

## CD4 T cells activated in the mesenteric lymph node mediate gastrointestinal food allergy in mice

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and M. Cecilia Berin<sup>1</sup>

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**Knight AK, Blázquez AB, Zhang S, Mayer L, Sampson HA, Berin MC.** CD4 T cells activated in the mesenteric lymph node mediate gastrointestinal food allergy in mice. *Am J Physiol Gastrointest Liver Physiol* 293: G1234–G1243, 2007. First published September 26, 2007; doi:10.1152/ajpgi.00323.2007.—A localized Th2 milieu has been observed in the intestine of subjects with food allergic disorders; however, the role of T cells in the pathophysiology of these disorders remains poorly understood. Our aim was to examine sites of T cell activation in response to food challenge, identify potential factors responsible for T cell recruitment to the gut, and determine the role of T cells in disease. BALB/c mice were systemically sensitized to ovalbumin (OVA) and repeatedly fed with OVA to induce allergic diarrhea. Local cytokine and chemokine expressions were assessed by quantitative PCR, and cytokine secretion levels in the mesenteric lymph node (MLN) were determined by ELISA. Homing molecule expression was determined by flow cytometry, and the role of CD4<sup>+</sup> T cells in promoting disease was tested by adoptive transfer. Mice developed diarrhea associated with changes in epithelial ion transport, mast cell infiltration, intestinal IgE secretion, and local upregulation of Th2 cytokines and the Th2 chemokines CCL1, CCL17, and CCL22 in the small intestine. T cell activation occurred in the MLN before symptom onset, and a single feed of OVA induced T cell proliferation,  $\alpha_4\beta_7$  upregulation, and CD62L downregulation. Cells from the MLN, including purified CD4<sup>+</sup> T cells, were able to transfer allergic diarrhea to naive mice. A gut-homing phenotype induced in the MLN and selective upregulation of Th2 chemoattractants are likely important factors in the gastrointestinal recruitment of pathological Th2-skewed CD4<sup>+</sup> T cells in food allergy.

chemokine; T lymphocyte; food allergy; diarrhea

FOOD ALLERGY IS A GROWING problem in pediatric populations. Two studies have demonstrated a doubling of peanut allergy in young children in the past two decades (20, 34), and similar increases may be occurring for other food allergens as well. It is estimated that 6% of young children have at least one food allergy (7, 14). Egg allergy is the second most common food allergy and occurs in 1.6% of 2-yr-old children (13). Presently, there are no treatments for food allergy, and strict avoidance is recommended.

Immune events in the gastrointestinal tract are central to the pathophysiology of food allergy. However, it is uncommon to obtain local tissue samples from allergic subjects because diagnosis is based on a combination of history, skin, or serum testing for food-specific IgE and clinical manifestations during a graded food challenge. Subjects with IgE-mediated food

allergies therefore do not routinely undergo endoscopy for diagnosis. One report carefully examined immune changes in the gastrointestinal mucosa of adult volunteers with food allergies (28). Lin et al. (28) obtained biopsies from a group of subjects with food allergies and normal volunteers. Subjects with food allergies, which were diagnosed by double-blind placebo-controlled food challenge, were biopsied after an extended oral food challenge (>7 days) and rebiopsied after a wash-out period of 2–3 mo during which their symptoms completely resolved. In their study, Lin et al. demonstrated that subjects with food allergies responded to allergen challenge with an increase in IgE-bearing cells in the intestinal mucosa and an increase in eosinophils, T lymphocytes (CD4<sup>+</sup> and CD8<sup>+</sup>), and IL-4 cytokine expression (28). Associated with this allergic inflammation were gastrointestinal symptoms, with diarrhea being the most common (12/14 subjects). The contribution of T lymphocytes to allergic disease in the gastrointestinal tract is not clearly understood, nor are the sequences of events from allergen challenge to T cell infiltration. Our aim was to use an animal model of gastrointestinal food allergy to understand the role of T cells and T cell activation in the generation of allergic symptoms in the gastrointestinal tract.

Two closely related models of allergic diarrhea have been described. Kiyono and colleagues (22, 23, 27) have used a model of allergic diarrhea in BALB/c mice that is generated by systemic priming with ovalbumin (OVA) in complete Freund's adjuvant followed by repeat feedings with OVA (50 mg  $\times$  10 feedings) to induce diarrhea symptoms. This model is dependent on Th2-cytokine-producing CD4<sup>+</sup> T cells, and allergic inflammation is localized to the large intestine (27). In contrast, Brandt et al. (9) used a modification of the widely used murine model of asthma to induce small intestinal allergic inflammation. After two systemic administrations of OVA in alum, exposure of mice to OVA by the oral route resulted in the generation of an acute antigen-specific diarrhea and allergic inflammation of the small intestine. This model was shown to be dependent on mast cells and IgE (9). We used this latter IgE-mediated food allergy model to determine the site of T cell activation in response to allergen exposure, the role of T cells in the generation of allergic diarrhea, and local factors in the gastrointestinal tract that could recruit proallergenic T cells to the intestinal mucosa.

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## METHODS

**Mouse model.** Female BALB/c mice were purchased from NCI (Frederick, MD) and maintained in filter-top cages under specific pathogen-free conditions. All experiments were performed with the approval of the Mount Sinai School of Medicine Institutional Animal Care and Use Committee. Mice were sensitized and challenged as previously described by Brandt et al. (9). Briefly, mice at 5–6 wk of age were sensitized to OVA (grade V; Sigma, St. Louis, MO) by intraperitoneal injection of 100 µg of OVA in alum (Alum Inject, Pierce, Rockford, IL) on *days 0* and *14*, followed by intragastric feedings of 1–50 mg of OVA in 0.2 M sodium bicarbonate on *days 28, 30, 32, and 34*.

**Ussing chamber.** Three to four days after the last feed of OVA, mice were euthanized, and 10 cm of jejunum were excised starting from the ligament of Treitz. Four segments per mouse were mounted in Ussing chambers (Physiologic Instruments, San Diego, CA), exposing an area of 0.3 cm<sup>2</sup>. Tissues were bathed in Krebs buffer containing 10 mM glucose on the serosal side of the tissue and either 10 mM glucose or 10 mM mannitol on the luminal side of the tissue. Tissues were voltage clamped and allowed to equilibrate for 20 min. OVA (200 µg/ml) was added to the luminal or serosal side of the tissue, and the short-circuit current (*I<sub>sc</sub>*) and resistance acquired at a data rate of 6/min. Data were acquired with Acquire and Analyze 2.3 (Warner Instruments, Hamden, CT). Thirty minutes after the addition of OVA, forskolin (1 µM) was added to verify the integrity of the tissue and the instrument setup. Only tissues with a normal *I<sub>sc</sub>* response to forskolin (mean change ± SE of 65 ± 8 µA/cm<sup>2</sup>) were analyzed.

**Cell culture.** Mesenteric lymph node (MLN) and spleen cells were isolated, and cells were cultured with medium alone (RPMI 1640 with 10% FCS and penicillin-streptomycin) or with OVA (100 µg/ml) for 72 h. Supernatants were collected for cytokine determination by ELISA (all from eBioscience, San Diego, CA).

**RT-PCR.** Segments of jejunum (2–3 cm; with Peyer's patches removed) were snap frozen in liquid nitrogen and stored at –80°C before RNA isolation. RNA was isolated with Trizol (Invitrogen, Carlsbad, CA) and DNase treated (Ambion, Austin, TX) to remove contaminating genomic DNA. RNA was reverse transcribed with Superscript II (Invitrogen), and cDNA was amplified in a real-time thermocycler (Applied Biosystems 7900 and 7300) using SYBR green master mix (Invitrogen). Primers are listed in Table 1 (all from Invitrogen). Amplification was performed on RNA isolated from individual mice, and data were normalized with the housekeeping gene GAPDH.

**Histology, mast cell staining, and CCL1 immunohistochemistry.** Jejunal segments were fixed in formalin and paraffin embedded for sectioning and histological examination. Sections were stained with hematoxylin and eosin for histological assessment. Jejunal mast cells were detected by chloroacetate esterase staining according to published protocols of mast cell detection (17). For CCL1 staining, 4-µm paraffin sections were dehydrated and endogenous peroxidase was quenched with H<sub>2</sub>O<sub>2</sub> (3% in methanol). Antigen retrieval was performed by microwave in a citrate buffer and endogenous avidin-biotin blocked (Vector Laboratories, Burlingame, CA). Anti-CCL1 antibody (20 µg/ml, R&D Systems, rat IgG<sub>2b</sub> monoclonal) or isotype control was added to the sections, followed by biotinylated goat anti-rat (Jackson ImmunoResearch, West Grove, PA), ABC detection kit (Vector Laboratories), and diaminobenzidine chromogen (Vector Laboratories), and finally counterstained with hematoxylin (DAKO, Carpinteria, CA).

**IgE detection.** Intestinal lavage fluid was collected immediately after mice were euthanized. The entire small intestine was removed and flushed with 4 ml of PBS containing complete protease inhibitor cocktail (Roche, Indianapolis, IN). Lavage fluid was centrifuged to remove debris and stored at –20°C before measurement of IgE. OVA-specific IgE in intestinal lavage fluid and serum was determined by ELISA. Briefly, plates were coated with monoclonal rat anti-mouse IgE (Pharmingen, San Diego, CA), followed by incubation with serial dilutions of lavage and serum. OVA-specific IgE was detected with DIG-labeled OVA (digoxigenin-labeling kit from Roche), followed by horseradish peroxidase-labeled anti-DIG Fab fragments (Roche).

**Cell transfer.** OVA-T cell receptor CD4<sup>+</sup> T cells were isolated from spleen and lymph nodes of D011.10 transgenic mice by negative selection (StemCell, Vancouver, BC) and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Carlsbad, CA). Cells (3–5 × 10<sup>6</sup>) were adoptively transferred into naive BALB/c mice by intravenous injection. Twenty-four hours after transfer, mice were fed 50 mg of OVA in PBS or left unfed (controls). MLNs were harvested 48–72 h later for analysis by flow cytometry. In additional experiments, MLN cells were isolated from mice with allergic diarrhea, cultured for 72 h with OVA as detailed above, and then transferred to naive mice at a dose of 5 × 10<sup>6</sup> cells by intraperitoneal injection. Alternatively, CD4<sup>+</sup> T cells were isolated by negative selection (StemCell) before transfer to naive mice (2 × 10<sup>6</sup> cells/mouse).

**Flow cytometry.** MLN cells were isolated and stained with antibodies (all from eBioscience, except anti-α<sub>4</sub>β<sub>7</sub> from BD Pharmingen) according to standard techniques. Cells were acquired on a LSRII flow cytometer (BD Pharmingen). Live (PI-negative) CD4<sup>+</sup>, KJ1-26<sup>+</sup> cells

Table 1. *Primer sequences*

Primer	Forward	Reverse
GAPDH	GGTGAAGGTCGGTGTCAACGGATTT	ATGCCAAAGTTGTCTATGGATGACC
IL-4	ATGGGTCTCAACCCAGCTAGT	GCTCTTTAGGCTTTCCAGGAAGTC
IL-13	mlux3306298 (D-Lux primer set; Invitrogen)	
IL-10	TGCTATGCTGCCTGCTCTTATGCTATGCTGCCTGCTCTTA	TCATTTCCGATAAAGGCTTGG
IFN-γ	TGCATCTTGGCTTTGCAGCTCTTCTCATGGC	TGGACCTCTGGGTTGTTGACCTCAAACCTTGGC
TNF-α	GTATGAGATAGCAAATCGGCT	TTCTGTCTACTGAACCTCCGG
CCL1	GGATGTTGACAGCAAGAGCA	CAGGGGTTCCACCTTCTTCAG
CCL17	GGGCCATTCCTATCAGGAGGGCCATTCCTATCAGGA	CTGTAGTGCATAAAGAGTCCA
CCL22	CTGTCTAGGGAGGAGGACTGTCTTAGGGAGGAGGA	GAGGCCAAGGAAGTCTGA
CCL25	CCTTCAGGTATCTGGAGAGGAGATC	CAAGATTCTTTATCGCCCTCTTCA
CXCL11	AGCTGCTCAAGGCTTCCTTAAGCTGCTCAAGGCTTCCTTA	CTGCATTATGAGCGGAGCTT
CCR4	AAGGCATTTGGGGAGGTCTTCC	AAGCCACCAGGTACATCCATG
CCR8	CGGGGCTGCCTGAACCACCGGGGCTGCCTGAACCAC	CCCACGGGCATTTGTCTCC
CCR9	AGCCAAGAAGTCAATCCAAGC	CCTTCGGAATCTCTCGCCAA
CXCR3	TGGGGTCTCTGTCTGCTCTTTGGGGTCTCTGTCTGCTCTT	TGCCACCACCACTACCACTA

CCR, chemokine receptor.

were gated, and proliferation was assessed by the CFSE dilution method. Analysis of flow cytometric data was performed with FlowJo software (Treestar, Ashland, OR).

**Statistics.** Data were analyzed by one-way ANOVA, followed by post hoc testing with Dunnett's test. For chemokine expression, nonparametric testing was used (Mann-Whitney test).  $P < 0.05$  was considered significant. Statistical analysis and graphing were performed with Prism (GraphPad Software, San Diego, CA).

## RESULTS

**Allergic diarrhea is associated with development of a Th2-biased local inflammation.** As previously described with this model (3, 9), the majority of OVA-sensitized mice developed severe acute diarrhea beginning after the third OVA feed (Fig. 1A). Sham-sensitized mice never developed diarrhea with OVA feedings. Although a dose of 50 mg of OVA was generally required to generate symptoms, mice could be dose-dependently "primed" with lower doses of oral OVA (5 or 1 mg) to respond to a subsequent 50-mg feeding of OVA with diarrhea symptoms. (Fig. 1B).

We performed Ussing chamber experiments to assess the electrophysiological changes associated with allergen challenge of OVA-sensitized and orally primed mice. Baseline

resistance of jejunum was not significantly different between unfed and OVA-fed mice ( $32.3 \pm 2.4$  vs.  $29.5 \pm 2.0 \Omega \cdot \text{cm}^2$  for unfed and OVA-fed mice, respectively). OVA challenge of jejunal segments *in vitro* resulted in a significant increase in  $I_{sc}$  within 3–5 min after allergen challenge (Fig. 1C), consistent with previous reports of allergen-induced changes in  $I_{sc}$  (12, 31). The addition of glucose to the luminal side is known to activate sodium-glucose cotransport, resulting in an increased  $I_{sc}$  and net absorption of sodium. In our studies, baseline  $I_{sc}$  in the presence of luminal glucose was significantly elevated compared with baseline  $I_{sc}$  in the absence of luminal glucose ( $244.6 \pm 27.9$  vs.  $32.4 \pm 5.8 \mu\text{A}/\text{cm}^2$ ), indicating active absorption. In the presence of luminal glucose, allergen challenge significantly suppressed  $I_{sc}$ , indicating a suppression of this active sodium absorption (Fig. 1D). In addition, allergen challenge resulted in a transient (within 5 min) increase in tissue resistance (Fig. 1E). These changes in epithelial transport function are all potential contributors to the acute allergen-specific diarrhea observed in this model.

Onset of diarrhea symptoms was associated with increased numbers of jejunal mast cells, elevated intestinal OVA-specific IgE, and local cytokine upregulation. Mast cells per high-

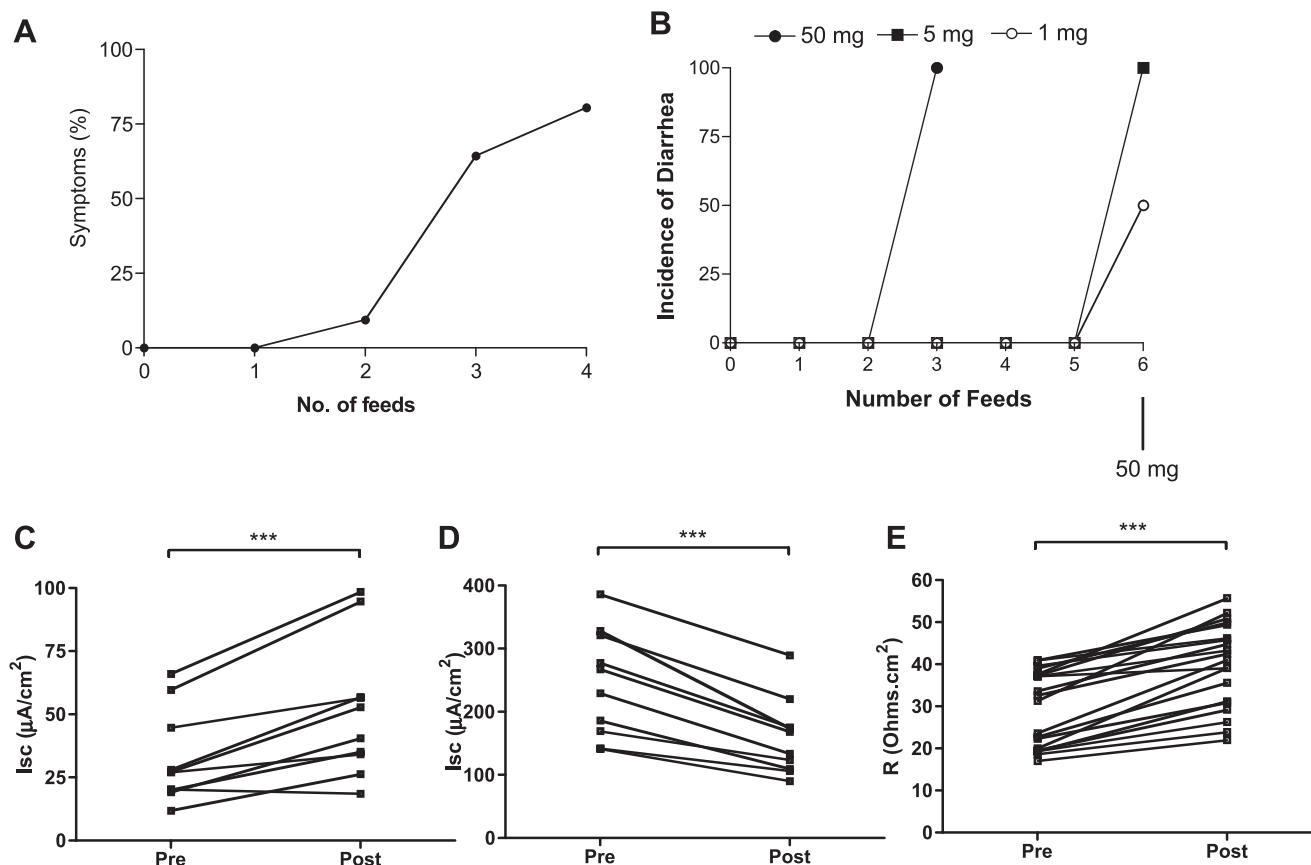


Fig. 1. Induction of allergic diarrhea by ovalbumin (OVA). **A:** development of acute diarrhea symptoms in mice sensitized to OVA and fed repeatedly with 50 mg OVA. (Cumulative data from 30 mice, with 4 mice killed after each time point, and the remainder killed after feed 4.) Data are cumulative incidence of diarrhea; therefore, no error bars are given. **B:** development of acute diarrhea symptoms in mice sensitized to OVA and fed with 50, 5, or 1 mg of OVA. Mice fed low-dose OVA did not develop symptoms but were primed to develop symptoms at the first high-dose OVA challenge. **C–E:** electrophysiology responses to *in vitro* OVA challenge in sensitized and fed (5 mg  $\times$  5 feedings) mice. **C:** short-circuit current ( $I_{sc}$ ) in jejunal segments before and after (peak, 3–5 min after challenge) addition of 200  $\mu\text{g}/\text{ml}$  of OVA to the Ussing chamber. **D:**  $I_{sc}$  in jejunal segments before and after (peak, 3–5 min after challenge) addition of OVA to the Ussing chamber in the presence of luminal glucose to induce sodium absorption. **E:** resistance ( $R$ ) of the jejunal segments before and after (peak, 3–5 min after challenge) addition of OVA to the Ussing chamber. \*\*\* $P < 0.001$ .

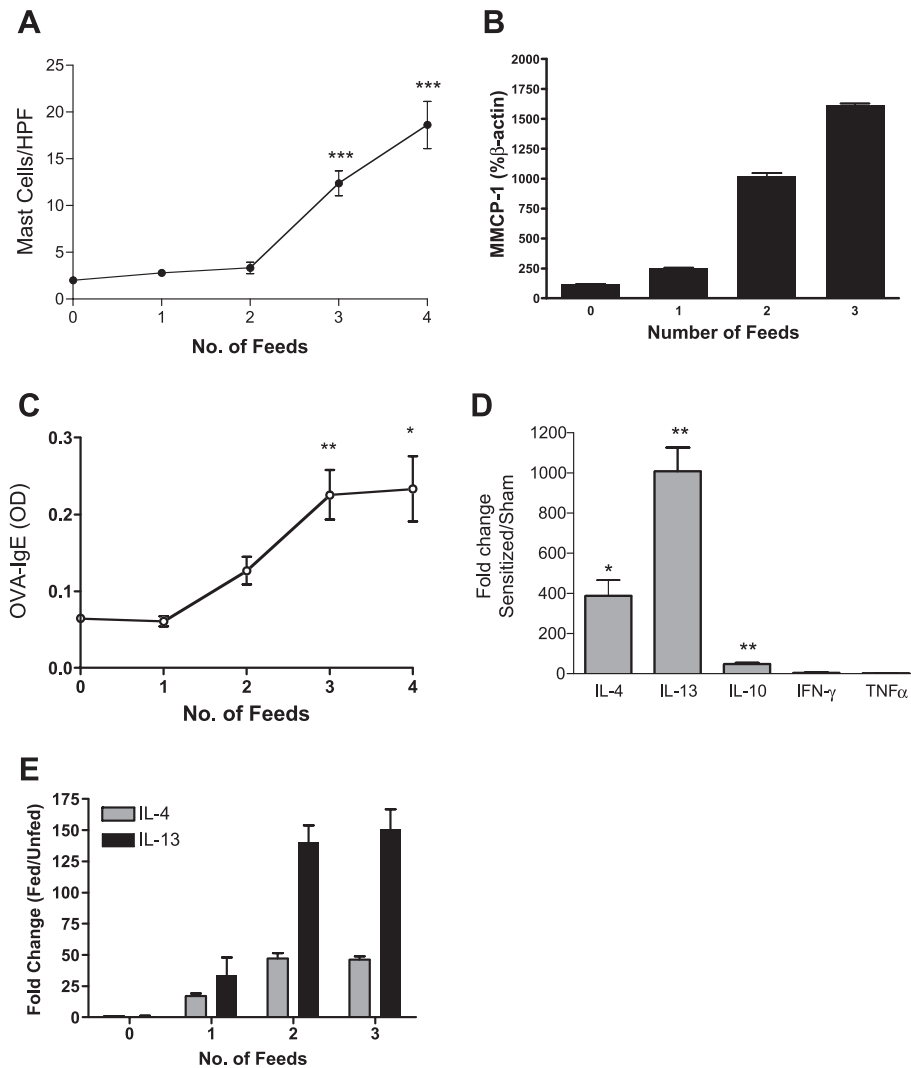


Fig. 2. Induction of jejunal allergic inflammation by OVA. **A:** number of mast cells in the jejunum of mice sensitized and fed with OVA. Mast cells were stained with chloroacetate esterase, and 10 high-power fields (HPF) were counted per mouse. Data are means  $\pm$  SE for 4 mice per time point. **B:** expression of mouse mast cell protease-1 (MMCP-1) by quantitative PCR using RNA isolated from whole thickness jejunum. Data are means  $\pm$  SE of triplicates from pooled RNA from 4 mice/time point. **C:** OVA-specific IgE in small intestinal lavage fluid. Data are means  $\pm$  SE for 4 mice per time point. OD, optical density. **D:** cytokine mRNA expression in the jejunum as measured by real-time PCR. Tissue was harvested immediately after the 4th feed of OVA. Data were normalized to GAPDH and then expressed as fold change of mice sensitized and fed with OVA ( $n = 12$ ) compared with mice sensitized to OVA but unfed ( $n = 3$ ). **E:** time course of IL-4 and IL-13 expression in the jejunum. Tissue was harvested immediately after 0–3 feeds of OVA, RNA was isolated, and quantitative PCR was performed on pooled RNA from 4 mice/time point. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

power field were significantly increased at the third feed of OVA (Fig. 2A). Mast cells were counted after chloroacetate esterase staining according to well-established protocols for intestinal mast cell staining (9, 15–17), and this increase was supported by real-time PCR showing an upregulation of the mouse mast cell protease-1 along the same time course (Fig. 2B). OVA-specific IgE within the intestinal lumen (intestinal lavage contents measured by ELISA) was also significantly increased from baseline (Fig. 2C) with the development of symptoms at the third feed.

We next assessed the expression of Th2, Th1, and proinflammatory cytokines in the jejunum of mice with allergic diarrhea compared with sensitized and unfed mice (Fig. 2D). RNA was isolated from jejunum removed from mice immediately after development of symptoms after the fourth feed. Increments in change (fold) were calculated by the comparative CT method, where the housekeeping control gene was GAPDH and the control CT value was obtained from sensitized but unfed (nonsymptomatic) mice. The highest upregulation of cytokine expression was observed with IL-13 (1,009-fold), followed by IL-4 (390-fold), IL-10 (49-fold), IFN- $\gamma$  (5-fold), and TNF- $\alpha$  (2.7-fold). IFN- $\gamma$  and TNF- $\alpha$  changes in the lamina propria did not reach statistical significance. There

was a clear Th2 bias in the expression of cytokines within the small intestine of mice with allergic diarrhea. A time course (Fig. 2E) showed that IL-4 and IL-13 expression paralleled the other inflammatory changes in the intestine and showed a cumulative increase with each OVA feeding. Therefore, when these results were compared with those found by Lin et al. (28) in human subjects after repeated allergen exposure, this model closely approximates the immune changes in the gastrointestinal tract of patients with food allergic disorders.

*Th2 activation in the MLNs precedes development of gastrointestinal symptoms.* Despite systemic sensitization associated with OVA-specific IgE in the serum and splenocyte cytokine reactivity, mice had a delayed development of symptoms and Th2-skewed inflammation that occurred only after multiple feeds of OVA. To determine where Th2 activation occurred in response to OVA feeding, we assessed the time course of cytokine secretion in spleen and draining MLN (Fig. 3). IL-4, IL-5, IL-13, IL-10, and IFN- $\gamma$  protein secretions in supernatants were measured by ELISA after in vitro stimulation with OVA. In the MLN, no antigen-specific cytokine secretion was detectable in mice sensitized to OVA but not yet fed with OVA. In contrast, mice systemically sensitized to OVA had significant spleen production of IL-5, IL-13, IFN- $\gamma$ ,



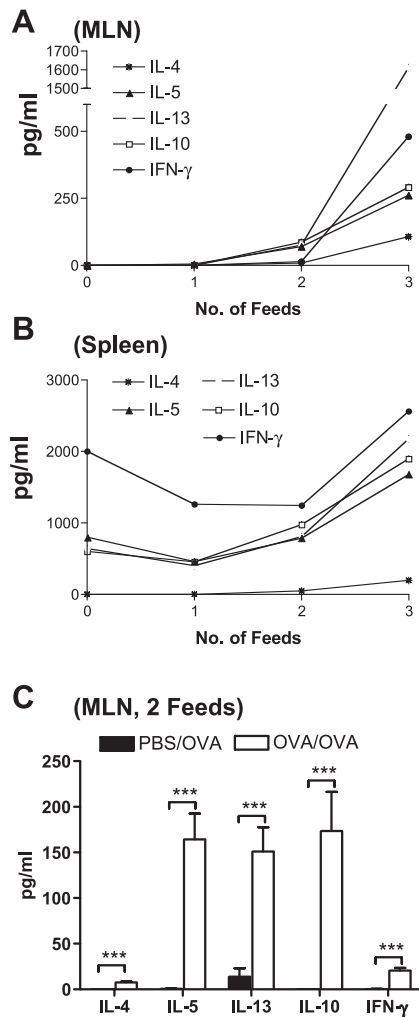


Fig. 3. OVA-specific cytokine response in mesenteric lymph node (MLN) and spleen. **A:** MLN production of cytokines in response to OVA restimulation in vitro. Cytokines were measured in triplicate cultures of cells pooled from 4 mice/time point and are representative of 3 similar experiments. **B:** splenocyte production of cytokines in response to OVA restimulation in vitro. Cytokines were measured in individual cultures from 4 mice/time point. **C:** detailed view of cytokine secretion in the MLN after 2 feeds of OVA, comparing mice that were unsensitized but fed OVA (PBS/OVA) with mice that were sensitized and fed OVA (OVA/OVA). Data are means  $\pm$  SE from 3 experiments, with each experiment representing pooled cultures from at least 4 mice. \*\*\* $P < 0.001$ .

and IL-10 before feeding. A dramatic increase in OVA-specific cytokine production in the MLN appeared after OVA feeding (Fig. 3A) and was first significantly elevated after the second feed of OVA (shown in greater detail in Fig. 3C). A less dramatic boosting was observed in the spleen (Fig. 3B), beginning after the third feed of OVA. These results show that T cell activation in the MLN preceded the development of gastrointestinal symptoms.

To more closely examine activation of antigen-specific T cells in the MLN after feeding, we transferred CFSE-labeled OVA-specific DO11.10 CD4<sup>+</sup> T cells to BALB/c mice. Recipient mice were fed OVA, and the MLN was harvested 48–72 h later for analysis. At 48 h after feeding, flow cytometric analysis of the CFSE content of DO11.10 T cells (gated by CD4<sup>+</sup> KJ1-26<sup>+</sup> staining) indicated significant cell proliferation in the MLN but not in the spleen in response to oral OVA

feeding at 48 h (Fig. 4A). At later time points (72–96 h), we begin to see low levels of proliferated cells in the spleen, which may be migrating to the spleen after activation in the MLN (data not shown). We examined expression of homing markers in the MLN at 72 h, when a greater percentage of cells had undergone expansion. Expression of CD62L and  $\alpha_4\beta_7$  was determined on the OVA-specific T cells at each round of division (Fig. 4B). There was a significant upregulation of the gut-homing marker  $\alpha_4\beta_7$  by the fourth round of division and a downregulation of L-selectin (CD62L) starting at the first round of division of the antigen-specific T cells. Together, these results indicate that, in sensitized mice, T cells reactivated in the MLN by oral antigen generate Th2 cytokine responses and acquire a gut-homing phenotype.

*Th2 chemoattractants are expressed in the jejunum of mice with allergic diarrhea.* Tissue-specific homing of lymphocytes is determined by a combination of homing molecule expression on the lymphocyte in addition to local tissue chemokine ex-

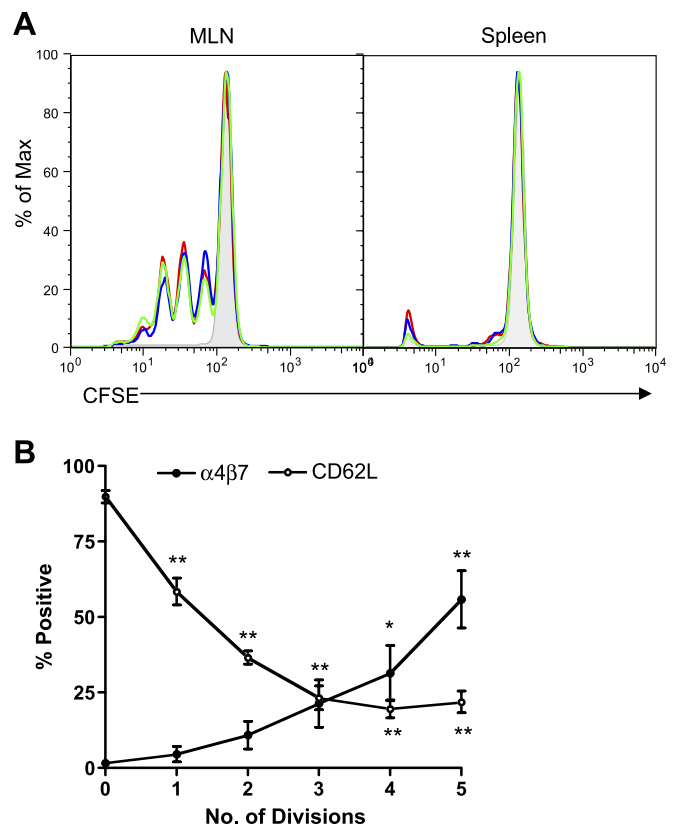


Fig. 4. Generation of a gut-homing phenotype by T lymphocytes in the MLN. CD4<sup>+</sup> T cells were isolated from DO11.10 OVA-TCR transgenic mice and transferred to naive BALB/c mice. Recipient mice were fed with a single feed of 50 mg OVA, and 48–72 h later mice were euthanized and MLNs and spleens were analyzed by flow cytometry. **A:** proliferation of DO11.10 cells in spleen and MLN 48 h after OVA feeding. Live CD4<sup>+</sup> cells were gated, and transgenic cells were identified by KJ1-26 staining. OVA feeding (+OVA) induced the robust proliferation of T cells in the MLN [loss of carboxyfluorescein diacetate succinimidyl ester (CFSE) staining], whereas cells from unfed mice (shaded histogram) did not proliferate. Transgenic cells from the spleen at this time point did not show proliferation in response to OVA feeding. Histograms from 3 fed mice (red, green, blue lines) are shown. **B:** analysis of  $\alpha_4\beta_7$  and CD62L staining of transgenic T cells gated at each cell division identified in MLN at 72 h after OVA feeding when a greater proportion of DO11.10 cells had undergone division. Data shown are means  $\pm$  SE from 5 mice. \* $P < 0.05$ , \*\* $P < 0.01$  compared with undivided cells.

pression. To determine the factors potentially involved in the recruitment of Th2 cells to the lamina propria, we examined the small intestinal expression of chemokines and chemokine receptors by real-time PCR. Results are summarized in Fig. 5. Th2 cells have been shown to selectively express the chemo-

kine receptors (CCR) CCR4, CCR8, and CCR3. Ligands for CCR4 and CCR8 were significantly upregulated in the small intestine of mice with allergic diarrhea (mice were euthanized immediately after becoming symptomatic after the fourth feed of OVA). CCL1 was upregulated more than 400-fold com-

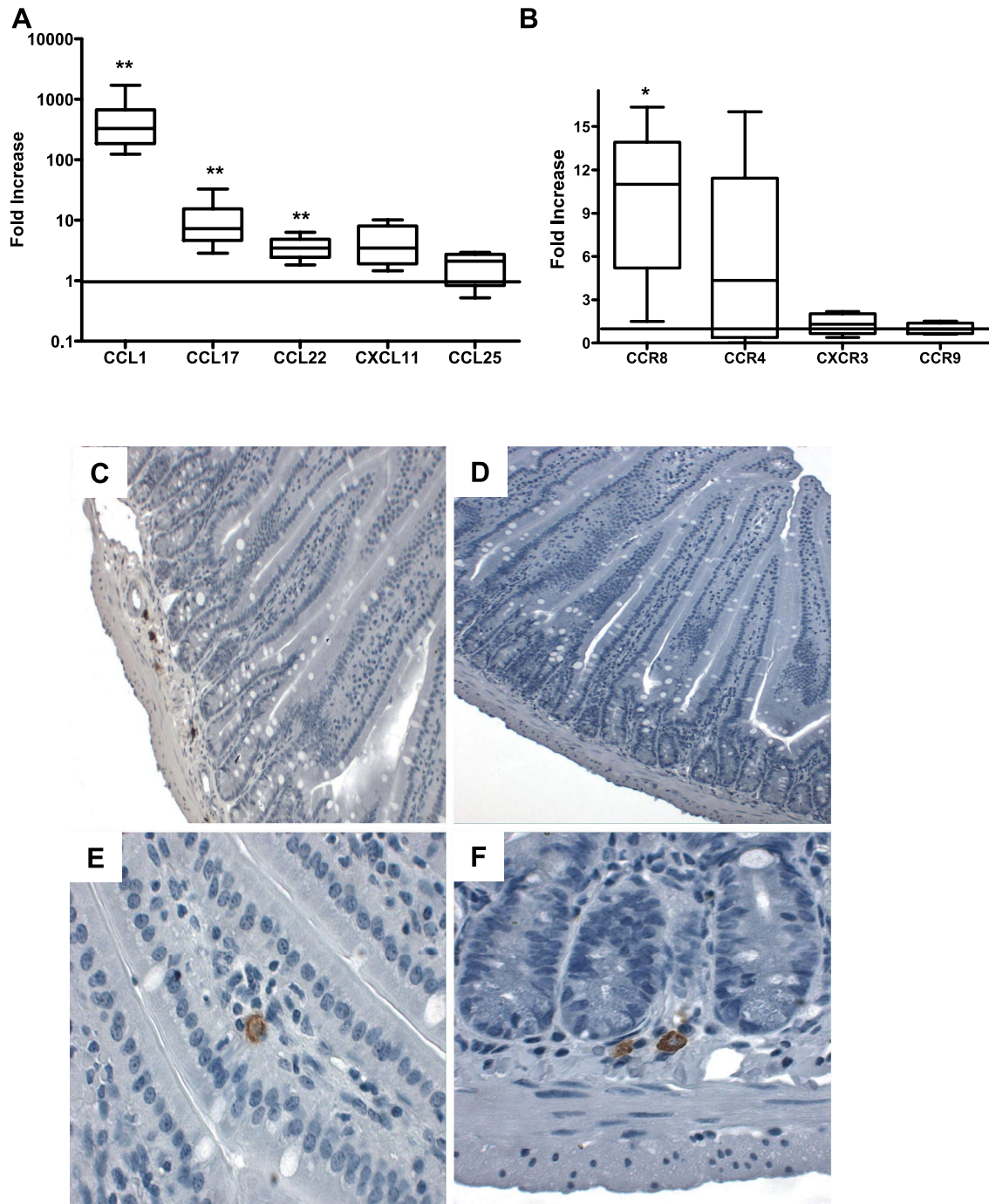


Fig. 5. Chemokine expression in the intestine of mice with allergic diarrhea. Mice were euthanized immediately after the 4th feed of OVA, and jejunum was removed for RNA isolation. Controls were mice systemically sensitized to OVA but unfed. Relative chemokine expression in mice with allergic diarrhea was determined by the comparative CT method, where GAPDH was the housekeeping gene. **A**: fold change in chemokine expression in the jejunum of mice with allergic diarrhea ( $n = 12$  sensitized and fed, 3 sensitized but unfed as control). **B**: fold change in chemokine receptor (CCR) expression in mice with allergic diarrhea. The control value (set at 1) is drawn. \*\* $P < 0.01$ , \* $P < 0.05$  compared with sensitized but unfed controls. **C–F**: immunostaining of CCL1 in the mouse intestine. **C**: jejunum stained with anti-CCL20 showing immunoreactive cells localized in the submucosa (original magnification = ×100). **D**: isotype control showing a lack of staining (original magnification = ×100). **E** and **F**: higher magnification micrographs showing CCL1 protein expression in the lamina propria in the villous (**C**) and in the region immediately below the crypt (**D**). Original magnification = ×400.

pared with sensitized but unfed mice, whereas CCL17 and CCL22 were significantly upregulated at 10- and 3.7-fold, respectively. The Th1 chemoattractant CXCL11 was also significantly upregulated (4.6-fold), but CCL25 was not significantly different from control. Chemokine receptor upregulation was much more modest, with only CCR8 reaching statistical significance, although CCR4 showed a trend toward increased expression. This modest receptor change may reflect receptor downregulation on entry into the tissue or delayed influx of cells in response to chemokine expression.

We next performed immunostaining for CCL1 in the mouse jejunum. CCL1 could be found expressed in relatively rare (2–3 per cross-section) cells of the submucosa and also in the lamina propria of the villi (Fig. 5E) and in cells immediately below the crypt epithelium (Fig. 5F). These results demonstrate that the chemokine CCL1 is expressed at the protein level in the small intestine. Isotype control (rat IgG<sub>2b</sub>) did not show any positive staining (Fig. 5D).

**Transfer of allergic diarrhea by MLN cells.** To determine whether primed MLN cells with a gut-homing phenotype could transfer allergic diarrhea to naive mice, MLN cells were isolated from mice with allergic diarrhea and injected into naive mice (Fig. 6). The proportion of cells in the MLN is ~50% CD4<sup>+</sup> T cells, 15% CD8<sup>+</sup> T cells, 30% B220<sup>+</sup> B cells, and 1–2% CD11c<sup>+</sup> dendritic cells. As determined by cytokine secretion after *in vitro* restimulation (Fig. 6A), the transferred cells had a dominant Th2 cytokine phenotype (IL-13 >> IL-4 >> IFN- $\gamma$ ) similar to the profile observed in the lamina propria after diarrhea onset. Naive mice did not develop diarrhea in response to MLN transfer in the absence of OVA administration. However, on initiation of administration of OVA by gavage every second day, mice developed allergic diarrhea despite the lack of systemic priming with OVA in alum (Fig. 6B). Mice without cell transfer did not respond to OVA feeding. We hypothesized that CD4<sup>+</sup> T cells were capable of disease transfer. In a separate experiment, naive mice were injected with MLN cells, or CD4<sup>+</sup> T cells purified from the same pooled MLN cells. Disease onset was similar whether mice were injected with unseparated MLN cells or CD4<sup>+</sup> T cells (Fig. 6C); therefore, we conclude that CD4<sup>+</sup> T cells are sufficient to transfer disease in this mouse model of gastrointestinal food allergy. Disease transfer correlated with the development of detectable OVA-specific IgE in the serum and antigen-specific Th2 cytokine production in the MLN. Thus CD4<sup>+</sup> T cell transfer can replace systemic sensitization with OVA and alum in the original model to generate mice susceptible to allergic sensitization after allergen exposure.

## DISCUSSION

Food allergic disorders are a poorly understood set of immune disorders triggered by food allergens. Although the gastrointestinal tract is a critical site for the induction of allergic sensitization and generation of symptoms, the mucosal immunology of food allergy is poorly understood because of the inaccessibility of the gastrointestinal tract for study. Thus animal models are critical for furthering our understanding of the mechanisms involved in both generation of allergic sensitization rather than tolerance to food proteins and in the pathophysiology of food allergen-triggered symptoms. In one study in which biopsies were obtained from subjects after

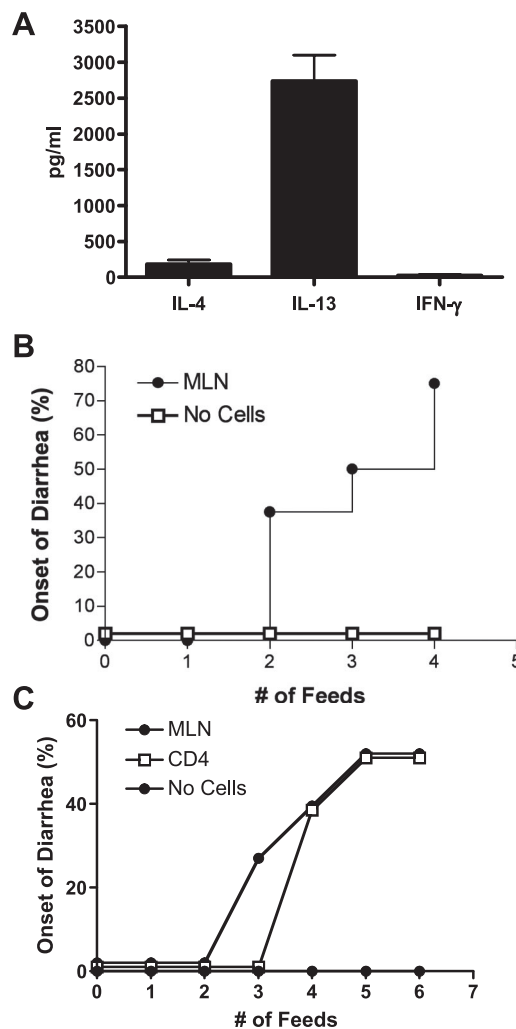


Fig. 6. Transfer of allergic diarrhea by MLN CD4<sup>+</sup> T cells. MLN cells were isolated from mice with allergic diarrhea, cultured as pooled cells (2 pools of 3 mice/pool) for 72 h with OVA, and transferred into naive mice ( $5 \times 10^6$  cells/mouse by intraperitoneal injection, 4 recipients/pool for a total of 8 mice). Two weeks after transfer, mice were fed with 50 mg of OVA every 2nd day. **A:** cytokine production from donor cells as measured in the supernatant collected immediately before transfer. **B:** symptoms (% of mice having diarrhea after each feed) of mice receiving MLN cells. **C:** MLN cells from mice with allergic diarrhea were prepared as in A. Before transfer, CD4<sup>+</sup> T cells were isolated from the cultured cells by negative selection. Purity was confirmed by fluorescence-activated cell sorting. Cells ( $4 \times 10^6$  MLN cells or  $2 \times 10^6$  CD4<sup>+</sup> T cells) were injected into naive recipients (8/group). Two weeks after transfer, mice were fed with 50 mg of OVA every 2nd day. Symptoms were monitored for 45 min after feeding.

repeated allergen challenge and after resolution of symptoms, investigators were able to characterize immune changes in the duodenum in response to allergen exposure in sensitized individuals (28). Independent of their serum IgE status, they observed an increase in IgE-bearing cells in the duodenum and infiltration of eosinophils and CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells. Staining for IL-4 and IFN- $\gamma$  showed an increase in IL-4 and a decrease in IFN- $\gamma$  in the duodenum of allergic subjects when symptomatic. Symptoms were predominantly gastrointestinal, and the majority (12/14) experienced diarrhea within a period of minutes after allergen exposure. These changes are closely paralleled by our observations in the murine model of allergic diarrhea. Mice develop allergic diarrhea after a priming period



of oral exposure to allergen. Although a high dose of OVA was required to induce diarrhea, lower doses of OVA could act to prime the mice to respond to subsequent high-dose OVA challenge. This priming period was not associated with gross changes in epithelial permeability, as baseline resistance levels were not significantly reduced in jejunal segments from mice before and after oral priming with antigen. In contrast, this priming was associated with immunologic changes within the gastrointestinal mucosa, including mast cell infiltration, appearance of local OVA-specific IgE, and development of a Th2 cytokine milieu. Brandt et al. (9), in their original description of the model, also showed that diarrhea was antigen specific, in that feeding OVA-sensitized mice with BSA could not trigger diarrhea symptoms. Antigen specificity has also been demonstrated in Ussing chamber studies, where challenge with a bystander antigen does not elicit changes in epithelial transport or barrier function (5). These changes in gastrointestinal physiology and immunology are consistent with the changes observed in human subjects with food allergy after repeated allergen exposure (28). Therefore, despite the fact that this model requires systemic sensitization to initiate the model, as has been widely used in murine models of asthma, the pathological features closely mimic those of human food allergy.

It has previously been shown that antigen-induced activation of mucosal mast cells is a driving force for chloride secretion in the mouse jejunum through the action of histamine, serotonin, prostaglandins, and mast cell sensory nerve cross-talk (12, 31). We show in this report that antigen challenge results not only in enhanced secretion but also in a significant impairment of glucose-dependent absorption and a transient increase in epithelial resistance. In vivo, each of these factors may contribute to a net fluid transport into (or fluid retention in) the intestinal lumen. Combined with the known effects of mast cell activation on intestinal motility (33), these changes would then lead to the outcome of diarrhea. Using this model of allergic diarrhea, Brandt et al. (9) have previously shown that symptoms are mast cell dependent. The mechanism of this suppression of absorption and increased resistance remains to be defined, but we hypothesize that, similar to the effects on chloride secretion, this is mediated by mast cell products. These results also suggest that there may be both secretory and osmotic components to the allergen-triggered diarrhea.

Gastrointestinal T lymphocytes producing Th2 cytokines have been shown to be associated with both human food allergy (6, 28) and experimental murine models of food allergy (9, 27). Transfer studies have shown that CD4<sup>+</sup> T cells from a sensitized mouse can transfer allergic diarrhea using a model of large-intestinal allergic inflammation and that these cells home specifically back to the large intestine (27). In this study, we show that activation of T cells in the draining MLN preceded the onset of symptoms and that recruitment of Th2-cytokine producing cells to the lamina propria was associated with the local upregulation of Th2 chemoattractants in the gastrointestinal mucosa. Murine models of asthma have indicated that Th2-cytokine-producing natural killer cells are an important component of the pathophysiology of allergic airway inflammation, and this has been verified in human asthma (1, 2). In addition, the oxazolone model of colitis has also been shown to be mediated by IL-13-producing natural killer T cells (21). In our transfer studies, we show that CD4<sup>+</sup> T cells from the MLN are able to transfer disease, supporting the conclusions that the

Th2 cytokine production in the intestine is derived at least in part from conventional CD4<sup>+</sup> T helper cells. However, Th2 cytokine and chemoattractant production in the lamina propria may also be derived from mucosal mast cells. Human mast cells isolated from bowel resections were shown to produce IL-5 and IL-13 in response to triggering by IgE (29). Mast cells have also been shown to produce the Th2 chemoattractants CCL1, CCL17, and CCL22 (18, 30). Thus mast cells may be necessary for recruitment of Th2 cells to the gut rather than Th2 cells promoting mast cell expansion in the gut or recruitment of mast cell progenitors. The cross-talk between T cells and mast cells in allergic gastrointestinal disease may be an important aspect of disease pathogenesis, as has recently been shown in allergic airway inflammation (19).

Activation within the MLNs is likely a key early event in the development of a Th2-biased inflammation within the gastrointestinal tract. We do not discount activation within the Peyer's patch, and likely immune activation in the Peyer's patch and MLN together contribute to allergic responsiveness to food allergens. As a soluble antigen, OVA would not be selectively taken up by M cells as is the case for particulate antigens; therefore, OVA would be taken up by lamina propria dendritic cells for transport to draining lymph nodes. It has recently been shown that lamina propria-derived dendritic cells expressing the adhesion molecule  $\alpha_E\beta_7$  (CD103) imprint T cells to home back to the gut by upregulating expression of  $\alpha_4\beta_7$  and CCR9 (24, 25, 35). In our studies with transfer of DO11.10 CD4 T cells into naive BALB/c mice, we observed that OVA feeding (in the absence of adjuvant) induced a significant proliferation of T cells within the MLN and that, with each cell division, the T cells lost expression of CD62L and gained expression of  $\alpha_4\beta_7$ . Thus these CD4 T cells became primed to home to the gut. In addition, when we transferred Th2 cytokine-secreting MLN cells from mice with allergic diarrhea to immunologically intact naive mice, we were able to transfer susceptibility to allergic diarrhea in the absence of priming with OVA-alum. It has been previously shown that CD4<sup>+</sup> T cells from spleen of systemically primed mice (using complete Freund's adjuvant-OVA) can transfer allergic diarrhea to severe combined immunodeficient mice (27). Alvarez et al. (3) have shown in studies of antigen-induced tolerance in the respiratory tract that induction of tolerogenic responses occurs in the draining lymph nodes. In addition, the spleen, but not the distal lymphoid compartments, contains a reservoir of tolerogenic T cells after antigen exposure. However, on exposure of antigen at distal sites (i.e., via the gastrointestinal tract), cells found systemically can home to those distal sites. This is supported by the finding that allergic inflammation of the gastrointestinal tract can induce susceptibility to allergic inflammation of the respiratory tract, even when gastrointestinal symptoms have resolved (8). Therefore, in models of gastrointestinal allergy, we hypothesize that, although T cells found systemically have the potential to transfer allergic disease, cells in the MLN are equipped with the appropriate homing markers to rapidly traffic back to the gastrointestinal tract and induce disease on reexposure to the allergen.

Tissue-specific lymphocyte homing has been shown to require the coordinated function of adhesion molecules and chemokine receptor expression (10). The accumulation of a Th2-skewed cytokine-expressing cell in the lamina propria led us to examine the expression of chemokines that are known to



play a role in Th2 recruitment. CCL17 [thymus and activation-regulated chemokine (TARC)] and CCL22 (MDC) acting on their cognate receptor CCR4 are perhaps the best defined of the Th2 chemoattractants and have been shown to play a role in allergic disorders of skin (32) and airways (26). CCL17 and CCL22 were significantly elevated in the jejunum of mice with allergic diarrhea; however, CCR4 upregulation did not reach statistical significance. It has previously been reported that gut-homing T cells in the circulation ( $\alpha_4\beta_7$ -expressing cells) do not express CCR4, whereas skin-homing T cells (CLA<sup>+</sup>) do express CCR4 (4, 11). Therefore, it is possible that, despite the upregulation of these Th2 chemoattractants in the gut, CCR4<sup>+</sup> T cells cannot be recruited because of the lack of appropriate adhesion molecules. CCR8 has also been reported to be preferentially expressed on Th2-skewed T cells (36). We observed both a significant upregulation of CCR8 and its ligand, CCL1, in the jejunum of mice with allergic diarrhea. In contrast, the Th1 chemoattractant CXCL11 and its receptor CXCR3 and the constitutive T cell homing chemokine CCL25 and its receptor CCR9 were not significantly altered in mice with allergic diarrhea. Thus we speculate that the coordinated function of CCR8 and  $\alpha_4\beta_7$  may play a critical role in the homing of pathogenic Th2 cells to the gastrointestinal tract in food allergy.

In summary, we have shown that MLN CD4<sup>+</sup> T cells can transfer susceptibility to food allergen-induced gastrointestinal pathophysiology. Therapies that target the local activation or homing of T lymphocytes to the gastrointestinal tract may therefore be of value in the treatment of food allergen-induced disease.

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