Systemically derived large intestinal CD4^{+} Th2 cells play a central role in STAT6-mediated allergic diarrhea

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Systemically primed BALB/c mice developed severe diarrhea after repeated oral administration of ovalbumin (OVA). Histological analysis demonstrated that dramatic infiltration of eosinophils and mast cells occurred in the large intestine but not in the small intestine of mice with diarrhea. Interestingly, CD4^{+} αβ T cells of the large intestine secreted IL-4 and IL-13 at high levels. Identically treated STAT6 gene-disrupted mice failed to develop OVA-induced diarrhea. Further, treatment of BALB/c mice with monoclonal anti–IL-4 antibody prevented the development of allergic diarrhea. An adoptive transfer study showed that systemically primed splenic CD4^{+} T cells were preferentially recruited into the large intestine upon exposure to oral OVA. These results strongly suggest that systemically derived CD4^{+} αβ T cells of the large intestine play a critical role in the onset of Th2-mediated intestinal allergic disorders via STAT6 signal transduction.

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Introduction

Diarrhea, one of the intestinal allergic symptoms provoked by ingested food allergens, is characterized by imbalanced ion exchange, excessive production of mucus, and high levels of serum IgE antibodies (Ab's; 1, 2). In fact, several animal models have been established to investigate the underlying mechanism for intestinal hypersensitivity such as diarrhea (3). These include murine models in which protein Ag was coadministered parenterally with adjuvants such as immune-stimulating complexes, aluminum hydroxide, and cholera toxin to produce high IgE immune responses (4–6). Further, an IgE-mediated anaphylactic murine intestinal model was developed by subcutaneous implantation of a murine hybridoma capable of producing monoclonal anti-TNP IgE Ab (7). Although such various murine hypersensitivity models have been developed, the underlying mechanism of intestinal allergic reactions remains to be elucidated. In the present study, we have developed a new experimental model where repeated oral challenge with OVA in systemically primed BALB/c mice with Ag in CFA leads to severe diarrhea. We have then used this murine diarrhea model to clarify the underlying mechanism of intestinal hypersensitivity.

Signal transducers and activators of transcription (STAT) 6 are essential for the induction of IL-4– and IL-13–mediated responses, such as class switching to IgE, as well as induction of Th2-type cells (8–10). In this regard, recent studies revealed that STAT6-deficient mice are protected from OVA-induced airway hypersensitivity resulting from low levels of serum IgE, eosinophil infiltration, and peribronchial inflammation (11, 12). Although the STAT6-mediated signaling pathway has been associated with the induction of airway hyperresponsiveness, it is still not known whether this STAT6-signaling cascade plays a critical role in the development of intestinal allergic reactions, such as food Ag-induced (e.g., OVA-induced) diarrhea.

Our present data demonstrate a central role for systemically originated IL-4– and IL-13–producing Th2 cells and the STAT6-mediated signal in the development of localized allergic responses in the large but not small intestine. Further, our murine diarrhea model provides a unique experimental tool for studying the underlying molecular and cellular mechanism of allergic disorders in the intestine.

Methods

Mice. BALB/c, C57BL/6, SJL/J, and C3H/HeJ mice, and SCID mice of BALB/c background were purchased from Japan Clea Co. (Tokyo, Japan). STAT6 gene-disrupted (*STAT6^{−/−}*) mice were purchased from The Jackson Laboratory Animal Resources Center (Bar Harbor, Maine, USA). Transgenic mice expressing the green fluorescence protein (GFP Tg mice) were kindly provided by M. Okabe of the Research Institute for Microbial Diseases, Osaka University (13). Given that the background of GFP Tg mice were BCF1 x BCF1, we back-

crossed these mice numerous times to BALB/c mice, to establish GFP Tg mice with BALB/c background. All mice were housed in the experimental animal facility at the Research Institute for Microbial Diseases, Osaka University, and received sterilized food (Certified diet MF; Oriental Yeast Co., Osaka, Japan) and autoclaved tap water ad libitum. All mice were 5–6 weeks of age at the beginning of individual experiments.

Antigen challenge. Mice were primed by systemically either once or twice with 1 mg of OVA (Fraction V; Sigma Chemical Co., St. Louis, Missouri, USA) in 100 μL of CFA (DIFCO Laboratories, Detroit, Michigan, USA). One week after the systemic priming, mice were repeatedly given 1, 10, 50, or 100 mg of OVA dissolved in 300 μL of PBS by gastric intubation three times per week. Whether systemically challenged once or twice, mice receiving 50 mg or 100 mg of oral OVA developed diarrhea. Thus, in this study, we selected a protocol of one dose of subcutaneous priming with 1 mg of OVA before oral feeding with 50 mg of OVA. For the control, mice were repeatedly given 50 mg of OVA in PBS by gastric intubation without any systemic challenge. Two hours after the last oral administration, mice were sacrificed and Ag-induced allergic reactions were analyzed.

Anti–IL-4 mAb treatment. In vivo Ab treatment was performed using a standard protocol as described previously (14). BALB/c mice were intraperitoneally injected with anti-mouse IL-4 mAb (BVD4-1D11, 1 mg/mouse) or isotype-matched control rat IgG2b mAb (R35-38, 1 mg/mouse) in 250 μL of PBS once a week for the duration of the experiment. Ab treatment was started 1 week before systemic priming with OVA in CFA.

Adoptive transfer experiment. Because GFP+ cells are considered to be the most suitable system for adoptive transfer experiments intended to address migration patterns of a specific subset of cells, GFP Tg mice were used as donor mice in our study (13). The BALB/c background GFP Tg mice were immunized via the subcutaneous route with 1 mg OVA in CFA as already described here. FACS-separated GFP+ CD4+ T (1×10^5 to 2×10^5) cells were resuspended in 200 μL of PBS and adoptively transferred to SCID mice of BALB/c background by tail vein injection. One week after reconstitution, the recipient SCID mice were repeatedly challenged with 50 mg of OVA by gastric intubation as already described here.

Histological evaluation. For histopathologic study, the small and large intestines from mice in each group were fixed in 4% paraformaldehyde and embedded in paraffin. The tissues were cut into 5-μm sections and stained with hematoxylin and eosin (H&E) for evaluation of general pathological changes and with Alcian blue and safranin dye for analysis of mast cells, respectively. For detection of GFP+ cells, the tissues were fixed in 4% paraformaldehyde and rapidly frozen in OCT embedding medium (Sakura Finetechnical Co., Ltd., Tokyo, Japan). Cryostat sections (7 μm) were prepared and analyzed under a confocal microscopy (Bio-Rad Laboratories Inc., Hercules, California, USA).

Immunohistochemical detection of IgE. Freshly obtained small and large intestines were rapidly frozen in OCT embedding medium and stored at –80° C until processing. Cryostat sections (7 μm) were prepared and stained with FITC-anti-IgE mAb's (R35-118; PharMingen, San Diego, California, USA) overnight at 4°C. Positive cells for IgE-specific mAb-staining were analyzed under a confocal microscopy.

ELISA for total and OVA-specific IgE Ab's in serum. For detection of total and OVA-specific IgE levels in sera, the immunoplates (Nalge Nunc International, Naperville, Illinois, USA) were coated with purified rat anti-mouse IgE mAb (R35-72; PharMingen) and incubated overnight at 4° C as described previously (15). After blocking with 3% BSA in PBS, serial dilutions of serum samples and standard mouse IgE (27-74; PharMingen) were added and incubated for 4 hours at room temperature. After extensive washing, biotinylated rat anti-mouse IgE mAb (R35-118; PharMingen) for total IgE Ab or biotinylated OVA for OVA-specific IgE Ab were added, respectively. After plates were incubated overnight at 4°C, peroxidase-labeled antibiotin mAb (Vector Laboratories, Burlingame, California, USA) for total IgE or 10 ng/mL AquaLite streptavidin (SeaLite Sciences, Notcross, Sweden) in 2 mM EGTA in PBS for OVA-specific IgE were added, respectively. After washing, the color reaction was developed with 3, 3′, 5, 5′-tetramethyl-benzidine (TMB; Moss Inc., Pasadena, California, USA) for total IgE. Light development for OVA-specific IgE was carried out in a luminometer (iEMS Reader; Labsystems Inc., Helsinki, Finland) by injection of Ca^{2+} buffer (50 mM Tris, 20 mM CaAc [pH 7.5]) (16). End-point titers of OVA-specific IgE were expressed as the reciprocal log_2 of the last dilution that showed a level of luminometric units twofold higher than the background. In no case did nonimmunized mice give titers greater than log_2 of 2.

Isolation of mononuclear cells. The spleen was removed aseptically, and single cell suspensions were prepared by the mechanical dissociation method as described previously (17). In addition, mononuclear cells were dissociated from small and large intestines using a collagenase (Type IV; Sigma Chemical Co.; 0.5 mg/mL of RPMI 1640) after removal of Peyer's patches and colonic patches (18). The single-cell suspensions were then pooled and further purified using a discontinuous Percoll gradient (Pharmacia Biotech AB, Uppsala, Sweden) as described previously (17, 18).

Enzyme-linked immunospot assay. An enzyme-linked immunospot (ELISPOT) assay was adopted to detect OVA-specific Ab-forming cells (AFCs) in spleen as well as in small and large intestines as described previously (19).

Cytokine analysis. Cytokine levels (including IFN-γ, IL-4, and IL-5) of culture supernatants of Ag-stimulated mononuclear cells isolated from spleen and from small and large intestine were measured by an ELISA as described previously (20). For the IL-13 assay, an ELISA Kit was purchased from R&D Systems Inc. (Minneapolis, Minnesota, USA).

FACS analysis and cell sorting. The PE-conjugated anti-CD4 (RM4-5) mAb from PharMingen was used in flow cytometry analysis using a FACScalibur (Becton Dickinson, San Jose, California, USA). In some experiments, the flow cytometry sorting separation was performed by FACS Vantage (Becton Dickinson) using FITC-conjugated anti-TCRαβ and PE-conjugated anti-CD4 mAb's.

Quantitative RT-PCR method. Total RNA was extracted by using TRIzol reagent (Life Technologies, Gaithersburg, Maryland, USA), and 5 μg/mL of extracted RNA was subjected to RT reaction using Superscript II Reverse Transcriptase (Life Technologies). The cDNA from 10 ng of RNA was used for each cytokine-specific PCR (21). In some studies, rapid-cycle DNA amplification was performed by a LightCycler (Boehringer Mannheim GmbH, Mannheim, Germany), which is a rapid thermal cycler using the double-strand-specific dye SYBE Green I (22). The PCR cycle was as follows: initial denaturation at 95°C for 2 minutes, followed by 45 cycles of denaturation at 95°C for 0 seconds and combined annealing-extension at 55°C for 5 seconds and 72°C for 10 seconds. Cycle-to-cycle fluorescence emission readings were plotted on the computer screen for continuous monitoring of PCR product using LightCycler software. Results are shown as fluorescence at 520 nm from 20 to 45 cycles of PCR reaction.

Intracellular cytokine assay. For intracellular cytokine analysis, mononuclear cells isolated from spleen, small and large intestine of SCID mice reconstituted with systemically primed GFP$^+$ CD4$^+$ T cells were cultured with complete RPMI medium containing 10% FBS, 5 μM 2ME, 10 U/mL penicillin, 100 μg/mL streptomycin, soluble anti-CD28 mAb (37.51, 2 μg/mL) and rmIL-2 (20 ng/mL) in 24-well flat-bottomed plates coated with anti-CD3ε mAb (145-2C11; 10 μg/mL) for 16 hours (23). GolgiStop (2 μM/mL; PharMingen) was added during the final four hours of incubation. Cytoplasmic staining was performed using Cytofix/Cytoperm Kits (PharMingen). PE-conjugated anti-mouse IFN-γ (XMG1.2), IL-4 (BUD4-1D11), and anti-rat IgG1 (R3-34) mAb's for isotype control were used. Specific mAb-labeled cells were analyzed in a flow cytometry analysis using FACScalibur.

Data analysis. Data were expressed as mean ± SE and evaluated by the Mann-Whitney *U* test for unpaired samples using a Statview II statistical program (SAS Inc., Cary, North Carolina, USA) designed for the Macintosh computer. *P* values of less than 0.05 were assumed to be statistically significant.

Results

Repeated oral administration with OVA resulted in severe diarrhea in systemically primed mice. To clarify the molecular and cellular mechanisms of intestinal hypersensitivity, we initially aimed to establish a model that would lead mice to develop severe intestinal allergic symptom such as diarrhea. To select the most suitable and reliable model, groups of BALB/c, C57BL/6, SJL/J, and C3H/HeJ mice were tested. Among these different mice

strains, we found that repeated oral administration to BALB/c mice of a high dose of OVA after previous subcutaneous immunization with OVA in CFA resulted in severe diarrhea (Figure 1a). In addition, a similar result was obtained in SJL/J mice. OVA-induced diarrhea was observed after eight to ten oral Ag administrations to the systemically primed mice; however, repeated oral administration of OVA without systemic priming did not induce any significant clinical sign of intestinal hypersensitivity. Diarrhea was observed within 30 minutes to 2 hours after the last oral Ag administration and decayed within 2 hours, suggesting that acute allergic responses had occurred in these mice. These results indicated that development of diarrhea was dependent on the mouse strains. Thus, we chose BALB/c mice for the remainder studies.

Eosinophils and mast cells dramatically infiltrated the large intestine but not the small intestine of mice with diarrhea. H&E staining was carried out to examine the histopathologic changes in the small and large intestines of mice with diarrhea. Interestingly, eosinophils infiltrated only the large intestine of mice with diarrhea, and only the epithelium and the crypt region of the large intestine, not the lamina propria and the submucosa (Figure 1b). As shown in Figure 1c, the number of infiltrating mast cells was greater in the large intestine of diarrhea-induced mice (Figure 1c, right) than those of control mice (Figure 1c, left). Further, increased numbers of mast cells were observed exclusively in the large intestine. Mucosal ulcers and hyperplasia of epithelial cells were not observed in either small or large intestines of diarrhea-induced mice (data not shown).

Selective induction of brisk Ag-specific B-cell responses in the large intestine of diarrhea-induced mice. Brisk levels of total and OVA-specific IgE Ab's were detected in serum of diarrhea-induced mice, whereas control mice that did not possess any sign of diarrhea showed either low or undetectable levels of total and OVA-specific IgE Ab's (Figure 2a). When the frequency of IgE AFCs was examined, IgE-producing cells were significantly more numerous in the large intestine of diarrhea-induced mice than in that of control mice without diarrhea (Figure 2a). Further, it is important to note that the number of IgE AFCs did not increase in the small intestine of mice with diarrhea (Figure 2a).

Once it had been demonstrated that high numbers of IgE-producing cells were present in the large intestine of diarrhea-induced mice, we next sought to determine whether IgG and IgA responses were similarly enhanced. Strikingly, high numbers of OVA-specific IgG and IgA AFCs were detected in the large intestine of diarrhea-induced mice, whereas no OVA-specific IgG and IgA AFCs were detected in the small intestine (Figure 2b). In addition, AFCs for OVA-specific IgG subclasses were also assessed, and IgG1 was found to be the most abundant IgG subclass in the large intestine of mice with diarrhea (Figure 2b, inset). Taken together, these results obtained by the

Figure 1
Induction of Th2-mediated allergic diarrhea. Diarrhea was induced in systemically primed mice by oral challenge with 50 mg of OVA (**a**, closed circle). Control mice were orally challenged with 50 mg of OVA without any systemic priming (**a**, open circle). Eosinophils (**b**) and mast cells (**c**, right panel) selectively infiltrated into the large intestines of diarrhea-induced mice. However, control group (**c**, left) did not show the increase of mast cells. Arrows point to eosinophils. Original magnification, × 200–400.
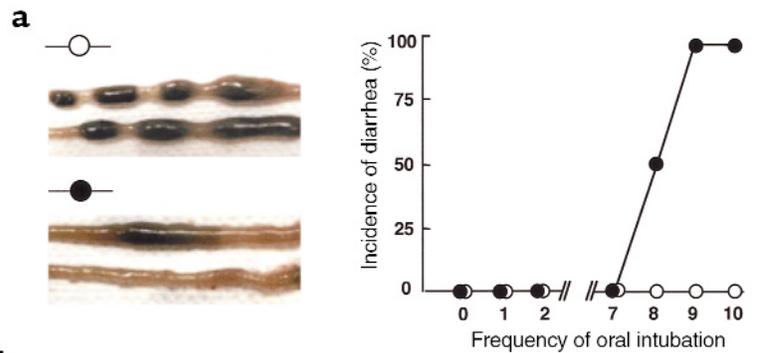
analysis of B-cell responses in the large intestine of diarrhea-induced mice may reflect the induction of localized dominant Th2-type responses.

Predominant Th2-type cytokine responses were selectively induced in the large intestine of diarrhea-induced mice. Large intestinal lymphocytes of mice with diarrhea elicited significant IL-4, IL-5, and IL-13 but little or no IFN-γ synthesis after restimulation with OVA in vitro, whereas small intestinal lymphocytes did not produce detectable levels of Ag-induced cytokines (Figure 3a). In contrast, splenic lymphocytes isolated from diarrhea-induced mice produced high levels of IL-4, IL-5, and IL-13 as well as IFN-γ (Figure 3a). These results clearly suggest that Th2-type cytokine responses are crucial to the development of IgE-, IgA-, and IgG1-producing cells in the large intestine of mice with diarrhea. To clarify further the role of large intestinal CD4+ αβ T cells in the development of diarrhea, we further examined the profile of Th1-type (IFN-γ) and Th2-type (IL-4 and IL-13) of cytokine synthesis by these cell subsets using cytokine-specific quantitative RT-PCR. The large intestinal CD4+ αβ T-cell fraction of diarrhea-induced mice possessed higher levels of IL-4 and IL-13 than did that of control mice (Figure 3b). These results suggest that the CD4+ αβ T cells of the large intestine are primarily committed to the generation of preferential Th2-type responses associated with allergic diarrhea.

STAT6−/− mice were protected from diarrhea induced by repeated oral challenge with OVA. To examine directly the role of predominant Th2-type cytokines (i.e., IL-4 and IL-13) in the development of diarrhea, specific gene-manipulated mice lacking STAT6 were used in the study of diarrhea induction. Interestingly, *STAT6−/−* mice were completely protected from the development of OVA-induced diarrhea, with no sign of eosinophil infiltration observed in their large intestines (Figure 4a). As one might expect in the mice lacking Th2 cytokine responses, no increase of total or OVA-specif-

ic IgE Ab's was detected in serum (Figure 4b). Moreover, significantly fewer OVA-specific IgG and IgA AFCs were detected in the large intestines of *STAT6−/−* mice than in those of wild-type mice (Figure 4c). To support this finding further at the level of the STAT6-mediated and -induced protein, two groups of BALB/c mice were treated with rat anti-mouse IL-4 mAb or isotype-matched control rat IgG2b mAb via the intraperitoneal route. The anti–IL-4 mAb–treated mice did not develop OVA-specific IgE–mediated diarrhea, whereas severe diarrhea was induced in the control group (Table 1). These results clearly demonstrate that the development of OVA-induced diarrhea is completely dependent on STAT6-regulated Th2-type responses.

Systemically primed splenic CD4+ T cells selectively migrated into the large intestine and expressed Th2-type cytokines in mice given a high dose of oral Ag. Because hyper Ag-specific T- and B-cell responses were seen in the large intestine and spleen but not in the small intestine of diarrhea-induced mice, we used an adoptive transfer experiment with the GFP Tg mice to investigate the
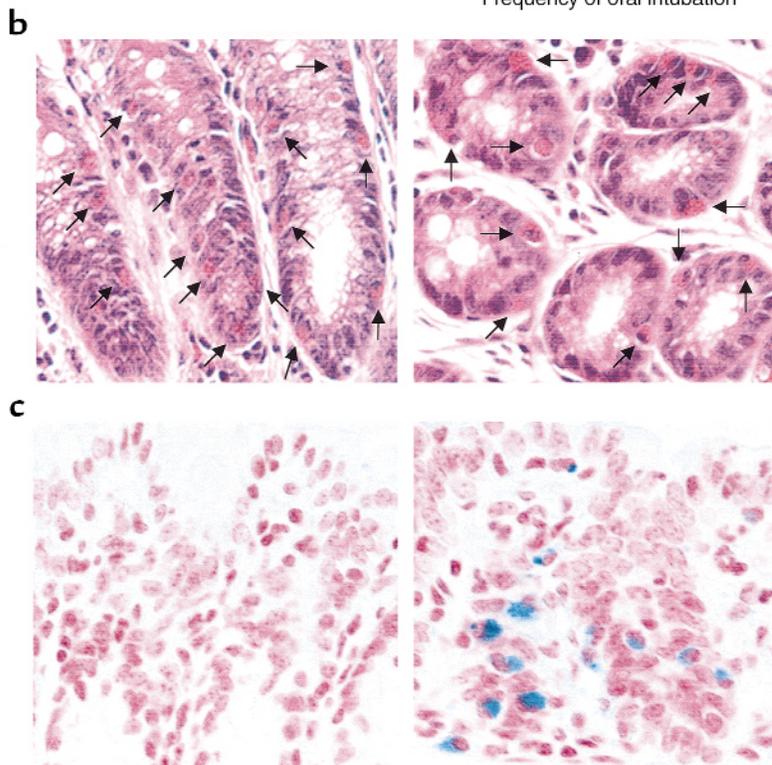
possibility that a cross-network system might exist between systemic (i.e., spleen) and mucosal (i.e., large intestine) compartments. For this purpose, splenic CD4+ T cells from systemically primed GFP Tg mice were adoptively transferred to SCID mice. As shown in Figure 5, a and b, significantly higher numbers of the reconstituted GFP+ CD4+ T cells were preferentially recruited into the large than into the small intestine of the recipient SCID mice after the administration of a high dose of oral Ag (54.3 vs. 16.1%). Further, the SCID mice that received systemically primed splenic GFP+ CD4+ T cells developed diarrhea upon oral challenge with OVA (data not shown).

Analysis of cytokine profiles of lymphocytes by RT-PCR methods showed that higher expressions of Th2-type cytokine (e.g., IL-4, IL-5, and IL-13) specific mRNA were detected in the large intestinal lymphocytes than in the small intestinal lymphocytes of the SCID mice that received GFP+ CD4+ T cells (Figure 5c). In contrast to the Th2-type cytokines, the intensity of IFN-γ message was similar among the lymphocytes isolated from spleen and small and large intestines. In the case of the spleen, a similar cytokine pattern of the large intestinal lymphocytes was seen. Characterization of IFN-γ– and IL-4–containing cells by intracellular cytokine staining supported the result obtained by the RT-PCR assay and showed that these cytokine-producing CD4+ T cells were predominantly found in the large intestine and spleen but not in the small intestine (Figure 5d). Taken together, these results clearly indicate that systemically primed splenic CD4+ T cells selectively migrate into the large intestine and that these systemically derived large intestinal CD4+ T cells produce Th2-type cytokines that play a critical role in the development of allergic responses.

Discussion

The main pathophysiological changes in intestinal allergic reactions include increased levels of IgE in serum and infiltration of mast cells and/or eosinophils into the intestine (24). These pathologic processes of allergic reaction are thought to be mediated by Th2 cells, which preferentially produce IgE-enhancing cytokines such as IL-4 and IL-13 (25, 26). Indeed, our present results provide direct evidence that cytokine synthesis by large intestinal CD4+ T lymphocytes at both protein and mRNA levels is predominantly of the Th2 type. Such Th2-type cytokine synthesis contributes to the high levels of local IgE production and infiltration of eosinophils and mast cells into the large intestine of diarrhea-induced mice. Of the Th2-type cytokines, IL-4 was thought to be a key cytokine for class-switching of μ to ε genes (27, 28). However, a recent study demonstrated that IL-4 gene–disrupted mice had comparable serum IgE levels to wild-type mice during the development of a murine retrovirus-induced immunodeficiency syndrome (29). In addition, it was reported that eosinophil infiltration and airway hyperreactivity were impaired but not com-

Table 1

Protection of OVA-induced diarrhea in anti–IL-4 mAb–treated mice

Treatment of mice	n	Percent of mice with diarrhea	Levels of IgE in serum		OVA-specific Ab responses in large intestine[C]	
			Total[A]	OVA-specific[B]	IgG	IgA
Rat IgG2b mAb	5	100	4150 ± 65	6.5 ± 0.5	633 ± 63	3631 ± 561
Anti–IL-4 mAb	5	0	ND[D]	ND[D]	ND[D]	ND[D]

[A]Total IgE levels are expressed as ng/mL. [B]OVA-specific IgE Ab's are expressed as reciprocal $\log_2$ titers. [C]Numbers of OVA-specific AFCs per 10^6 cells. [D]$P <$ 0.01 vs. rat IgG2b mAb–treated mice. ND, not detectable.

pletely abolished in the $IL\text{-}4^{-/-}$ mice as a result of the inhalation of an allergen (30). These findings suggest that IL-4 is not the only cytokine that is involved in the induction of allergic reactions associated with predominantly Th2-type cytokine expression, tissue eosinophilia, and high levels of IgE Ab's.

IL-13, which shares a receptor component (i.e., IL-4R) and signaling pathways (i.e., IRS-2, Jak1, Jak3, or STAT6) with IL-4, is thought to be heavily involved in the development of allergic responses (31, 32). Repeated stimulation of allergen-specific memory CD4+ T cells induces IgE production via IL-13 in patients with atopy (33). Significant elevations of IL-4 as well as IL-13 expression were simultaneously found in the airway of a patient with allergic asthma (34). The overlapping biologic properties of IL-4 and IL-13 can then be
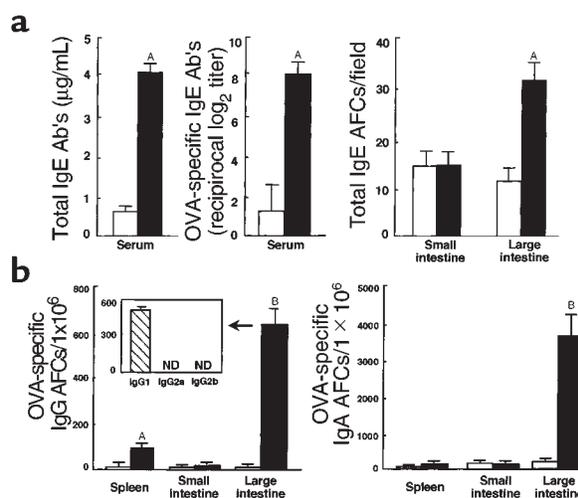
Figure 2

Selective induction of brisk B-cell responses in the large intestine of mice with diarrhea. (**a**) Total and OVA-specific IgE Ab's in serum and total IgE AFCs in the small and large intestines. For evaluation of the numbers of IgE positive–staining cells in the large intestine, ten randomly selected fields were counted under ×200. (**b**) Represents OVA-specific IgG and IgA AFCs in the spleen and in the small and large intestines. The inset shows the frequency of OVA-specific IgG subclass AFCs in the large intestines of mice with diarrhea. Mononuclear cells were isolated from spleen and small and large intestines of control (open bars) and diarrhea-induced mice (filled bars). These data are representative of three independent experiments containing five to seven mice in each group. [A]$P < 0.05$, [B]$P < 0.01$ versus control mice. ND, not detectable.
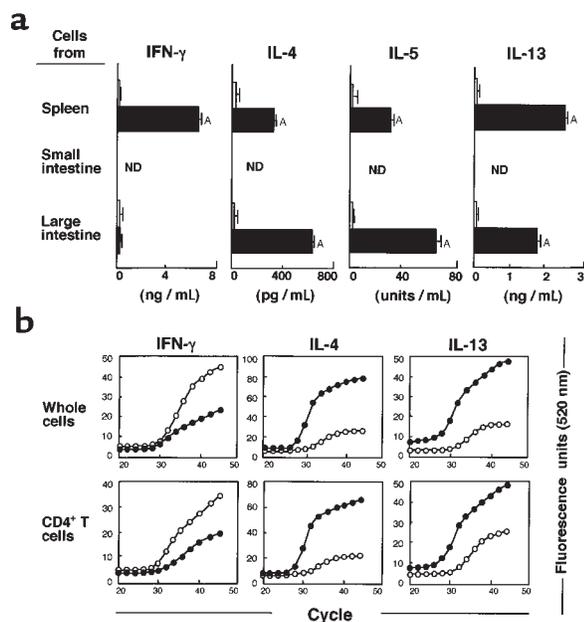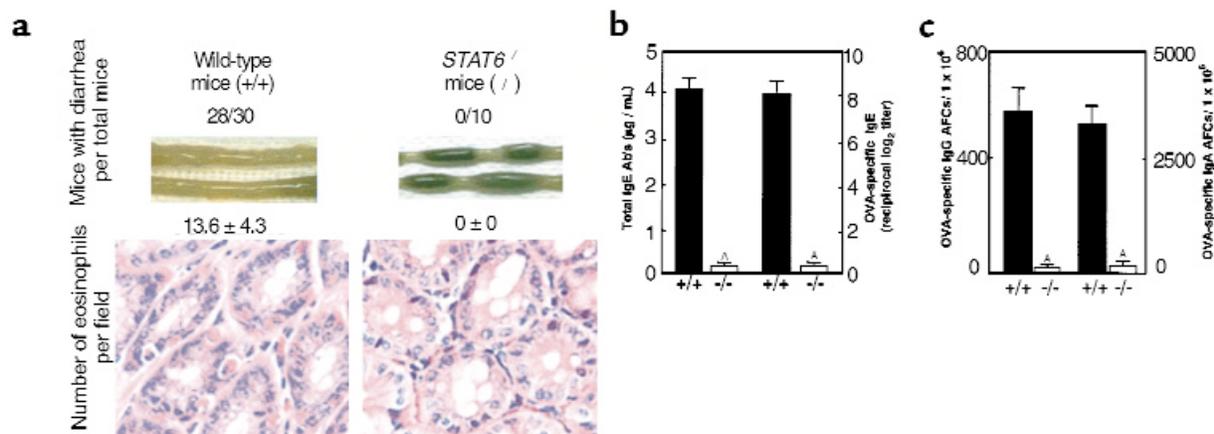
Figure 3
Induction of predominant Th2 cytokine response by large intestinal lymphocytes from mice with OVA-induced diarrhea. (**a**) Cytokine synthesis by the mononuclear cells isolated from the spleens and from the small and large intestines of control (open bars) or diarrhea-induced (filled bars) mice. The mononuclear cells were cultured in the presence of 100 μg/mL of OVA for 4 days, and then culture supernatants were analyzed by the respective cytokine-specific ELISA. (**b**) Expression of Th1 (IFN-γ) and Th2 (IL-4 and IL-13) cytokine-specific mRNA by whole lymphocytes and CD4+ αβ T cells from large intestines of control (open circle) and diarrhea-induced (closed circle) mice. Quantitative RT-PCR methods were performed using the LightCycler. These data are representative of two independent experiments containing five to seven mice in each group. ^A$P < 0.05$ versus control mice. ND, not detectable.

explained by the sharing of signal pathways via STAT6 (8–10). We therefore examined the role of STAT6 signaling on the development of OVA-induced diarrhea using STAT6 gene-disrupted mice. Of interest was the finding that *STAT6*−/− mice were completely protected from the development of OVA-induced diarrhea upon repeated challenge with oral Ag. Moreover, this failure to develop OVA-induced diarrhea in *STAT6*−/− mice was associated with the inability of these mice to produce Th2-type cytokines and IgE Ab's and to promote infiltration of eosinophils. In this regard, it was recently shown that *STAT6*−/− mice failed to develop airway hypersensitivity reactions after repeated aerosol exposure with OVA (12). *STAT6*−/− mice that inhaled allergen showed only low levels of eosinophil infiltration and of Th2-type cytokine synthesis and no Ag-specific IgE (11). Our present study is the first demonstration to our knowledge that STAT6 signaling plays an essential role in the development of intestinal allergic diarrhea. Further, localized specific disruption of this signal pathway may provide a strategy for the treatment of patients with food allergies who suffer from persistent diarrhea, vomiting, and dehydration.

Interestingly, we found localized infiltration of eosinophils in the large intestine of diarrhea-induced mice. Previous studies also demonstrated that intestinal allergic reactions were characterized by high numbers of eosinophils in jejunum and cecum upon chronic oral Ag exposure (7, 35). Eosinophil degranulation may contribute to the development of intestinal allergic reaction, which liberates proinflammatory mediators such as major basic protein, eosinophil cationic protein, and eosinophil peroxidase (36). Although IL-5 has been shown to be an important factor in migration and activation of eosinophils (37, 38), recent studies revealed that IL-4 and IL-13 also contribute to activation of these cells by promoting IL-5 production (11, 31). In this study, Ag-specific large intestinal CD4+ T cells were found to produce high levels of IL-4, IL-5, and IL-13, which may contribute to localized infiltration as well as to activation of eosinophils in mice with OVA-induced diarrhea. It was recently suggested that there are both STAT6-dependent and -independent mechanisms for Ag-induced eosinophilia, as *STAT6*−/− mice were found to only partially suppress eosinophils in the bronchoalveolar lavage of mice with airway hyperresponsiveness (11). In contrast to these results, the fact that no eosinophils were detected in the large intestine of *STAT6*−/− mice upon repeated oral administration with OVA suggests that infiltration and activation of eosinophils in diarrhea-induced mice are completely dependent on STAT6 signaling.

An interesting and important aspect of our study is the fact that systemically primed Ag-specific allergic responses selectively occurred in the large intestine but not in the small intestine of mice with diarrhea. This finding suggests the possible existence of a tightly regulated cross-network system between systemic and selected mucosal compartments (e.g., spleen and large intestine, respectively). To test this possibility, an adaptation of the adoptive transfer experiment using GFP Tg mice as donors and SCID mice as recipients demonstrated preferential migration of systemically primed splenic CD4+ T cells into the large intestine upon exposure to a high dose of oral OVA. Further, these systemically derived large intestinal CD4+ T cells were shown to produce Th2-type cytokines, which in turn further promote allergic reactions such as dramatic increases in the numbers of IgE-producing cells, eosinophils, and mast cells. The distinct tissue migration pattern of systemically derived CD4+ T cells is presumably due to different environmental factors and/or to differing expressions of adhesion molecules in the small and large intestine. For example, there is a higher concentration of enteric bacteria in the large than in the small intestine. Thus, in selected types of inflammatory bowel diseases, colonic inflammation could be abolished under germ-free conditions (39). In support of this, antibiotic-treated mice were partially protected from the development of diarrhea (M.-N. Kweon et al., unpublished observation). These results suggest that microflora in the large intestine may influence the migration of systemically derived

Figure 4
Protection of OVA-induced diarrhea in *STAT6*^{–/–} mice. No sign of diarrhea or of eosinophil infiltration was observed in the large intestines of *STAT6*^{–/–} mice (**a**). Significantly lower levels of total or OVA-specific IgE Ab's in serum (**b**) and of OVA-specific IgG and IgA AFCs in the large intestines (**c**) of *STAT6*^{–/–} mice (–/–) were noted when compared with those of wild-type mice (+/+). ^A*P* < 0.01 versus wild-type mice.

CD4+ T cells for the induction of Th2 cell–derived allergic diarrhea. Alternatively, our previous study demonstrated that mucosal T-cell subsets isolated from the small and large intestines markedly differed, especially in the frequency of αβ T cells, CD4+ T cells, and the expression of LFA2 molecules, the levels of which were significantly higher in the large intestine (40). In addition, large intestinal intraepithelial T lymphocytes secreted mainly Th2-type cytokines, such as IL-4 and IL-6, whereas small intestinal T lymphocytes mainly produced IFN-γ (40). Further, it has been shown that transfer of CD4+ CD45RB^{high} cells isolated from the spleen of naive healthy donor mice into SCID mice leads to inflammatory bowel disease, whereas transfer of CD4+ CD45RB^{low} and CD8+ CD45RB^{high} cells does not (41, 42). These findings also suggest that selected populations of spleen-derived CD4+ T cells play a crucial role in the induction of localized immune responses in the large intestine.

Taken together, the results of this study invite consideration of the critical contribution of systemically primed splenic CD4+ T cells to the development of localized Th2-type cell-mediated hypersensitivity in the large intestinal immune system and point to the possible existence of a unique cross-talk immune pathway between systemic (e.g., spleen) and mucosal (e.g., large intestine) compartments. Further, they suggest that a part of the large intestine may be totally independent from the common mucosal immune system (CMIS).
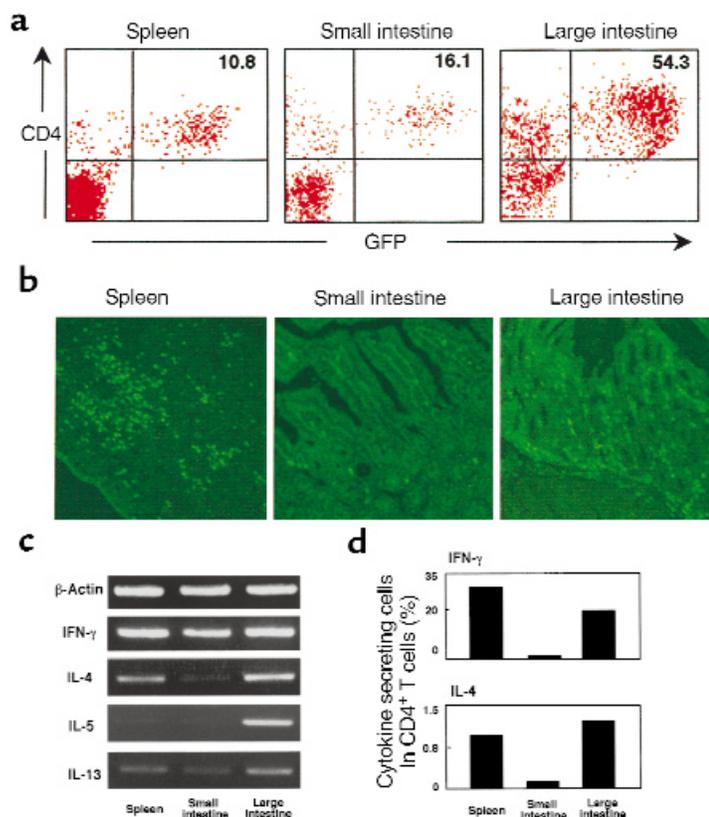
Figure 5
Preferential migration of systemically primed splenic GFP+ CD4+ T cells into large intestines of SCID mice. (**a**) Migration pattern of GFP+ T cells obtained from the spleen and small and large intestines of SCID mice to which GFP+ CD4+ T cells have been adoptively transferred. (**b**) Histological analysis of spleen and small and large intestines isolated from recipient SCID mice. Original magnification of ×40 or ×100 was used. (**c**) Expression of Th1 (IFN-γ) and Th2 (IL-4, IL-5, and IL-13) cytokine–specific mRNA in lymphocytes obtained from the spleen and small and large intestine of recipient SCID mice. (**d**) Represents the frequency of IFN-γ– and IL-4–secreting cells as determined by intracellular cytokine staining. Representative results from two individual experiments are shown.

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