since the production of Th2 cytokines was significantly decreased in the large intestine. Overall, IL-12p40-supported, Th2-type cytokine synthesis plays a critical and pathological role in the induction of allergic reactions in large intestinal tissues. Although we do not have any specific explanation for the generation of IL-12p40 at the disease site, one possibility could be antigen overload in the intestinal tract. Our previous study demonstrated that oral administration of high doses of OVA induced Th2-mediated allergic diarrhea in systematically pre-sensitized BALB/c mice.¹ In contrast, low doses of oral OVA failed to induce allergic diarrhea. It was also shown that high doses of OVA peptide increased the numbers of naive CD4⁺ T cells with Th2-like phenotype, which in turn produced dramatically large amounts of IL-4.³² Therefore, high doses of oral antigen may create an immunological environment favoring Th2 cell development. To support this view, it has also been shown that high doses of oral antigen preferentially inhibit IFN- γ -producing Th1-type cells.¹ Further, the dose of antigen can determine whether Th1- or Th2-type cells are generated by antigen-presenting cells including DC.³³ Taken together, these findings allow us to postulate that an overload of oral antigen may direct mucosal antigen-presenting cells, including DC and MØ, and epithelial cells, to produce monomeric or dimeric forms of IL-12p40 instead of IL-12p70.

IL-12 has been considered as an inhibitory factor for allergic responses induced by preferential Th2-cytokine production. Indeed, endogenous rIL-12 decreased IgE levels and Th2 cytokine production induced by allergic reaction.³⁴ In contrast, IL-12 has also been shown to be involved in the pathological phase of mucosa-associated allergic diseases of the respiratory tract. In the murine asthma model, IL-12 contributed to the recruitment of eosinophils into the respiratory tract via the induction of VCAM-1 on local vascular epithelial cells.³⁵ Thus, the deletion of the IL-12 gene (p40) resulted in a substantial reduction in the airway recruitment of eosinophils and in the expression of VCAM-1 when compared with wild-type

mice exhibiting an asthma-like reaction induced by systemic sensitization followed by nasal OVA.³⁶ In addition, selective overexpression of IL-12p40 was noted in airway epithelial cells and bronchoalveolar lavage fluids of patients with asthma.²⁰ Our present findings also demonstrate that the locally produced p40 form of IL-12 was associated with the development of OVA-induced allergic diarrhea. Thus, IL-12p40 was preferentially expressed only in the large intestine of allergic diarrhea mice. In addition to these results generated through the characterization of an asthma model, our present finding suggests a critical role for IL-12, especially that of p40-associated molecules, for the development of allergic diseases including asthma and food allergy.

The treatment with anti-IL-12p40 mAbs effectively reduced the incidence as well as the severity of allergic diarrhea, an effect most likely due to an alteration in the dominant immunopathological Th2-type response to a Th1-type environment. To support this view, locally overexpressed IL-12p40 may compete with the well-known Th1 promoter IL-12p70 and IL-23 (p40/p19).³⁷⁻³⁹ To this end, it has been shown that endogenous IL-12p40 can overcome the Th1-promoting activity of IL-12p70 and/or IL-23.¹⁸ In this regard, our recent and separate study showed that IL-23p19-specific mRNA expression was not detected in either diseased or healthy BALB/c mouse groups (data not shown). The results suggest that IL-23 dose not play an important role in the development of our diarrhea model. Therefore, treatment with anti-IL-12p40 antibody likely eliminated the antagonistic effect of IL-12p40 at the local site, perhaps leading to the creation of an IL-12p70 environment for the initiation of down-regulation of Th2 responses. An alternative explanation would be that anti-IL-12p40 mAb used in this experiment may possess a higher affinity for the monomeric or dimeric form of IL-12 than for the IL-12p70 heterodimer. Although our emphasis has been on the inhibitory effects of anti-IL-12p40 mAb for the prevention of allergic diarrhea, one must accept the fact that complete prevention of disease development was never achieved through use of mAbs. A possible explanation for this finding could be that anti-IL-12p40 mAb inhibited Th1 induction of IL-12p70 in addition to IL-12p40. Thus, this alteration of a Th2 dominant environment and shift to one of a Th1-type may partially occur in the large intestine of mice with allergic diarrhea. To support this possibility, the mAb used in these experiments has been shown to neutralize IL-12p70 in addition to IL-12p40.^{13,14} In addition, the experiments using IL-12p40 KO mice suggest that the absence of IL-12p40 results in a complete failure to develop allergic diarrhea. It clearly shows that IL-12p40 play a critical role in the development of this disease. However, one alternative and simple expectation would be that an IL-12p40 deficiency may lead to the creation of Th2 environment due to the lack of Th1 inducing IL-12p70. Thus, it may lead to the more susceptible condition for the development of Th2-mediated diarrhea. Although we do not have any specific data to negate the latter possibility, one possible explanation would be that the deficiency of IL-12p70 formation in IL-12p40 KO mice lead to the lack of ability to active antigen presenting cells. IL-12p70 deficiency may

result in the absence of induction antigen-specific T cell response including the pathological Th2-type cells. It has been shown that IL-12 or IL-12-induced IFN γ can directly activate antigen presenting cells.⁴⁰ To address the issue, a series of interesting experiment would be the adaptive transfer of large intestinal M ϕ , DC, and epithelial cells into IL-12p40 and/or p35 KO mice. These experiments are, of course, planned for our future study.

Recently, it has been suggested that IL-12 is also one of the key cytokines for the regulation of the intestinal immune response.⁴¹ Mouse IL-12p40 is produced as monomer and homodimer five to ninety times as frequently as IL-12p70 *in vivo* and *in vitro*,^{40,42} implying the existence of additional immunological roles for IL-12p40. An interesting possibility would be that excess production of the monomeric and/or the homodimeric form of IL-12p40 could be a key contributing factor to the maintenance of immunological homeostasis at the mucosal compartment. Interestingly, our present findings demonstrate that over-expression of IL-12p40 occurred only in the large but not the small intestine following oral exposure to high doses of protein antigen. At the present time, we cannot offer any specific explanation for this distinct localization of IL-12p40. However, an interesting possibility would be that the expression of negative regulators for IL-12, including sCD40L and IL-10R,^{14,43} could differ between the small and large intestine. To support this possibility, epithelial cells have been shown to express CD40 and IL-10R.^{34,44} Since the large intestinal tract is continuously exposed to overloaded microflora, the level of co-stimulatory molecule expression such as CD40 by large intestinal epithelial cells could be lower to avoid unnecessary inflammatory responses. Thus, the large intestinal tract may form an immunological environment favoring the generation of IL-12p40. This interesting possibility is currently being tested in our laboratory.

In summary, our results demonstrated that locally produced IL-12p40 contribute to the Th2 cell generation of pathological polarization in the large intestine of OVA-induced allergic diarrhea. This study provides the first evidence for the association of over-expressed IL-12p40 from intestinal epithelial cells, DC and M ϕ , in the development of allergic diarrhea. Thus, the application of anti-IL-12p40 mAb resulted in the reduction of disease incidence and severity. Further, the disease development was completely eliminated in the deletion of IL-12p40 gene. Taken together, our studies provide an opportunity to consider that anti-IL-12p40 mAbs may be an alternative therapeutical regimen for the control of allergic intestinal disease.

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