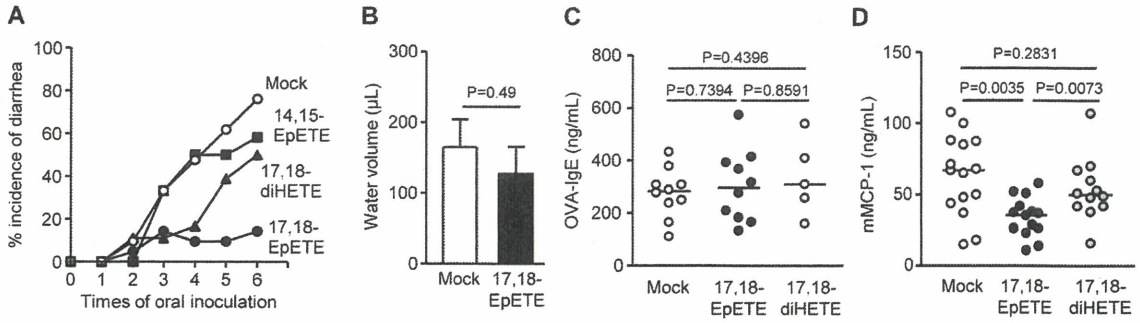

Figure 6 | 17,18-EpETE prevents the development of allergic diarrhea by impairing MC degranulation. (A) Mice were injected i.p. without (mock) or with 100 ng 17,18-EpETE, 14,15-EpETE, or 17,18-diHETE 30 min before systemic priming and oral challenge with OVA, after which the incidence of allergic diarrhea was measured (n = 16 per each group). (B) Mice were injected i.p. without (mock) or with 100 ng 17,18-EpETE at 24 and 1 hr before oral inoculation of 25 μg cholera toxin. Fifteen hours after oral administration of cholera toxin, water volume in the intestinal lumen was measured. The data represent the mean ± 1 SD (n = 4). (C, D) One day after the eighth oral challenge with OVA, serum was collected for the measurement of OVA-specific IgE (C) and mMCP-1 (D) levels. Graphs show data from individual mice from 2 individual experiments, and bars indicate median values.

St. Louis, MO) in complete Freund's adjuvant (Difco Laboratories, Detroit, MI). One week after systemic priming, mice were challenged orally with 50 mg OVA and continued to be challenged 3 times each week. We assessed allergic diarrhea 30 to 60 min after oral inoculation with OVA.

Cholera diarrhea was induced by oral administration of 25 μg cholera toxin (List Biological Laboratories, Campbell, CA)[44]. Fifteen hours later, we examined the water volume in the intestinal lumen.

Cell isolation. Cells were isolated from the large intestine as previously described[44,45]. Briefly, intestines were opened longitudinally, washed with RPMI-1640, cut into 2-cm pieces, and stirred for 20 min at 37°C in RPMI-1640 containing 0.5 mM EDTA and 2% FCS to remove epithelial cells and intraepithelial lymphocytes. The tissues were then stirred three times (20 min each) in 1.6 mg/ml collagenase (Wako, Osaka, Japan).

Flow cytometry. Cells were pre-incubated with 10 μg/mL anti-CD16/32 antibody (Biolegend, San Diego, CA) and then stained with an antibody specific to c-kit (BD Biosciences, San Diego, CA) and FcεR1α (eBioscience, San Diego, CA) for 30 min at 4°C. We used FSC-H and FSC-A discrimination to exclude doublet cells and Viaprobe Cell-viability Solution (BD Biosciences) to discriminate dead and living cells. Flow-cytometric analysis was performed by using a FACSCantoII (BD Biosciences).

Measurement of mMCP-1, OVA-specific IgE, and IgG by ELISA. OVA-specific IgE and mMCP-1 production in serum was measured by using DS Mouse IgE (OVA) ELISA kit (DS Pharma Biomedical Co., Osaka, Japan) and Mouse MCP-1 ELISA kit (eBioscience), according to the manufacturers' protocols. OVA-specific IgG1 and IgG2a were measured as previously reported[44]. Briefly, plates were coated with 1 mg/mL OVA in PBS; this was followed by blocking for 1 hr at room temperature with 200 μL PBS containing 1% (w/v) BSA per well. After extensive washing of the plates with PBS containing 0.05% Tween 20, serial serum dilutions were added for incubation overnight at 4°C. Samples were then incubated for 1 hr at room temperature with optimally diluted HRP-conjugated goat anti-mouse IgG1 or IgG2a (SouthernBiotech, Birmingham, AL). After sample washing, the color reaction was developed at room temperature by using 3,3′,5,5′-tetramethylbenzidine (KPL, Baltimore, MD) and terminated by adding 0.5 M HCl. We measured the color reaction as the absorbance at 450 nm.

Gas chromatography. We extracted lipids from serum and large intestine by using chloroform–methanol and chloroform solutions. The specimens were dried in nitrogen gas and dissolved in 0.4 M potassium methoxide in methanol and 14% boron trifluoride in methanol. The FA concentrations in the solutions were measured by using gas chromatography (model GF 17A; Shimazu, Kyoto, Japan) at SRL Inc. (Tokyo, Japan).

MALDI-IMS. Large intestines within 2 cm from the ileal end were isolated. After the intestinal lumen was washed with PBS, the mesenteries were removed, and the intestines were cut into 2-cm lengths. The intestines were frozen in 2% carboxymethylcellulose (Wako, Osaka, Japan) dissolved in ultra-pure water. Before sectioning, the frozen samples were kept for 30 min at −20°C. The 10-μm sections were thaw-mounted onto an indium–tin–oxide-coated glass slide (Bruker Daltonics, Bremen, Germany) and dried at room temperature. The sections were placed in a polycarbonate tube and stored at −20°C until IMS analysis.

We performed the matrix deposition of 9-aminoacridine (Merck Schuchardt, Hohenbrunn, Germany) onto a slide in a sublimation apparatus (Shimadzu, Kyoto, Japan). IMS was performed with a MALDI TOF/TOF-type instrument, the Ultraflex II (Bruker Daltonics Bremen, Germany), which was equipped with a 355-nm Nd/YAG laser with a repetition rate of 200 Hz. All pixel sizes of imaging were 100 μm. The MS parameters were set in the range of *m/z* (200–400) in negative-ion mode. Automatic acquisition of the mass spectra and reconstruction of the ion images were performed by using FlexImaging software (Bruker Daltonics), which normalized all mass spectra based on total ion current.

Detection of FAs and their metabolites in the large intestine. LC-MS/MS-based lipidomics was performed to measure the amounts of lipid mediators as previously reported[13]. Briefly, lipids were collected by solid-phase extraction using Sep-Pak C18 cartridge (Waters) with a deuterium-labeled internal standard (AA-d8, 15-HETE-d8, LTB4-d4, and PGE2-d4). We used a triple quadrupole linear ion trap mass spectrometer (QTRAP5500; AB SCIEX) equipped with a 1.7 μm, 1.0 × 150 mm Acquity UPLC™ BEH C18 column (Waters). The MS/MS analyses were performed in negative ion mode, and FA metabolites were identified and quantified by multiple reaction monitoring.

Statistics. Results were compared by non-parametric Mann–Whitney's *U*, two-tailed unpaired *t*, and One-way ANOVA tests (GraphPad Software, San Diego, CA).

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Author contributions

J.K. planned and performed immunologic experiments, analyzed data, and wrote the paper; M.A. planned and performed lipidomic experiments and analysis and analyzed data and wrote the paper; T.H., G.H., and R.I. performed lipidomic experiments and analyzed data; R.N., Y.S., S.S., E.H., I.I., Y.K., T.N. and H.S. performed immunologic experiments and analyzed data; and H.A., M.S. and H.K. were involved in data analysis, discussion, and writing the paper.

Additional information

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RESEARCH ARTICLE SUMMARY

MUCOSAL IMMUNOLOGY

Innate lymphoid cells regulate intestinal epithelial cell glycosylation

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INTRODUCTION: The combination of food intake and the resident gut microbiota exposes the gastrointestinal (GI) tract to numerous antigens. Intestinal epithelial cells (ECs) compose a physical barrier separating the internal organs from the gut microbiota and other pathogenic microorganisms entering the GI tract. Although anatomically contained, the gut microbiota is essential for developing appropriate host immunity. Thus, the mucosal immune system must simultaneously maintain homeostasis with the gut microbiota and protect against infection by pathogens. Maintenance of the gut microbiota requires epithelial cell-surface glycosylation, with fucose residues in particular. Epithelial fucosylation is mediated by the enzyme fucosyltransferase 2 (Fut2). Polymorphisms in the *FUT2* gene are associated with the onset of multiple infectious and inflammatory diseases and metabolic syndrome in humans.

RATIONALE: Despite its importance, the mechanisms underlying epithelial fucosylation in the GI tract is not well understood. In particular, although commensals such as *Bacteroides thetaiotaomicron* induce epithelial fucosylation, how mucosal immune cells participate in this process is unknown. We used a combination of bacteriological, gnotobiological, and immunological techniques to elucidate the cellular and molecular basis of epithelial fucosylation by mucosal immune cells in mice, especially innate lymphoid cells (ILCs). To explore the role of ILCs in the induction and maintenance of epithelial fucosylation, we used genetically engineered mice lacking genes associated with the development and function of ILCs. To investigate the physiological functions of ILC-induced epithelial fucosylation, we used a Fut2-deficient mouse model of *S. typhimurium* infection.

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RESULTS: The induction and maintenance of Fut2 expression and subsequent epithelial fucosylation in the GI tract required type 3 ILCs (ILC3s) that express the transcription factor ROR t and the cytokines interleukin-22 (IL-22) and lymphotoxin (LT). Commensal bacteria, including segmented filamentous bacteria (SFB), induced fucosylation of intestinal columnar ECs and goblet cells. Expression of IL-22 by ILC3 required commensal bacteria, whereas LT was expressed in a commensal-independent manner. Ablation of IL-22 or LT in ILC3 resulted in a marked reduction in epithelial fucosylation, demonstrating that both cytokines are critical for the induction and regulation of epithelial fucosylation. Fucosylation of ECs in response to the intestinal pathogen *S. typhimurium* was also mediated by ILC3. Compared with control mice, Fut2-deficient mice were more susceptible to pathogenic inflammation as a result of *S. typhimurium* infection, suggesting that epithelial fucosylation contributes to host defense against *S. typhimurium* infection.

CONCLUSION: We demonstrate the critical role of the cytokines IL-22– and/or LT-producing ILC3 in the induction and regulation of intestinal epithelial fucosylation. We also show that ILC3-mediated epithelial fucosylation protects the host from invasion of *S. typhimurium* into the intestine. Our results provide important details of the glycosylation system and homeostatic responses created by the trilateral ILC3–EC–commensal axis in the intestine. Modulation of mucosal immune cell–mediated epithelial glycosylation may provide novel targets for the treatment or prevention of infectious diseases in humans. ▯■

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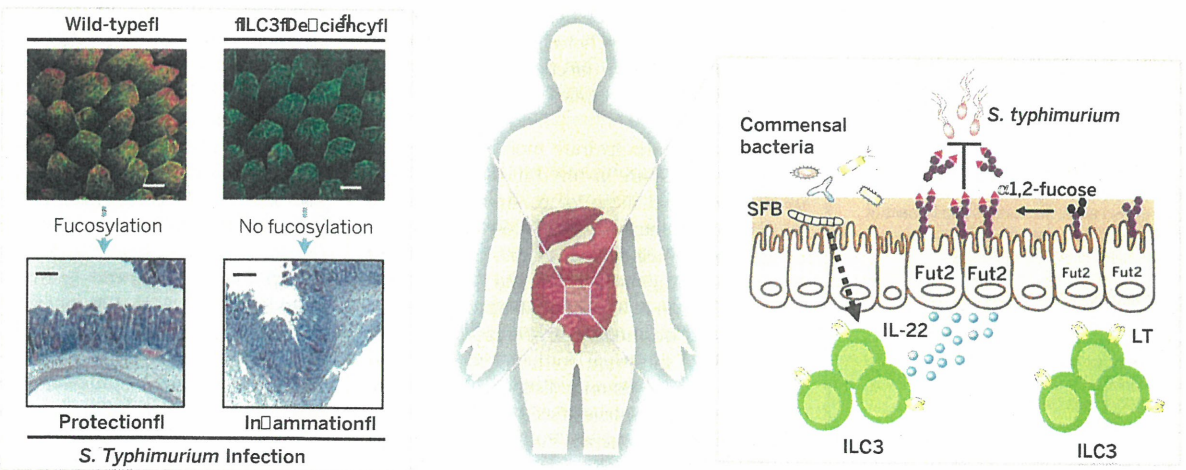

ILC3s regulate epithelial glycosylation. Commensal bacteria, including segmented filamentous bacteria (SFB), induce IL-22 production by ILC3. LT is produced by ILC3 in a commensal bacteria–independent manner. ILC3-derived IL-22 and LT cooperatively induce the production of Fut2 and subsequent epithelial fucosylation, which protects the host against *Salmonella typhimurium* infection.

RESEARCH ARTICLE

MUCOSAL IMMUNOLOGY

Innate lymphoid cells regulate intestinal epithelial cell glycosylation

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Fucosylation of intestinal epithelial cells, catalyzed by fucosyltransferase 2 (Fut2), is a major glycosylation mechanism of host–microbiota symbiosis. Commensal bacteria induce epithelial fucosylation, and epithelial fucose is used as a dietary carbohydrate by many of these bacteria. However, the molecular and cellular mechanisms that regulate the induction of epithelial fucosylation are unknown. Here, we show that type 3 innate lymphoid cells (ILC3) induced intestinal epithelial *Fut2* expression and fucosylation in mice. This induction required the cytokines interleukin-22 and lymphotoxin in a commensal bacteria–dependent and –independent manner, respectively. Disruption of intestinal fucosylation led to increased susceptibility to infection by *Salmonella typhimurium*. Our data reveal a role for ILC3 in shaping the gut microenvironment through the regulation of epithelial glycosylation.

In the gastrointestinal tract, bilateral regulation between the gut microbiota and the host creates a mutually beneficial environment. The intestinal epithelium is a physical barrier that separates the environments inside and outside the mucosal surface. Intestinal epithelial cells (ECs) are the first line of defense against foreign antigens, including those from commensal and pathogenic bacteria. ECs play key roles in initiating and maintaining an immunologically appropriate and balanced environment in reaction to constant foreign stimulation (*1*). Resident commensal bacteria support the development of this functional mucosal immune system, and in turn, mucosal immune cells control the homeostasis of the gut microbiota and protect against pathogenic bacterial infection through intestinal ECs. In particular, type 3 innate lymphoid cells (ILC3) produce interleukin-22 (IL-22), which not only regulates the homeostasis of the commensal microbiota but also protects against *Citrobacter rodentium* infection, presumably by inducing EC-derived antimicrobial molecules such as RegIIIγ (*2*–*5*).

Fucosylated carbohydrate moieties expressed on intestinal ECs are involved in the creation of an environmental niche for commensal bacteria in mice and humans (*6*–*10*). Fucosylated glycans are generated by the addition of an L-fucose residue via an α1-2 linkage to the terminal β-D-galactose residues of glycan in a process catalyzed by fucosyltransferase. Two fucosyltransferases, Fut1 and Fut2, mediate intestinal epithelial fucosylation, and each enzyme acts on a distinct subset of epithelial cells. Fut1 regulates fucosylation of Peyer's patch (PP) M cells, whereas Fut2 is a key enzyme regulating intestinal columnar epithelial fucosylation and the production of secretory fucosylated ABO(H) histo-blood group antigens (*11*). Defective Fut2 has been shown to result in susceptibility to *Candida albicans* infection in mice (*12*). In addition, inactivating polymorphisms of *FUT2* are associated with metabolic abnormalities and infectious and inflammatory diseases in humans (*13*–*19*).

The importance of epithelial fucose has been explored through studies of host–microbe interactions. Signals from commensal bacteria are required for epithelial fucosylation (*6*). Specific commensals, in particular *Bacteroides*, have been shown to induce epithelial fucosylation and are able to catabolize fucose for energy or incorporate it into bacterial cellular components—capsular polysaccharides—that give microbes a survival advantage in competitive environments (*8*, *9*). Indeed, a lack of Fut2 alters the diversity and composition of the fecal microbiota in humans and mice (*20*, *21*). Therefore, epithelial fucose functions as a mediator between the host and commensal microbiota. Although a previous report proposed a model in which *Bacteroides*–EC interaction mediates epithelial fucosylation (*7*), the precise mechanisms by which Fut2 regulates fucosylation remain largely unknown.

Microbiota induces epithelial fucosylation

Epithelial fucosylation, a major glycosylation process, occurs in the small intestine (*10*, *11*). To assess the inductive mechanism of intestinal epithelial fucosylation, we first investigated the localization of fucosylated ECs (F-ECs) along the length of the small intestine, divided equally into four parts from the duodenum (part 1) to the terminal ileum (part 4), in naïve mice (Fig. 1A). The frequency of F-ECs, detected with the α(1,2)-fucose–recognizing lectin *Ulex europaeus* agglutinin-1 (UEA-1), was low in the duodenum and jejunum (part 1 and a portion of part 2; <15% F-ECs) and gradually increased toward the ileum (part 4; 40 to 90% F-ECs) (Fig. 1, A to C). Consistent with epithelial fucosylation, epithelial Fut2 expression was also higher in the ileum (Fig. 1D). Because greater numbers of microorganisms are present in the distal ileum than in the duodenum (*22*), it may be possible that high numbers of ileal F-ECs are induced and maintained through microbial stimulation. To test this hypothesis, we examined the fucosylation status of ileal ECs (part 4) in mice treated with a mixture of antibiotics (AB), as well as in germ-free (GF) mice. The number of F-ECs was dramatically reduced in AB-treated and GF mice (Fig. 2A and fig. S1A). Furthermore, expression of epithelial *Fut2* was also reduced in AB-treated mice (Fig. 2B). Epithelial fucosylation was restored after cessation of AB treatment and in conventionalized GF mice (Fig. 2A and fig. S1A). In addition, fucosylation of goblet cells, but not Paneth cells, was lost in AB-treated and GF mice (Fig. 2C), indicating that commensal bacteria induce fucosylation of columnar epithelial cells and goblet cells, but not Paneth cells.

It has been shown that epithelial fucosylation can be induced by the mouse and human commensal *Bacteroides thetaiotaomicron* (*6*). However, on the basis of bacterial 16*S* ribosomal RNA (rRNA) gene clone library data obtained from

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