

Doctorate Dissertation

博士論文

Title of Dissertation C-type lectin receptor Dectin-1
regulates the balance of mouse intestinal microbiota
(論文題目 C 型レクチン受容体 Dectin-1 を介した
マウス腸内細菌叢制御機構の解析)

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Abstract

In recent years, the functional roles of commensal bacteria in the intestinal immune homeostasis have drawn attention. There are countless symbiotic organisms in the gastrointestinal tract that are closely related to the host's immune system. The immune-modulatory effects of intestinal bacteria spread from the intestine to the systemic immune system and impact the immune homeostasis of not only the intestine, but also other organs. The collapse of intestinal homeostasis, including the balance of intestinal commensal microflora, causes serious effects on the human body and induces various diseases such as colitis. There are several physiological systems that play vital roles to prevent dysbiosis, and recent studies have revealed pattern recognition receptors as one of these control systems.

Dectin-1 (gene symbol: *Clec7a*) is a member of c-type lectin receptor family, a pattern recognition receptor family. Dectin-1 is a receptor for β -glucans and plays an important role for the host defense against fungal infection. Although recent reports indicate that β -glucans activate the intestinal immune system, their

activation mechanism has not been elucidated completely. This study aimed to analyze and clarify the effect of β -glucans on the intestinal immune homeostasis.

I showed that Dectin-1 is expressed in intestinal myeloid-derived cells and *Clec7a*^{-/-} mice are highly resistant to dextran sodium sulfate (DSS)-induced colitis compared with wild-type (WT) mice because of the expansion of regulatory T (Treg) cells in the colon. Because involvement of intestinal microbiota is suggested to be involved in the development of DSS-colitis[1], the composition of the intestinal microbiota was analyzed. I found that the proportion of a bacterial strain, *Lactobacillus murinus* NBRC14221 (*L. murinus*), is increased in the feces of *Clec7a*^{-/-} mice compared to WT mice. Next, with the use of gnotobiotic mice harboring only *L. murinus*, I found that *L. murinus* causes to increase Treg cell population in the large intestine, indicating that Dectin-1 regulates Treg cell differentiation in the intestine through regulation of *L. murinus* growth.

Next, I investigated how Dectin-1 controls *L. murinus* growth. To examine the possibility that antimicrobial peptides control the bacterial growth, the effects of Dectin-1-deficiency on the expression of antimicrobial peptides

were analyzed. I found that antimicrobial peptide S100A8, which specifically suppresses *L. murinus* proliferation by forming a complex with S100A9, decreased in the colonic epithelial cells of *Clec7a*^{-/-} mice. Further analysis revealed that Interleukin (IL)-17F is induced in intestinal myeloid cells by β -glucans through activation of Dectin-1, and resulting IL-17F promotes *S100a8* expression in colonic epithelial cells, explaining why the relative content of *L. murinus* increases in the feces of IL-17F-deficient mice. Thus, I showed that Dectin-1 regulates Treg population in the intestine by modulating the IL-17F-S100A8-*L. murinus*-Treg axis.

Ordinary diets and various food components, such as bread and mushrooms, contain β -glucans. β -glucans are also contained in some intestinal microorganisms. Therefore, it is important to identify which sources of β -glucans regulate the intestinal immune system. Using germ-free, gnotobiotic, and SPF mice, Dectin-1-dependency of S100A8 induction was examined. Intestinal microbiota and the bacterial β -glucan synthase only marginally affected Dectin-1 stimulation in the intestine. Then, to examine the effect of β -glucans in the diet,

β -glucan-free diet-fed mice were analyzed. I found that relative expression of the *S100a8* gene, relative contents of *L. murinus*, and the severity of DSS-induced colitis in mice fed with β -glucan-free diet tend to be similar to that in Dectin-1-deficient mice, indicating that diet-derived β -glucans mostly contribute Dectin-1 activation in the intestine.

From these results, I concluded that orally ingested β -glucans induce IL-17F via Dectin-1 expressed on intestinal myeloid-derived cells and promote the production of S100A8 in colonic epithelial cells. Furthermore, it was suggested that S100A8/9 regulates the proliferation of *L. murinus*, which induces Treg cell differentiation and suppresses DSS-colitis in *Clec7a*^{-/-} mice. Thus, in this study, I have succeeded in unraveling the mechanism how orally ingested β -glucans regulate intestinal immune responses.

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3. Abbreviation

A. faecalis : *Alcaligenes faecalis*

AMPs : antibacterial proteins/peptides

β -glucans : β -1, 3-linked glucans with β -1, and 6-linked branches

BGFD : β -glucan-free diet

BMDCs : bone marrow-derived DCs

cECs : colonic epithelial cells

cLP : colonic lamina propria

CLR : C-type lectin receptor

DCs : dendritic cells

DSS : dextran sodium sulfate

ELISA : Enzyme-Linked Immunosorbent Assay

EpCAM : epithelial cell adhesion molecule

FBS : fetal bovine serum

GPR : G protein-coupled receptor

IFN : interferon

IL : interleukin

ILC : innate lymphoid cell

ITAM : immunoreceptor tyrosine-based activation motif

L. murinus : *Lactobacillus murinus* NBRC14221

LAB : lactic acid bacteria

LP : lamina propria

LPS : lipopolysaccharide

Mφs : macrophages

ND : normal diet

NOD : nucleotide-binding oligomerization domain

PGLYPRs : peptidoglycan recognition proteins

Pi-PGRP : β -glucan-recognizing protein derived from *Plodia interpunctella*

PRR : pattern recognition receptor

qRT-PCR : quantitative real-time PCR

rDNA : ribosomal DNA

ROS : reactive oxygen species

SCFAs : short-chain fatty acids

SFB : segmented filamentous bacteria

SPF : specific pathogen-free

Syk : spleen tyrosine kinase

TGC : thioglycolate

Th17 : T helper 17

TLR : toll-like receptor

TNF : tumor necrotic factor

Treg : regulatory T

WT : wild-type

4. Introduction

Intestinal microbiota

Humans have a symbiotic relationship with a large number of microorganisms.

The luminal side of the digestive tract directly contacts the outer world, and commensal organisms grow on the surface of epithelial cells in these organs [2]–

[4]. Viruses, bacteria, and fungi are members of symbiotic microorganisms. Many studies have reported symbiotic relationships between microorganisms and hosts.

Symbiotic relationships vary depending on various environmental factors, such as daily diet, and genetic factors [5], [6]. Accordingly, commensal bacteria in the intestine and their influence on the host have been studied by many researchers

(Fig. 1). For example, certain chloroform-resistant bacteria metabolize short-chain fatty acids (SCFAs) from dietary fiber and induce the differentiation of regulatory T cells (Treg) through G protein-coupled receptor (GPR) 43. As a result, Treg cells suppress colonic inflammation [7]–[9]. Additionally, GPR43 is also expressed in adipose tissues, and is expected to prevent fat accumulation [10].

As another example, it has been reported that segmented filamentous bacteria

(SFB) colonizing the small intestine of mice influence the intestinal immune system. SFB colonize the surface of small intestinal epithelial cells and promote serum amyloid A production from epithelial cells, thereby inducing the differentiation of T helper 17 (Th17) cells in lamina propria (LP) [11]–[13]. SFB also participate in the regulation of innate lymphoid cell (ILC) 3, promoting the glycosylation of small intestinal epithelial cells and preventing the invasion of pathogenic bacteria [14]. Functionality of probiotic bacteria in food and their role in intestinal homeostasis have been widely studied. Some strains of lactic acid bacteria (LAB) have long been believed as probiotics. Recent research reported that double-stranded RNAs from LAB promote the production of interferon (IFN)- β from intestinal dendritic cells (DCs) via toll-like receptor (TLR) 3, which is a pattern recognition receptor (PRR), and IFN- β suppresses intestinal inflammation [15]. It was also reported that lipoteichoic acid produced by *Lactobacillus* species suppresses the development of inflammation-dependent colorectal cancer [16], [17]. Prevention of preterm delivery by orally ingested probiotics is also reported, although the mechanism is uncertain [18]. Recent studies have revealed the

relationship between nervous diseases, such as mental stress disorder and depression, and intestinal bacteria. This phenomenon is thought to be caused by an action of bacteria on the intestinal nervous system, explaining why the intestine is called as second brain [19]–[21]. The effect of bacteria on the nervous system is partially explained by the fact that intestinal bacteria metabolize γ -aminobutyric acid, a nerve-activating substance [22], [23]. There are hundreds of species of human intestinal bacteria (on the order of 10^7 in the small intestine and 10^{12} in the large intestine). However, most of these bacterial functions and their influence on the human body remain unknown. This research aims to further elucidate relationships among the host, intestinal microbiota, and immunity.

Antibacterial substances

Commensal and pathogenic organisms affect host immunity. Conversely, the growth, colonization, and invasion of pathogens are affected by the host immune system. A host possesses the mechanisms to fight against pathogenic bacteria.

Epithelial cells of mucosal tissue secrete antibacterial proteins/peptides (AMPs) [24], [25], and immunoglobulins to suppress colonization and growth of bacteria [26], [27]. Mucus layer also physically blocks the invasion of bacteria [28]. AMPs directly bind bacteria. Many AMP families are involved in the host defense against pathogens including cationic AMPs such as defensins [29], [30] and cathelicidin [31], peptidoglycan binding proteins such as Reg family [28] and peptidoglycan recognition proteins (PGLYPRs) [32], [33], S100 family [34], [35], and lysozymes. AMPs bind to the target bacteria through electrostatic interaction or recognition of the carbohydrate structure of the bacterial cell wall [24], [36] (Fig. 2). Alpha-defensins electrostatically bind to the bacterial cell walls and form dimeric ion channel in the cell membrane, resulting in an antibacterial effect by changing bacterial membrane permeability [37]. The Reg3 family member binds to peptidoglycans in gram-positive bacteria, forms a hexamer, and kills bacteria by affecting the permeability of the cell membrane [38]. It was reported that cytokines are involved in the induction of AMPs in epithelial cells.

Induction of AMPs and regulation of commensal bacteria by cytokines

Cytokines are signaling molecules secreted mainly by immune associated cells and exert various biological functions through directly inducing cellular growth or differentiation and indirectly including inflammation and suppressing inflammation [24], [39]. AMPs are also one of these molecules induced by cytokines. Epithelial cells express specific cytokine receptors and produce AMPs upon cytokine stimulation [40], [41] (Fig. 3). Interleukin (IL) -1, IL-10, and IL-17 are suggested to induce AMPs [42]–[44]. IL-22, an IL-10 subfamily, is induced in T cells, DCs, and ILCs, and acts on epithelial cells and induces Reg3 γ , S100, and β -defensin production in a STAT3-dependent manner [44]–[46]. Particularly, recent studies suggest that proliferation of intestinal epithelial cells is established by AMPs and maintained by IL-22 from ILC3 [14], [47]. Interestingly, IL-22-producing ILC3 strongly contributes to the regulation of commensal bacteria, and the ILC3 population decreases in germ-free mice [48]. IL-1 β promotes the production of lipocalin and PGLYPRs in human epithelial cells [49], and IL-23 induces Reg3 γ , and commensal bacteria invade into intestinal crypts in IL-23-deficient mice [28],

[50]. Change of intestinal flora was also observed upon protozoan infection [51].

Protozoan infection causes the IFN- γ production from Th1 and suppresses the development and activation of Paneth cells, and IFN- γ induces the production of defensins, thereby altering the intestinal microflora.

IL-17 family and mucosal immunity

The roles of IL-17 family members have drawn much attention recently because this family plays very important roles in the homeostasis of the intestinal immunity.

Previous reports showed that IL-17A, IL-17C, and IL-17F induce the production of AMPs [46], [52]. The IL-17 family is composed of IL-17A, B, C, D, E, and F. IL-17A and IL-17F bind to the IL-17 receptor consisting of IL-17RA and IL-17RC, IL-17B and IL-17E bind to IL-17RA and IL-17RB complex and IL-17C binds to IL-17RA and IL-17E complex, although the receptors for IL-17D is not known yet [53], [54]. IL-17A and IL-17F have high amino acid identity (50%), form heterodimer and bind to the same receptor. The IL-17A/F receptor is expressed on epithelial cells, T cells, B cells, and other types of cells. IL-17A/F receptor

signaling induces the production of defensins from epithelial cells [52] (Fig. 3). Both IL-17A and IL-17F are produced by Th17 and gamma delta T cells, while IL-17F is also produced by macrophages and epithelial cells [55]. Interestingly, although IL-17A and IL-17F bind to the same receptor, their biological activities are not identical. A detailed mechanism of such different effects between IL-17A and IL-17F is presently not well understood. IL-17C is reported to work with IL-22 and leads to the production of various AMPs in intestinal epithelial cells [46].

PRRs

PRRs recognize pathogen-associated molecular patterns and play important roles in the host mechanisms by promoting cytokine and chemokine production [56], [57] (Fig. 4). PRRs also directly protect from infection via production of antimicrobial substances. A nucleotide-binding oligomerization domain (NOD)-like receptor 2 (NOD2), one of PRRs, suppresses intestinal inflammation by suppressing the expansion of commensal bacteria, *Bacteroides vulgatus*, via induction of AMPs Reg3 β and Relm- β [58], [59]. NLRP6, one of NOD-like

receptors, does not recognize intestinal bacteria, but it forms inflammasomes in epithelial cells by the stimulation with intestinal bacteria. NLRP6 inflammasome is essential for the secretion of mature IL-18 from epithelial cells, which promotes the production of AMPs and regulates the balance of intestinal microflora [60].

The TLR family molecules also play important roles in the production of antimicrobial molecules. TLR5, which recognizes bacterial flagellin, is expressed on DCs; it promotes IL-23 and IL-22 production and induces AMPs, such as Reg3 γ , from epithelial cells [61]. Most of the PRRs are commonly expressed in myeloid cells such as DCs and macrophages [62], but several PRRs are also expressed on epithelial cells [63]. It is reported that the PRRs expressed on epithelial cells induce the production of cytokines and AMPs in an autocrine manner [64]. MyD88 is an adapter molecule of the TLR family that has been widely studied in the context of the intestinal immune system and microflora. MyD88-deficient mice develop dysbiosis, which affects fat metabolism and leads to intestinal microflora changes [65].

Dectin-1

The C-type lectin receptor (CLR) is one of PRRs and recognize specific carbohydrate structures on pathogens, such as fungi, mycobacteria, and house dust mites, in a Ca^{2+} -dependent manner [66]. It provides an important mechanism for protection against pathogens [67]–[70]. Dectin-1 (gene symbol: *Clec7a*) is a type II CLR. Dectin-1 has a carbohydrate recognition domain in its extracellular carboxyl terminus, and a hemi-immunoreceptor tyrosine-based activation motif (ITAM) in its intracellular amino terminus [71] (Fig. 5). Dectin-1 is highly expressed in myeloid-derived cells, such as DCs, macrophages (Mφs), and neutrophils [72]. Hemi-ITAM activation results in the recruitment and activation of spleen tyrosine kinase (Syk) and triggers the activation of the Syk/BCM complex signaling pathway, composed of CARD9, Bcl10, and Malt1 [70], [73], [74]. This pathway finally activates NF-κB, which produces several cytokines and chemokines. Previously, Brown et al. and Iwakura et al. showed that Dectin-1 is the receptor for β-1, 3-linked glucans with β-1, and 6-linked branches (β-glucans), and plays a critical role in the host defenses against fungi by promoting cytokine

and reactive oxygen species production [75], [76]. Although Dectin-1 is expressed in the colonic lamina propria (cLP) in the intestine and β -glucans are contained in many food components, such as mushrooms [77], yeasts, and seaweeds, the role of Dectin-1 in the intestinal immune system has not been completely elucidated.

The purpose of this study

Since Dectin-1, the receptor for β -glucans, is highly expressed in DCs and macrophages in the intestine and β -glucans are contained in various foods, I thought that β -glucans might influence the intestinal immunity through activation of Dectin-1. Therefore, in this study, I investigated the role of exogenous β -glucans on the intestinal immunity to understand the roles of PRR signalings in the intestinal immune homeostasis. I find that the β -glucan-Dectin-1 axis is crucial for the homeostasis of the intestinal immune system by regulating commensal microbiota in the intestine.

5. Materials and Methods

Mice.

Clec7a^{-/-}, *Il17a*^{-/-} and *Il17f*^{-/-} mice were generated as previously described [52], [75], [78], and were used for experiments after backcrossing for 9 generations to C57BL/6J mice. Control C57BL/6J mice were bred in the same animal room, and age- and sex-matched mice were used. All mice - except for germ-free and bacteria mono-colonized mice- were kept under specific pathogen-free (SPF) conditions with a γ-ray sterilized diet, acidified (0.002 N HCl, pH 2.5) tap water, and an autoclaved wood chip bed in environmentally controlled clean rooms at the Research Institute for Biomedical Sciences, Tokyo University of Science. Mice were fed with a normal diet (F1, Funabashi Farm, Chiba, Japan), except for the experiments in which they were fed with a β-glucan-free diet (AIN-93G, Oriental Yeast Co., LTD, Tokyo, Japan) and housed with a wood chip bed. The experiments were carried out according to the institutional ethical guidelines for animal experiments and the safety guidelines for gene manipulation experiments, and were approved by the institutional committees of the Tokyo University of

Science (animal experiment number S12054, S13002, S13005, S14019).

Bacterial strain and preparation.

Lactobacillus. murinus (NBRC 14221), *Lactobacillus. johnsonii* (NBRC 13952), *Escherichia. coli* (NBRC 102203), *Lactococcus lactis* subsp. *cremoris* (NBRC 100676), *Lactococcus lactis* subsp. *lactis* (NBRC 100933), and *Alcaligenes. faecalis* (NBRC 13111) were purchased by the National Institute of Technology and Evaluation Biological Resource Center (Kisarazu, Japan). *L. murinus*, *L. johnsonii* and *Lactococcus lactis* were cultured in Lactobacilli Man-Rogosa-Sharpe broth (Difco, Detroit, MI, USA) at 37°C for 18 h and anaerobic incubation, and *E. coli* and *A. faecalis* were cultured in lysogeny broth at 37°C for 18 h before use.

Fecal microbiota 16S rDNA analysis.

Fresh feces were collected from mice just after compelled defecation and were digested with 1 mg/ml Protease K (Sigma-Aldrich; St. Louis, MO, USA) in a buffer

containing 50 mM Tris (pH7.5), 100 mM EDTA, and 0.5% SDS for 5 h at 55°C.

Fecal microbiota DNA was then isolated and purified by phenol/chloroform extraction, followed by ethanol precipitation.

For quantitation of bacteria in feces, a total of 16S rDNA was amplified from extracted DNA using bacterial universal PCR primers Bact-27F (5'-AGRGTTTGATYMTGGCTCAG-3') and Bact-1492R (5'-GGYTACCTTGTTACGACTT-3'). The reaction conditions were as follows: 10X PCR buffer (TaKaRa-Bio; Shiga, Japan), 25 mM dNTP (Takara-Bio), 10 pmol/μl primer (each), 5 U/μl (ExTaq DNA polymerase, Takara-Bio), template DNA, and DNase free water. PCR was performed using an iCycler system (Bio-Rad; Hercules, CA, USA). The following cycling parameters were used: 30 s of initial denaturation at 96°C, 20 s annealing at 56°C, and elongation (90 s at 68°C), with a final extension at 72°C for 10 min. Amplified products from all samples were verified by gel electrophoresis using 1 μg of the PCR reaction mixture in 1.0% agarose gels. PCR products were cloned into pCR-4-TOPO vectors (Invitrogen; Carlsbad, CA, USA), and DH12S competent *E. coli* (Invitrogen) were transformed

using the TOPO-TA cloning kit for sequencing (Invitrogen). Next, 96 colonies were randomly isolated from each bacterial PCR product. Sequencing templates were prepared by colony PCR using primers M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3'). PCR products were then treated with exonuclease I and shrimp alkaline phosphatase (GE Healthcare; Waukesha, WI, USA). The 16S sequences of the inserts were determined by cycle sequencing using BigDye Terminator (Applied Biosystems; Carlsbad, CA, USA) and 3.2 pmoles of T7 (5'-TAATACGACTCACTATAGGG-3'), T3 (5'-AATTAACCCTCACTAAAGGG-3'), and Bact-357F (5'-CCTACGGGAGGCAGCAG-3') sequencing primers. The DNA was cleaned with ethanol precipitation and sequenced with an automated ABI 3730 capillary sequencer (Applied Biosystems). Each clone's data were assembled with the Phred-Phrap program. The sequences assembled with Phrap were aligned by Clustalw analysis, and the multiple-aligned sequences were calculated for the distance-matrix of whole sequences aligned to the 16S rRNA sequences of the Ribosomal Database Project II (RDP) database running in the NCBI BLAST

(<https://rdp.cme.msu.edu>).

For the relative quantity of *L. murinus*, 2 ng of total fecal DNA was used as the template for qRT-PCR. The following bacteria-specific primers were used: 16S (8F&R357) Forward 5'-AGAGTTTGATCMTGGCTCAG-3', Reverse 5'-CTGCTGCCTYCCGTA-3'; *L. murinus* (NBRC14221) Forward 5'-CGTGGGTAGCAAACAGGATT-3', and Reverse 5'-TAAGGTTCTTCGCGTTGCTT-3'. Cycle numbers of PCR were used to calculate the relative quantity by normalizing to the amount of total bacterial 16S rDNA.

Isolation of cLP cells.

Mouse colons were cut out and opened longitudinally. They were washed with PBS to remove luminal contents, and then the tissues were cut into small pieces. Tissue pieces were first incubated in an RPMI1640 (Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS), 1% penicillin+streptomycin, and 20 mM EDTA for 30 min at 37°C in a shaking water bath. Epithelial cells in the culture supernatant were discarded. After centrifugation (1500 rpm/min), a tissue pellet

was further incubated with an RPMI containing 10% FBS, 300 U/ml collagenase type VIII (Sigma-Aldrich), and 5 U/ml DNase I (Sigma-Aldrich) for 2 h at 37°C in a shaking water bath. The digested tissues were then vortexed for 1 min to obtain a single cell suspension. CD11b⁺ and CD11c⁺ cells from cLP cells were purified to >90% by positive selection using an auto MACS Pro Separator (Miltenyi Biotec; Bergisch Gladbach, Germany) according to the manufacturer's directions.

Isolation and culture of intestinal epithelial cells.

Mouse colons were isolated and cut into 5mm × 5mm pieces and washed with ice-cold PBS. Tissue pieces were incubated with an RPMI1640 containing 10% FBS, 1% penicillin+streptomycin, and 20 mM EDTA at 37°C for 30 min in a shaking water bath. Then, the released epithelial cells were collected, washed three times with PBS, and passed through a 200 µm nylon mesh. Cells were counted, and 5×10⁵ colonic epithelial cells (cECs) were resuspended with Matrigel (BD Biosciences; San Jose, CA, USA) and then placed into a pre-chilled 48-well plate. After the Matrigel solidified, the culture medium was added and the

samples were incubated at 37°C with 5% CO₂. Six hours later, Dispase (BD Biosciences) was added on the Matrigel medium, and after 1 h, cECs were collected for qRT-PCR analysis.

***In vitro* culture and measurement of cytokine concentration.**

Purified cLP-derived CD11b⁺ CD11c⁺ cells (3×10⁵) were cultured with curdlan (Sigma-Aldrich) for 6 h followed by qRT-PCR analysis, or the supernatant of the culture was harvested after 72 h and co-cultured with cECs in Matrigel medium.

Colonic epithelial cell line CMT93 was cultured with recombinant cytokines for 6 h followed by qRT-PCR analysis. Cytokine concentration was measured by an Enzyme-Linked Immunosorbent Assay (ELISA) development kit for mouse tumor necrotic factor (TNF) (R&D systems, Minneapolis, MN, USA).

Preparation of cells for *in vitro* assays.

For thioglycolate-elicited peritoneal macrophages preparation, mice received an intraperitoneal injection of 1 ml of 4% thioglycolate (TGC) (Nissui, Tokyo, Japan).

Peritoneal cells were collected 3 days after TGC injection and washed with PBS, and resuspended cells were used as TGC-Mφs. To prepare bone marrow-derived DCs (BMDCs), bone marrow cells were collected after hemolysis and cultured in an RPMI1640 (Wako) containing 10% FBS, 1% penicillin+streptomycin, and recombinant mouse GM-CSF (20ng/ml, Peprotech, Inc., CT, US). On day 7, non-adherent cells were collected as BMDCs.

Flow cytometry.

Antibodies to mouse CD3 (145-2C11), CD4 (RM4-5), CD11b (M1/70), CD11c (HL3), CD19 (1D3/CD19), CD25 (PC61), CD45 (30-F11), NK1.1 (PK136), Ly6G (1A8), and Ly6C (HK1.4) were obtained from BioLegend (San Diego, USA); antibodies to IL-17F (18F10), Foxp3 (FJK-16s), and EpCAM-1 (G8.8) were obtained from eBioscience (San Diego, USA); and antibodies to Dectin-1 (2A11) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). The 2.4G2 (anti-FCγRII/III-specific mAb, rat IgG1, producing hybridoma) was obtained from American Type Culture Collection (Manassas, VA, USA). For intracellular

detection of cytokines or AMPs, cells were stimulated with 100 µg/ml curdlan and 5 µg/ml Brefeldin A (BD Biosciences) for 3 h at 37 °C and 5% CO₂. After cell surface staining, the Cytofix/Cytoperm kit (BD Biosciences) and antibodies were used to detect the intracellular proteins. For intracellular detection of Foxp3, after the cell surface staining the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and antibodies were used to detect intracellular proteins. The stained cells were analyzed with FACS Canto II and FACS Diva software (BD Biosciences), and Flow Jo software (Tree Star, Ashland, OR, USA) was used for data analysis.

Quantitative real-time PCR (qRT-PCR).

Total RNA from colon pieces or cLP or cEC cells was extracted with Sepasol-RNA I Super G (Nacalai tesque, Kyoto, Japan) according to the manufacturer's instructions. RNA was denatured in the presence of an oligo dT primer and then reverse-transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA USA). qRT-PCR was performed with a

SYBR Premix Ex Taq (Takara, Kusatsu-shi, Shiga, Japan) and an iCycler system (Bio-Rad, Hercules, CA, USA), and the expression of each mRNA encoding cytokine was normalized with the *Gapdh* expression level. The sequences of primer used in this study are described in Table 1.

Anti-bacterial effects of AMPs.

L. murinus (NBRC 14221), *L. johnsonii* (NBRC 13952), *E. coli* (NBRC 102203), and *Lactococcus lactis* subsp. *lactis* (NBRC 100933) were cultured in the presence of recombinant S100A8, S100A8 + S100A9 (1:1 mixture, 5 μ g/ml of each protein, Abcam; Cambridge, UK), or recombinant Lipocalin-2 (5 μ g/ml, R&D, Minneapolis, MN, USA) for 3 or 9 h at 37°C, and bacterial growth was measured with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Yokohama, Japan).

DSS-colitis.

For the induction of acute colitis, the mice were administered 2% (weight/volume)

DSS (molecular weight 36–50 kDa; MP Biomedicals, Illkirch, France) in their drinking water, and the survival rate was observed daily. In the other experiments, the mice were sacrificed on day 7 after DSS administration, and the cLP and cEC cells were prepared for flow cytometry.

Histological analysis.

The distal colon of the mouse on day 10 after DSS administration was removed, fixed with 10% neutral buffered formalin, and embedded in paraffin. After being cut into round slices, 5- μ m-thin tissue sections were stained with haematoxylin and eosin. The histological score was calculated using a method from previous research: epithelium (E), 0=normal morphology; 1 = loss of goblet cells; 2 = loss of goblet cells in large areas; 3 = loss of crypts; 4 = loss of crypts in large areas; and infiltration (I), 0 = no infiltrate; 1 = infiltrate around the crypt basis; 2 = infiltrate reaching the lamina muscularis mucosae; 3 = extensive infiltration reaching the lamina muscularis mucosae and thickening of the mucosa with abundant oedema; and 4 = infiltration of the lamina submucosa. The total histological score

was given as E+I [79].

Statistical analysis.

Differences in parametric data were evaluated by the Student *t* test. Differences in survival rates were evaluated by the log-rank test (Mantel-Cox). Histological scores were analyzed using the Mann-Whitney *U* test. Differences of $p < 0.05$ were considered statistically significant.

6. Results

I . Regulation of *L. murinus* colonization by Dectin-1 in the colon

i . Dectin-1 is expressed on myeloid-derived cells of the cLP and regulates colonic immunity by modifying commensal microbiota, *Lactobacillaceae*.

Dectin-1 is expressed on DCs and macrophages (Mφs) in cLP but not on epithelial cells and ILCs.

Dectin-1 is previously reported to be expressed on myeloid cells[57] [67], [80], but the cells expressing this molecule in the mouse colon is not known completely.

Then, I examined cell types on which Dectin-1 is expressed in the colon. Dectin-1 expression was checked using flow cytometry and cells from Dectin-1-deficient mice (*Clec7a*^{-/-}) were used as negative controls. The results showed that Dectin-1 was highly expressed on CD11b⁺CD11c⁻ and CD11b⁺CD11c⁺ cells of wild-type (WT) cLP (Fig. 6A), but not on cECs (Fig. 6B). Although a few studies reported expression of PRRs on innate lymphoid cells (ILCs), I could not detect Dectin-1 expression on ILCs in cLP (Fig. 7). Because CD11b and CD11c are myeloid cell markers [81], I concluded that Dectin-1 is expressed in myeloid-derived cells.

DSS-colitis is suppressed in Clec7a^{-/-} mice in which Treg cells are increased

Then, I examined sensitivity of *Clec7a^{-/-}* mice to DSS-colitis to examine possible effects of Dectin-1-deficiency on intestinal immunity. The survival rate in *Clec7a^{-/-}* mice was found to be better than that of the control animals, suggesting that Dectin-1 signaling exacerbates colitis development (Fig. 8A). Indeed, histological analyses showed a reduction of the disease score (Fig. 8B and C). Regulatory T (Treg) cells, which suppress inflammation, increased in the colon of *Clec7a^{-/-}* mice (Fig. 9).

The proportion of Lactobacillaceae in colonic microbiota is increased in Clec7a^{-/-} mice

I next analyzed the mechanism how Dectin-1 regulates the intestinal immune responses. Because it is suggested that intestinal microbiota play important roles in the regulation of intestinal immune responses, I examined the microflora of *Clec7a^{-/-}* mice. The colonic microflora populations in fresh feces of WT and

Clec7a^{-/-} mice were analyzed using bacterial 16S ribosomal DNA (rDNA) sequence analysis. I found that the proportion of the *Lactobacillaceae* family was five-fold higher in the fecal microflora of *Clec7a*^{-/-} mice than that of WT mice (Fig. 10A). The proportion of the specific *Lactobacillus* strain, *Lactobacillus murinus* NBRC14221 (*L. murinus*), in total *Lactobacillaceae* dramatically increased in *Clec7a*^{-/-} mice, while this strain was barely detected in WT mice (Fig. 10B). The proportion of *Lactobacillus johnsonii* GAL-2 also increased in *Clec7a*^{-/-} mice (Fig. 10B).

L. murinus induces Treg cells in the colon

Next, to analyze the influence of *L. murinus* on the intestinal immune system, we prepared *L. murinus* mono-colonized mice (gnotobiotic mice), and the intestinal immune cells were analyzed. I found that the proportion of Treg cells increased in these *L. murinus* gnotobiotic mice compared with germ free mice (Fig. 11). These results suggest that *L. murinus* can induce preferential differentiation of Treg cells in the intestine, leading to the suppression of the development of DSS-

colitis.

ii . Production of AMP S100A8, which specifically suppresses *L. murinus* replication, is reduced in *Clec7a*^{-/-} cECs.

Expression levels of S100a8 mRNA are reduced in cECs of Clec7a^{-/-} *mice*

Next, I investigated the mechanism for the increase of *Lactobacillaceae* in the colon of *Clec7a*^{-/-} mice. Because AMPs have potent antimicrobial activities [24], [82], AMP gene expression in the cECs of WT and *Clec7a*^{-/-} mice was first analyzed. The expression levels of S100A8 (*S100a8*), but not S100A9 (*S100a9*) or other AMPs such as RegIII family members (*Reg3b* and *Reg3g*), α-defensin family member (*Defa1*), β-defensin family member (*Defb4*), or Lysozyme family members (*Lyz1* and *Lyz2*), were significantly lower in cECs of *Clec7a*^{-/-} mice than that of WT mice (Fig. 12). Lipocalin-2 (*Lcn2*)-encoding gene expression, α-defensin 4 (*Defa4*), and β-defensin 1 (*Defb1*) were also relatively decreased in *Clec7a*^{-/-} cECs.

S100A8 protein expression is reduced in $Clec7a^{-/-}$ cECs

The cEC layer is consisted of enterocytes, goblet cells, and others [83]. I examined S100A8 protein expression using flow cytometry, and I showed that S100A8 was mainly expressed in CD45-EpCAM (epithelial cell adhesion molecule)-1⁺ cells (Fig. 13A). CD45 is a lymphocyte marker [84], and EpCAM1 is a transmembrane glycoprotein in the epithelia [85]. This result is consistent with previous reports stating that S100A8 is produced by myeloid cells and epithelial cells [43], [86], [87]. This S100A8⁺ cEC population was decreased in $Clec7a^{-/-}$ mice compared with WT mice (Fig. 13B). *S100a8* expression in $Clec7a^{-/-}$ cLP also showed a tendency to decrease, although not statistically significant (Fig. 13C,). A fewer number of S100A8-expressing cells were observed in the cLP layer compared with the cEC layer (Fig. 13D). These results indicate that Dectin-1-induced S100A8 is mainly produced by cECs.

*S100A8/A9 heterodimer suppresses the growth of *L. murinus**

Next, the relationship between the AMPs and the microflora, both of which were

changed in *Clec7a*^{-/-} mice, was examined. S100A8/A9 heterodimer (calprotectin), but not S100A8 monomer, was reported to suppress the growth of gram-positive bacteria such as *Staphylococcus* spp [88]. S100A8+A9, but not S100A8, significantly suppressed the growth of *L. murinus* (Fig. 14A), while S100A8+A9 did not suppress the growth of *Escherichia coli*, *Lactococcus lactis*, or even *L. johnsonii* (Fig. 14C). Furthermore, Lipocalin-2 had no *L. murinus*-suppressing activity (Fig. 14B). These results suggest that calprotectin preferentially controls the *L. murinus* population.

iii. Dectin-1 signaling induces cytokines including IL-17F in the cLP.

Gene expression of Il17f is lower in the cLP layer of Clec7a^{-/-} *mice*

Lipopolysaccharide (LPS), a toll-like receptor 4 (TLR4) ligand, is reported to induce *S100a8* expression in macrophages [89] as well as cytokine expression, such as IFN- γ , IFN- β , and TNF. IL-17C can also induce S100A8 [46], [90]. As Dectin-1 signaling can induce the production of various cytokines [75], [80], I examined whether or not colonic S100A8 induction is mediated by these

cytokines. Because the IL-17 family cytokines are reported as an important inducer of the skin and mucosal AMPs [40], [44], I closely examined the expression of this family. Using a real-time quantitative polymerase chain reaction, the gene expression of *Il17f*, but not *Tnf*, *Il23p19*, *Il10*, or other IL-17 family members (*Il17a*, *Il17b*, *Il17c*, *Il17d*), was found to be significantly lower in the cLP layer of *Clec7a*^{-/-} mice than that of WT mice (Fig. 15). The expression levels of the IL-22 gene also tended to be lower in *Clec7a*^{-/-} colons, but this was not statistically significant. This cytokine interacts with epithelial cells together with IL-23 to induce the production of AMPs [43].

Dectin-1 signaling induces IL-17F in cells of cLP.

As Dectin-1 was highly expressed on CD11b⁺CD11c⁻ and CD11b⁺CD11c⁺ myeloid cells in cLP, I hypothesized that S100A8 induction in colonic epithelial cells by Dectin-1 is indirectly mediated by another factors. Therefore, I investigated cytokine production in the curdlan-treated cLP cells. Curdlan is a high molecular weight β -glucan from bacteria and usually used as a Dectin-1

ligand for *in vitro* and *in vivo* assays. The cytokines known to be induced by Dectin-1 signaling and to be able to induce AMP expression were measured, and Dectin-1-deficient cells were used as a negative control. As shown in Figure 16A, the expression of *Il17f* and *Tnf*, but not *Il22* or *Il23p19*, in cLP CD11b⁺ cells was significantly induced by Dectin-1 signaling. CD45⁺ leukocytes in cLP were the main producer of IL-17F, and IL-17F⁺ CD45⁺ leukocytes significantly decreased in *Clec7a*^{-/-} mice (Fig. 16B).

iv. *S100a8* gene expression is induced by specific cytokines in the colon.

S100a8 gene expression is induced by several cytokines in the colon: *ex vivo* analysis

Next, I examined *S100a8*-inducing activity of various cytokines. I used the colon *ex vivo* culture system which is generally used to examine the reaction of the organ to exogenous stimulation [91], [92]. *S100a8* expression was examined in colon organ pieces from WT mice after stimulation with recombinant mouse IL-10, IL-17F, IL-22, IL-23, or TNF. I found that *S100a8* expression was upregulated

by IL-17F, IL-22, and IL-23 stimulation, but not by IL-10 or TNF (Fig. 17A). By testing a variety of concentrations of cytokines, I found that IL-17F, IL-22, and IL-23 upregulated *S100a8* gene expression in a dose-dependent manner (Fig. 17B and C).

S100a8 gene expression is induced by IL-17F and IL-22 in cEC primary culture

Furthermore, to examine the induction of S100A8 in cells, S100A8 induction was examined in cEC line CMT93. Upon stimulation of CMT93 cells with several cytokines, only IL-17A and F induced *S100a8* gene expression at the indicated concentrations (Fig. 18). In addition, primary culture of colonic epithelial cells also was performed. To create the conditions that are similar to the basal membrane of the colon, three-dimensional cultivation conditions using a specific gelled medium with growth factors was used [93]. These results clearly showed that only recombinant mouse IL-17F and IL-22 induce the gene expression of *S100a8* in cECs (Fig. 19A and B).

S100A8 production in the colon is reduced in $Il17f^{-/-}$ mice

Because only IL-17F, but not IL-22, expression was significantly reduced in $Clec7a^{-/-}$ colon (Fig. 15) and IL-17F could induce *S100a8* gene expression in cECs, the AMP expression in IL-17-deficient mice was investigated. IL-17A is the most studied IL-17 family member and induces the production of several AMPs [40], [44]. Interestingly, I found that the *S100a8* expression in cECs significantly decreased in $Il17f^{-/-}$ mice, but did not change in $Il17a^{-/-}$ mice (Fig. 20A). No significant changes for other AMP expression were observed in $Il17f^{-/-}$ and $Il17a^{-/-}$ mice (Fig. 20B and C).

v . Dectin-1-induced IL-17F regulates *L. murinus* via induction of S100A8.

*Percentage of S100A8⁺ epithelial cells is reduced and the content of *L. murinus* is increased in $Il17f^{-/-}$ mice*

Next, S100A8 production in cECs of $Il17f^{-/-}$ mice was examined to estimate the contribution of IL-17F in the induction of S100A8 *in vivo*. S100A8-producing cECs were significantly, but not completely, decreased in $Il17f^{-/-}$ mice compared with

those in WT mice (Fig. 21A and B). Because a reduction of S100A8-expressing cECs was detected in *Il17f*^{-/-} mice, the composition of commensal bacteria was analyzed. I found that relative contents of *L. murinus* were significantly increased in the *Il17f*^{-/-} mouse colon feces (Fig. 21C), as in *Clec7a*^{-/-} mice (Fig. 10).

β-glucan stimulation promotes S100A8 production in cECs via CD11b⁺ myeloid cells of cLP

In Figure 16, I showed IL-17F induction by Dectin-1 signaling in colonic myeloid cells. IL-17F stimulation promoted *S100a8* gene expression in cECs (Fig. 17A). By combining these culture systems, I checked whether or not Dectin-1 ligand induces *S100a8* gene expression in cECs of WT mice via the activation of cLP cells. I then purified cLP CD11b⁺ cells from WT mice, cultured them with curdlan, and then harvested the culture supernatants to stimulate mouse cECs. The AMP-inducing activity was examined after 6-h stimulation of cECs with the supernatants. As expected, *S100a8* expression was specifically induced in cECs by the conditioned medium (Fig. 22A), but the induction of other AMPs such as

S100a9 and *Defb1* was not observed (Fig. 22A and B).

Dectin-1 signaling promotes S100A8 production through induction of IL-17F in cLP CD11b⁺ myeloid cells.

Because S100A8 production from cECs was induced by the culture supernatant of curdlan-stimulated CD11b⁺ WT cells from the cLP, the dependency of Dectin-1 and IL-17F of S100A8 production was examined. The purification of cLP CD11b⁺ cells from WT and *Clec7a*^{-/-} mice was carried out as shown in Figure 16A. Lower levels of *S100a8* gene expression were observed in cLP CD11b⁺ cells from *Clec7a*^{-/-} mice compared with those from WT mice (Fig. 23A). Furthermore, the induction of this AMP was not observed in CD11b⁺ cells from IL-17F gene-deficient mice (Fig. 23B). These results further support the notion that S100A8 production in cECs is mainly caused by IL-17F which is induced by β -glucan-stimulated Dectin-1 signaling in cLP cells.

II . Dectin-1 ligands in the mouse intestine

i . Commensal microbiota do not express Dectin-1 ligands.

Dectin-1 ligands are not detected in intestinal commensal microbiota

The main source of Dectin-1 ligands in mouse intestines was investigated. β -glucans exist everywhere in the environment in which we live: in fungi, food products, and some bacteria. A recent study reported the effects of β -glucans on intestinal inflammation [94]. It was reported that the mouse colon is inhabited by commensal fungi, such as *Candida tropicalis*, which expresses β -glucans as a cell wall component. To search for Dectin-1 ligands in the mouse intestine, the colonic microbiota from WT mouse feces were incubated with a β -glucan-recognizing protein derived from *Plodia interpunctella* (Pi-BGRP) [95] and the binding of Pi-BGRP was analyzed by flow cytometry. Although Pi-BGRP binding resulted in a slight shift in the fluorescent intensity in non-blocking groups, this shift disappeared after pre-treatment with a non-immunized goat serum (Fig. 24A). Because *Candida albicans* was efficiently recognized by Pi-BGRP (Fig. 24A), these results suggested that no commensal fungi or other Dectin-1 ligand-expressing microorganisms are present in the mouse intestine.

Alcaligenes faecalis (*A. faecalis*) var. *myxogenes* was found to produce curdlan [96], and is used in the industry to produce this molecule in large amounts [97], [98]. However, whether or not *A. faecalis* subsp. *faecalis* isolated from mouse intestines could produce curdlan is unknown. Because it was thought that Dectin-1 signaling by β -glucan stimulation is related to intestinal immunity and may result in the induction of S100A8, I checked the population of S100A8⁺ cECs in germ-free mice, SPF mice, and *A. faecalis*-mono-colonized mice. The S100A8⁺ cEC population in *A. faecalis*-mono-colonized mice was found to be similar to that in germ-free or SPF mice (Fig. 24B). These results support the notion that *A. faecalis* and other commensal microbiota do not express Dectin-1 ligands.

In addition, I checked Dectin-1-stimulating activity of bacteria which have β -glucan synthase. *L. lactis cremoris* and *lactis* have β -glucan synthase, while *A. faecalis* and *L. murinus* were the control groups and they were co-cultured with TGC-M ϕ (Fig. 25A) and BMDCs (Fig. 25B) derived from WT and *Clec7a*^{-/-} mice. Results showed that cytokine production was not enhanced by the coculture with β -glucan synthetase-positive bacteria.

ii . β -glucans from food are the main source of Dectin-1 ligands

*S100a8 expression is decreased and *L. murinus* is increased in β -glucan-free diet (BGFD)-fed mice*

Mouse food commonly contains yeast extract as a nutrition. Thus, the possibility that food component-derived β -glucans activate Dectin-1 in the mouse intestine was examined. I analyzed colonic AMP expression in mice fed with a BGFD or normal diet (ND) and found that *S100a8* expression was significantly decreased in cECs of BGFD-fed mice compared with those of ND-fed mice (Fig. 26A). *L. murinus* rDNA contents were increased in BGFD-fed mice (Fig. 26B), as in *Clec7a*^{-/-} or *Il17f*^{-/-} mice. These results suggest that β -glucans from food regulate the *L. murinus* population by inducing S100A8 in the intestine.

S100a8 expression is induced by β -glucans

Furthermore, to examine the ability of β -glucans to induce *S100a8* gene expression in the intestine, mice were fed with a BGFD with additional β -glucans.

After 3 weeks with the BGFD supplemented with β -glucans (Fig. 27A), *S100a8* gene expression in cECs was increased to the same levels as in the ND-fed group (Fig. 27B). From these results, I concluded that food-derived β -glucans are responsible for the induction of *S100a8* in the intestine.

iii. BGFD-fed mice are resistant to DSS-colitis.

A BGFD prevents mice from DSS-colitis

A previous report indicated that DSS-colitis was suppressed by the blockade of Dectin-1 signaling [1]. Thus, I examined whether removal of β -glucans from food could inhibit the development of DSS-colitis. Continuous administration of a BGFD for 3 weeks before treatment with DSS significantly improved the survival rate of mice compared with those fed with an ND (Fig. 28). After colitis induction, enlargement of the spleen was suppressed, and edema and shortening of the colon, secondary to inflammation, were also suppressed (Fig. 29A). The histological severity scores of the inflammatory lesions in the colons of BGFD-fed mice were also significantly lower than these in ND-fed mice (Fig. 29B and C).

Neutrophil infiltration in both cEC and cLP layers is suppressed in BGFD-fed mice

Because the inflammation score was suppressed in BGFD-fed mice, I checked neutrophil infiltration, which is known as an indicator of inflammation, in the intestine of DSS-colitis mice. The neutrophil infiltration in the inflamed colon was estimated by FACS [99]. Ly6G⁺Ly6C⁺ neutrophil population was significantly decreased in BGFD-fed mice in both cEC and cLP layers (Fig. 30A and B). These findings suggest that orally taken β -glucans regulate colonic inflammation.

7. Figures and Table

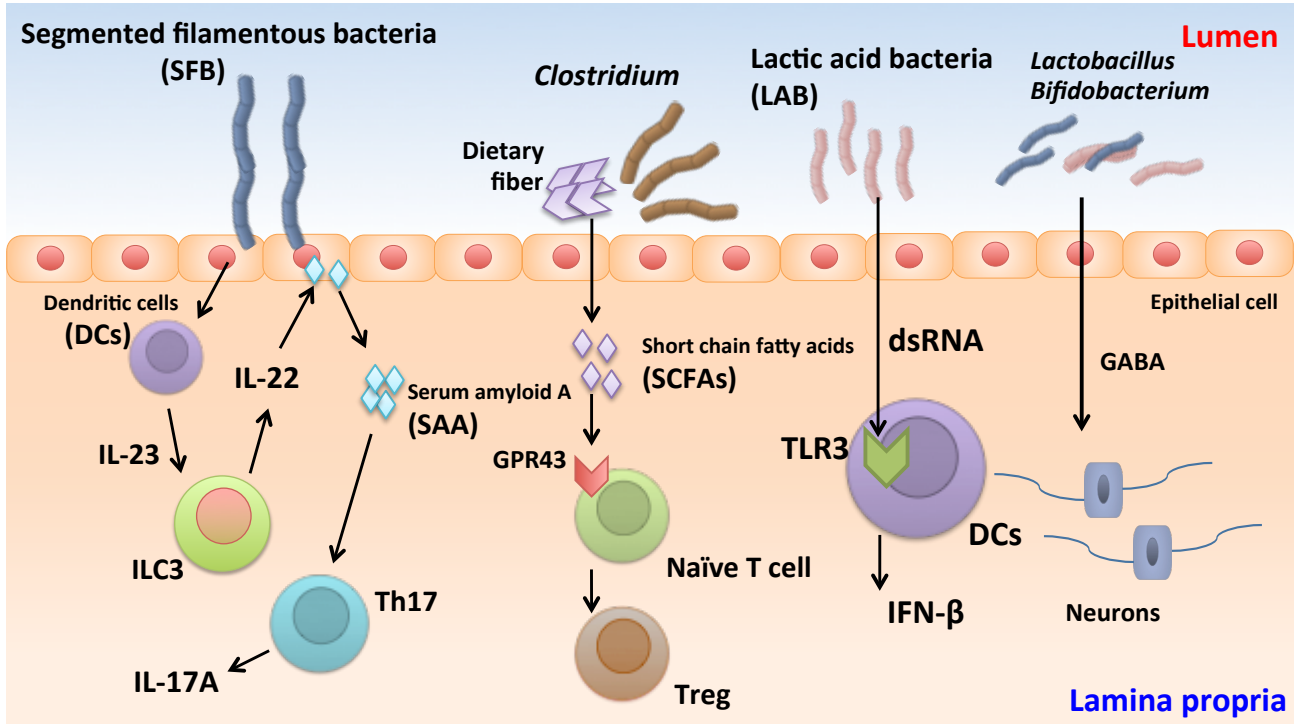
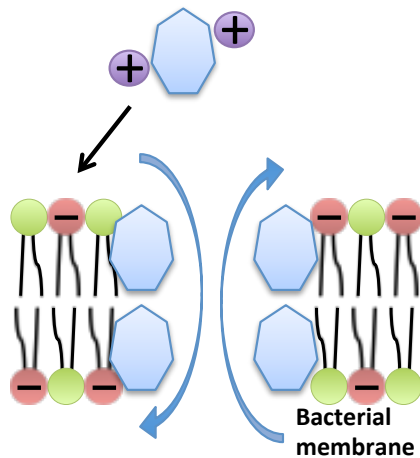


Figure 1. Effects of intestinal bacteria on intestinal host cells.

SFB; segmented filamentous bacteria, SAA; serum amyloid A, ILC3; innate lymphoid cell 3, Th17; T helper 17, SCFAs; short-chain fatty acids, GPR43; G protein-coupled receptor 43, Treg; T-regulatory cell, LAB; lactic acid bacteria, TLR3; toll-like receptor 3, DCs; dendritic cells, IFN-β; interferon-beta, GABA; gamma-aminobutyric acid.

(1) Cationic AMPs



(2) carbohydrate binding AMPs

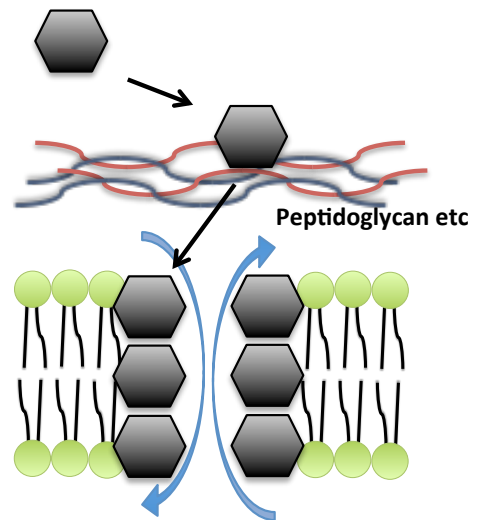


Figure 2. The function of AMPs.

AMPs kill the target bacteria by directly binding to the bacterial cell wall with (1) electrostatic interaction or (2) recognition of specific carbohydrate structure.

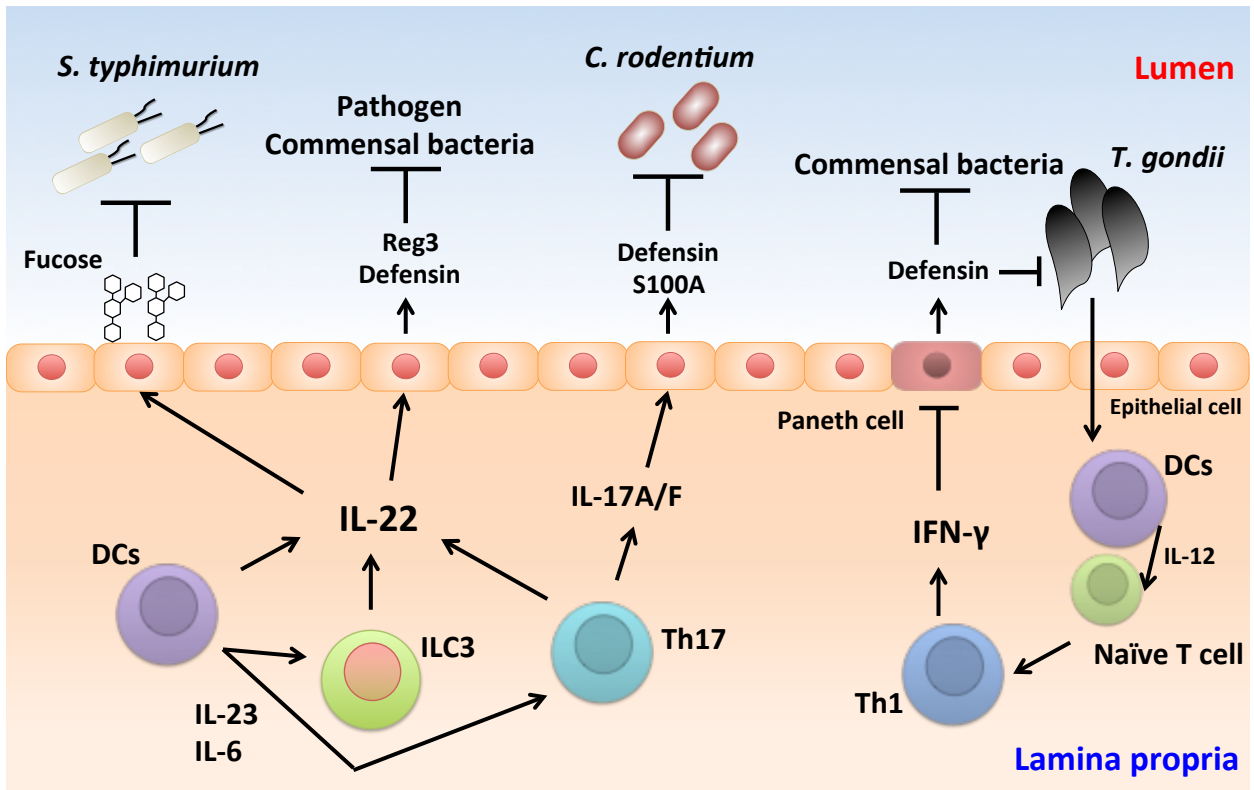
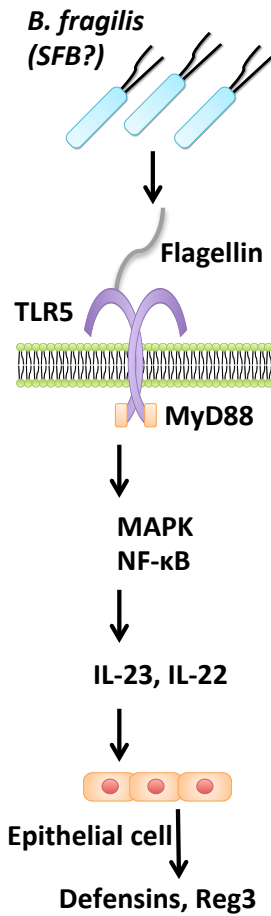


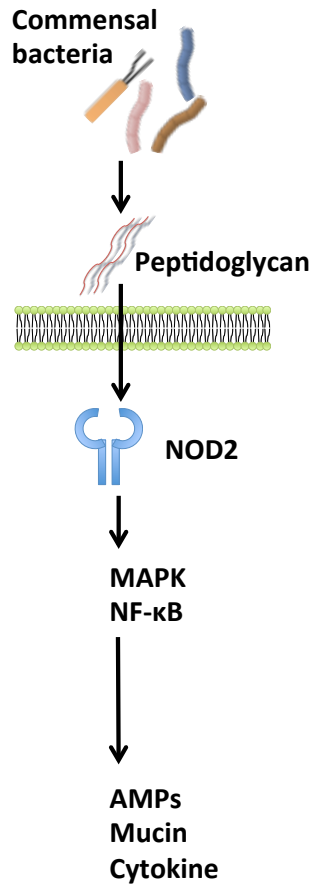
Figure 3. Regulation of intestinal bacteria and fungi by the intestinal immune system.

S. typhimurium; *Salmonella typhimurium*, *C. rodentium*; *Citrobacter rodentium*, *T. gondii*; *Toxoplasma gondii*.

(1) TLR5



(2) NOD2



(3) NLRP6

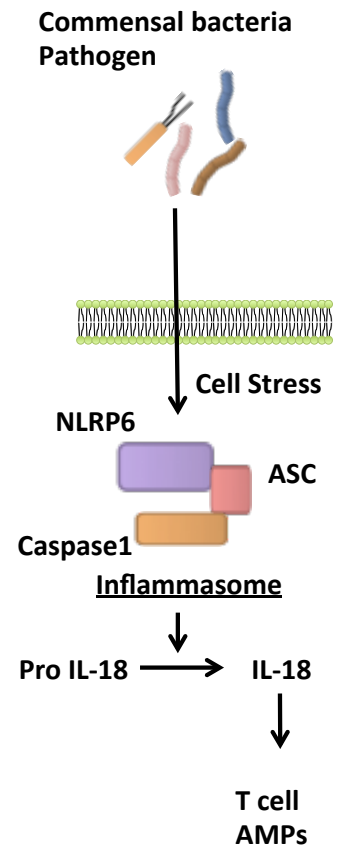


Figure 4. The role of pattern recognition receptors in the intestine.

TLR5; toll-like receptor 5, NOD2; nucleotide-binding oligomerization domain receptor 2, NLRP6; NOD-like receptor protein 6.

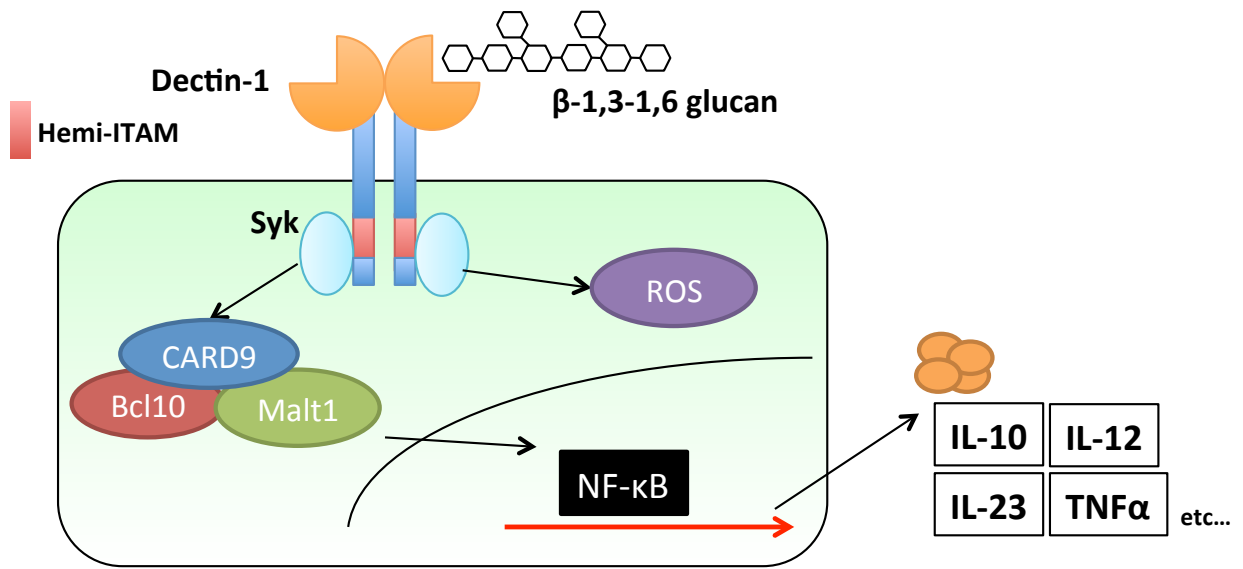


Figure 5. Dectin-1 signaling pathway.

β-1,3-1,6-glucans bind the carbohydrate recognition domain of Dectin-1 and activate the signaling pathway in the downstream. Hemi-ITAM (immunoreceptor tyrosine-based activation motif) recruits Syk and the BCM complex (Bcl10, CARD9, and Malt1), followed by the activation of a transcriptional factor NF-κB. Many cytokines are produced by NF-κB activation. Reactive oxygen species (ROS) are also produced by Dectin-1 signaling.

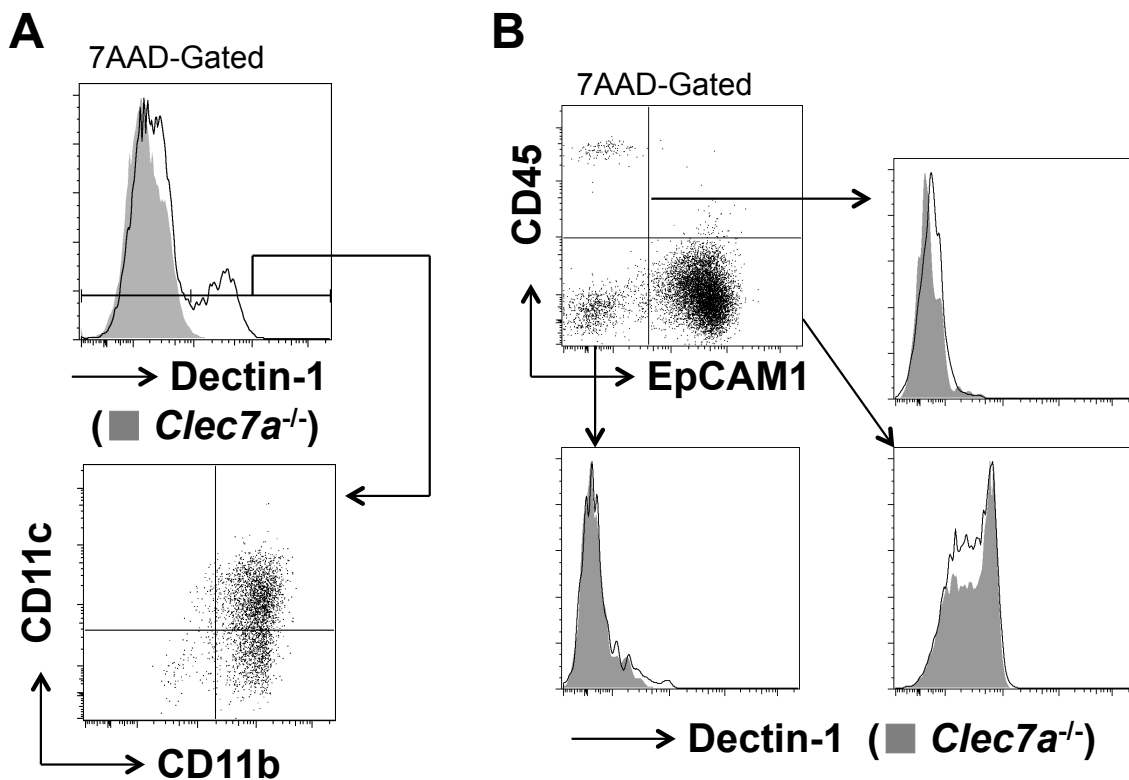


Figure 6. Dectin-1 is expressed on myeloid-derived cells in cLP.

(A) CD11b and CD11c expression on Dectin-1⁺ cLP cells from WT or *Clec7a*^{-/-} (used as a negative control) mice were analyzed by flow cytometry. (B) CD45⁺ and CD45-EpCAM1⁺ colonic epithelial (cEC) cells from WT or *Clec7a*^{-/-} mice were analyzed for expression of Dectin-1 by flow cytometry.

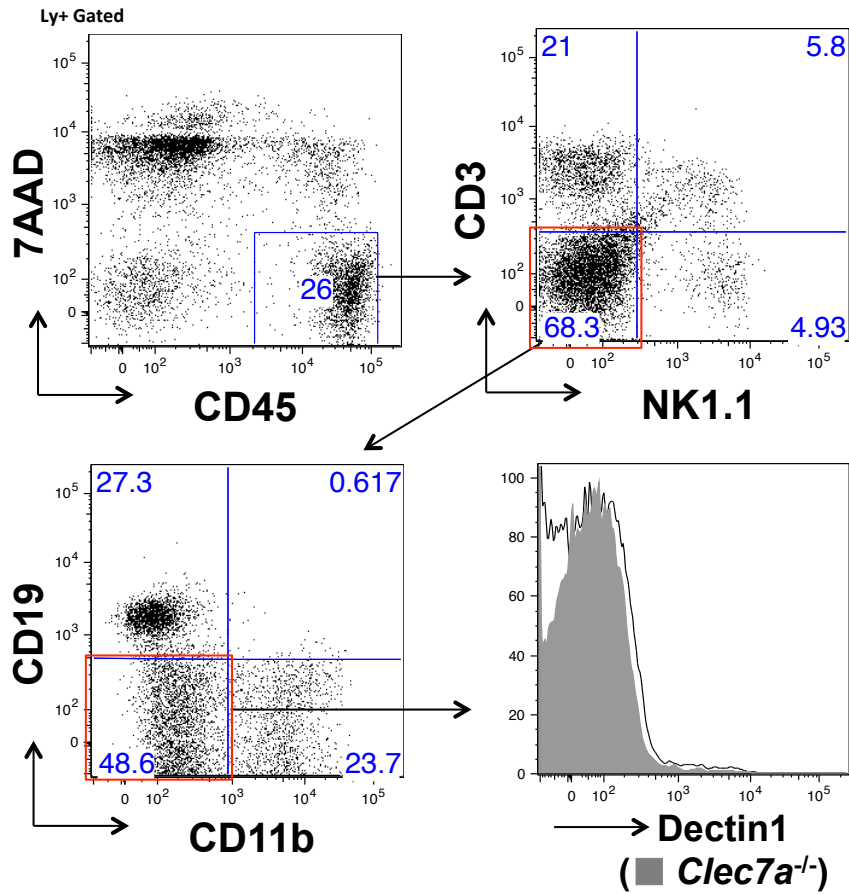


Figure 7. Dectin-1 is not expressed on innate lymphoid cells in cLPs. The CD45⁺7AAD⁻ cells of cLP cells from WT or *Clec7a*^{-/-} mice were stained with indicated markers, and the negative fraction of the four markers was compared by flow cytometry.

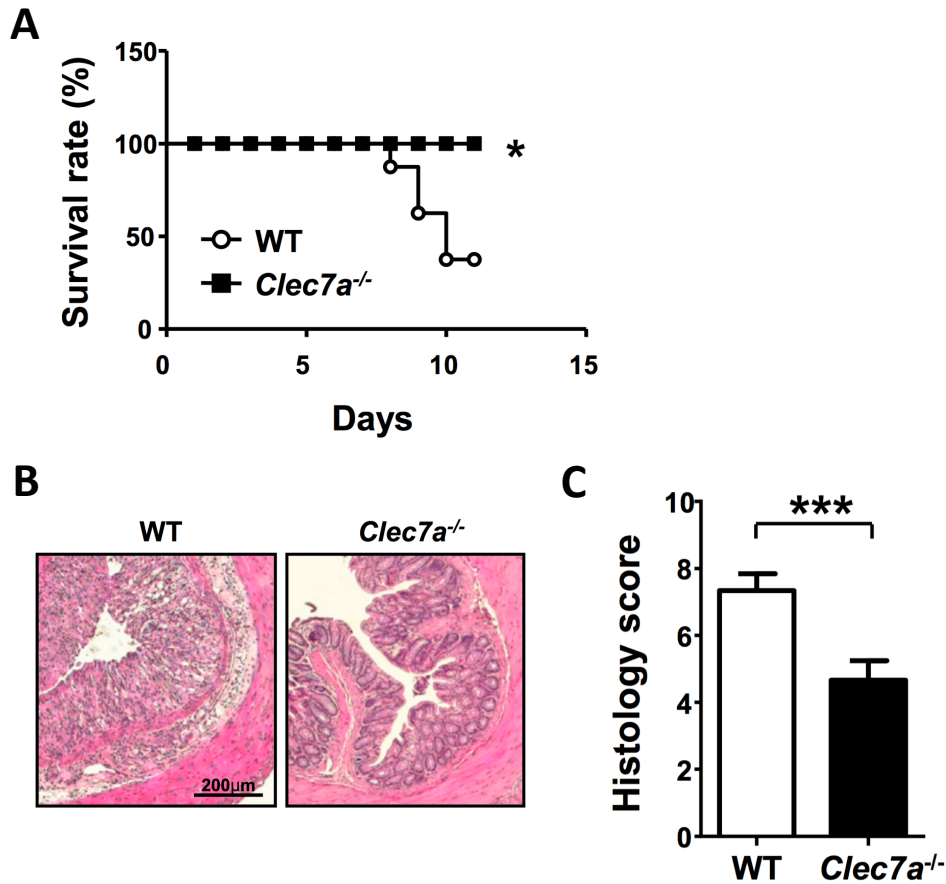


Figure 8. *Clec7a*^{-/-} mice are resistant to DSS-colitis.

(A) WT and *Clec7a*^{-/-} mice were administered 2% dextran sodium sulfate (DSS) in drinking water, and the survival rate was evaluated daily ($n = 8/\text{group}$). (B, C) On day 11, after 2% DSS treatment, mice were sacrificed, and the gross pathology of the colon and histological analysis of the distal colon (H&E staining) were examined. Data are representative of three independent experiments and expressed as means \pm SD. * $p < 0.05$, *** $p < 0.001$ vs. control.

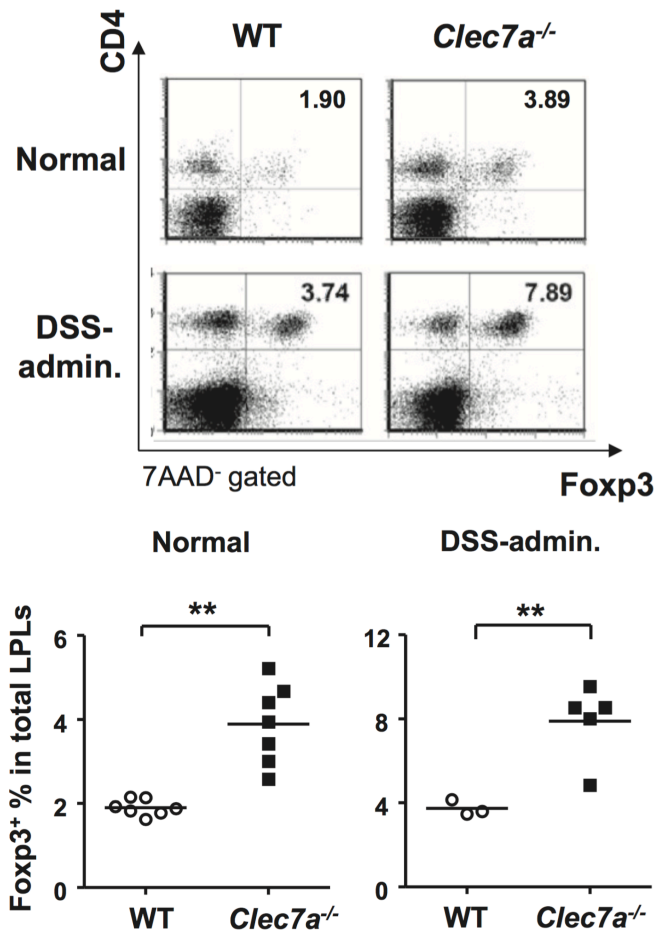


Figure 9. Treg cells are expanded in cLPs of *Clec7a*^{-/-} mice. Frequencies of Foxp3⁺CD4⁺ cells (Treg) in cLP cells from a steady state or DSS-administered mice (Day 10) were evaluated by flow cytometry. Data are representative of three independent experiments. ** $p < 0.05$ vs control.

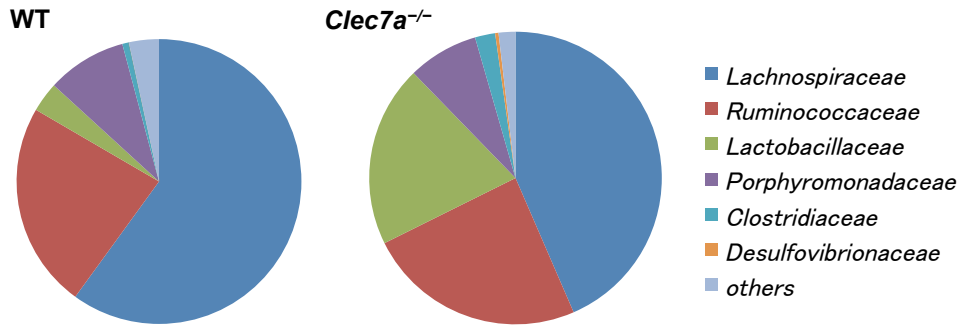
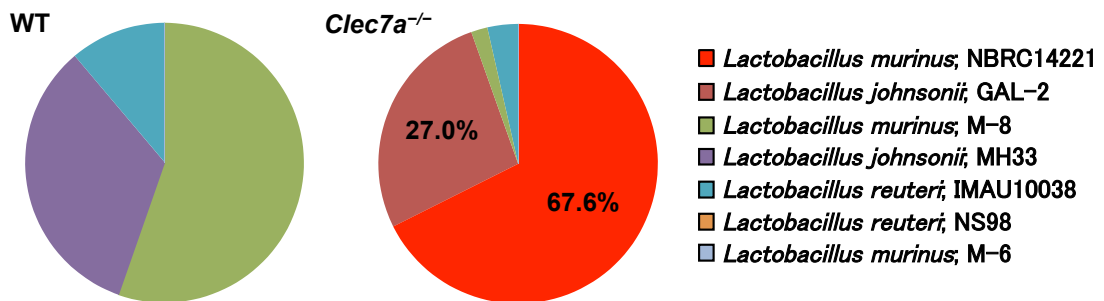
A**B**

Figure 10. The proportion of *L. murinus* in colonic microbiota is increased in *Clec7a*^{-/-} mice.

(A) Fecal microflorae from WT and *Clec7a*^{-/-} mice were analyzed by the 16S rDNA sequence method, and proportions of major commensal bacteria are presented ($n = 3/\text{group}$). (B) Frequencies of major *Lactobacillus* strains in total *Lactobacillaceae* from WT and *Clec7a*^{-/-} mice are presented ($n = 3/\text{group}$).

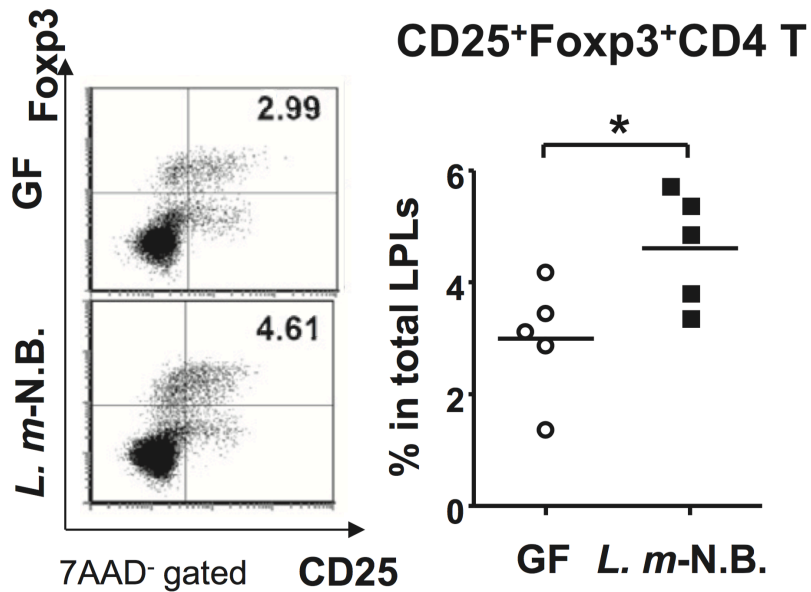


Figure 11. Treg cells are increased in *L. murinus* mono-colonized mice. Germ-free WT mice were mono-colonized with 10^6 CFU of *L. murinus* (*L. m-N.B.*) for 5 weeks, and the percentage of CD25⁺Foxp3⁺CD4⁺ cells in cLP cells was evaluated by flow cytometry. Data are representative of three independent experiments. * $p < 0.05$ vs control.

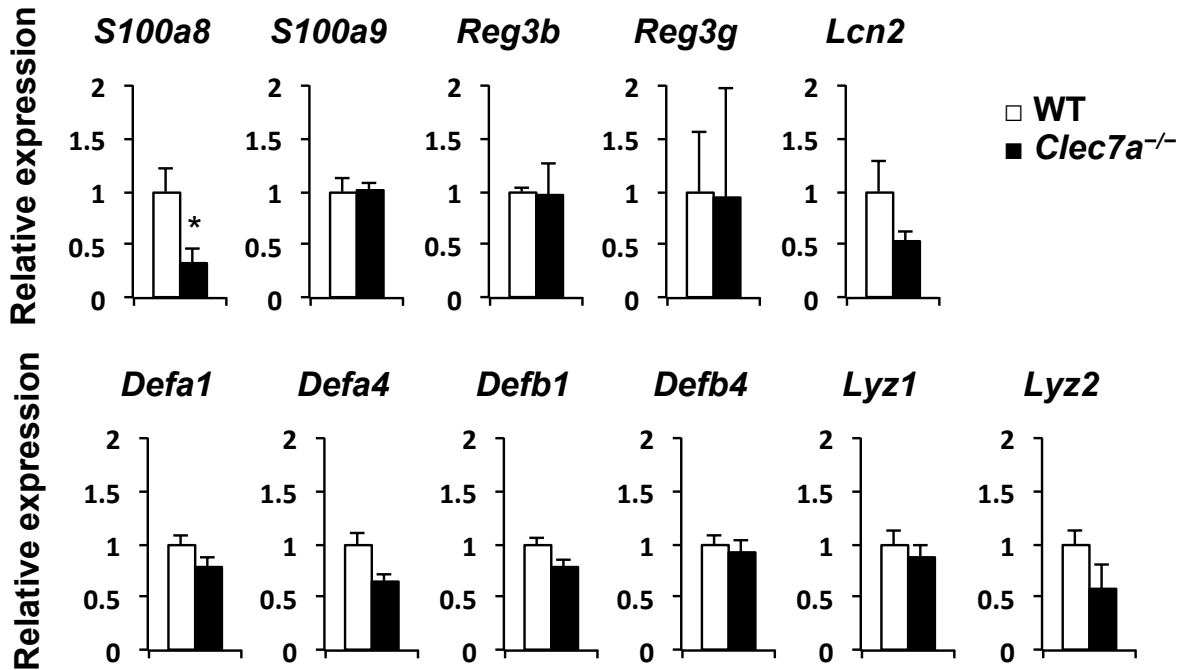


Figure 12. Expression levels of the *S100a8* gene, but not other AMPs, are significantly lower in the cEC layer of *Clec7a*^{-/-} mice than in that of WT mice. Expression of AMP genes [Lipocalin-2 (*Lcn2*), α - and β -defensins (*Defa1*, *Defa4*, *Defb1*, and *Defb4*), S100A8 and 9 (*S100a8* and *S100a9*), RegIII β and γ (*Reg3b* and *Reg3g*), and Lysozyme-1 and 2 (*Lyz1* and *Lyz2*)] in the cEC layer of WT and *Clec7a*^{-/-} mice was measured by quantitative real-time PCR (qRT-PCR) ($n = 3-4$ /group). Data are representative of three independent experiments and expressed as means \pm SD. * $p < 0.05$ vs. control.

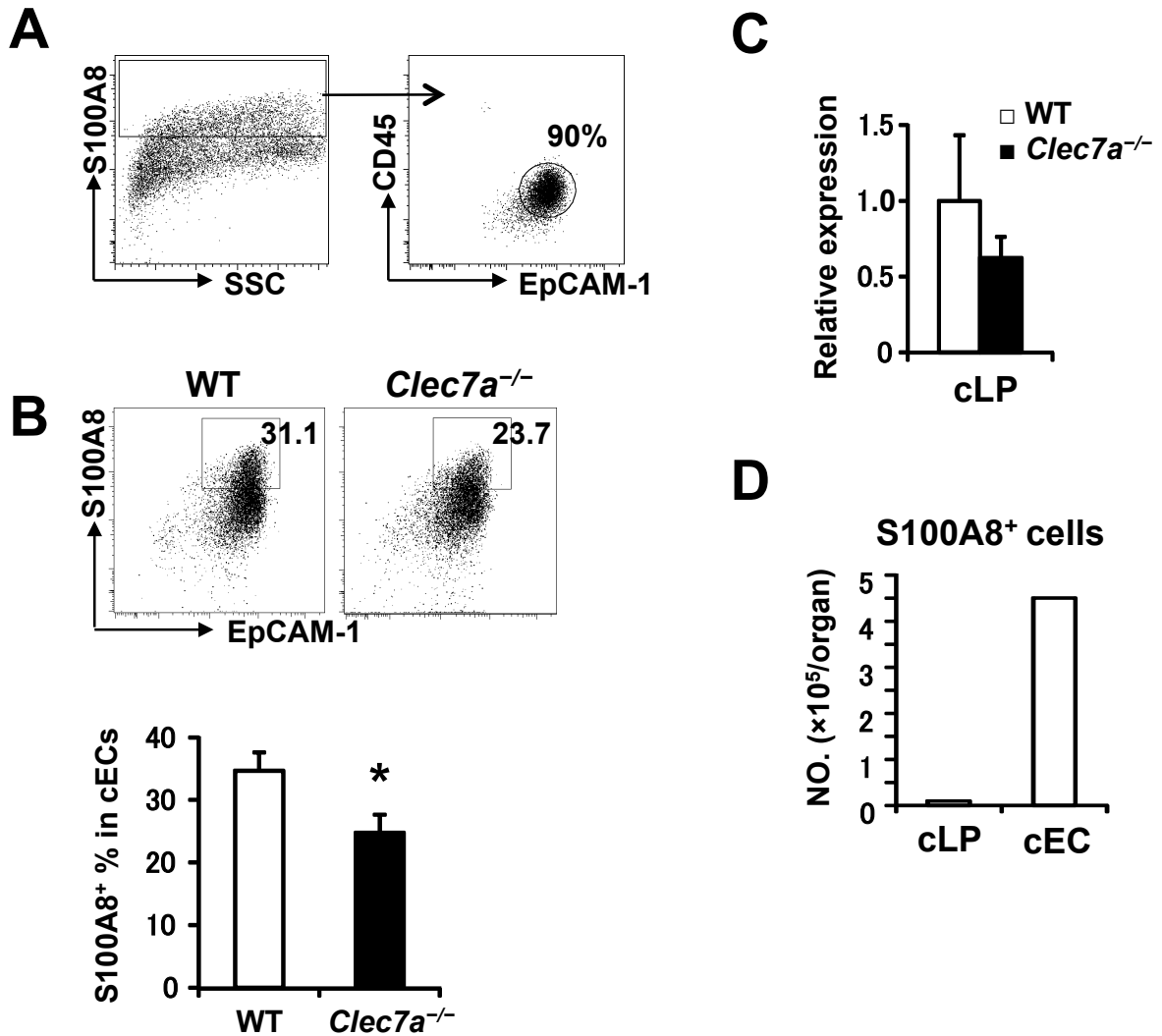


Figure 13. Production of AMP S100A8 is reduced in *Clec7a*^{-/-} colonic epithelial cells (cECs).

(A) Cells from the cEC layer of WT mice were analyzed for the S100A8-producing population by flow cytometry. (B) S100A8-producing epithelial cells from WT or *Clec7a*^{-/-} mice were analyzed by flow cytometry ($n = 3/\text{group}$). (C) *S100a8* mRNA expression in the cLP layer of WT or *Clec7a*^{-/-} mice was examined by qRT-PCR. (D) The number of S100A8⁺ cells in WT cLP or cEC layers was measured by flow cytometry. Data in B are representative of two independent experiments and expressed as means \pm SD. * $p < 0.05$ vs. control.

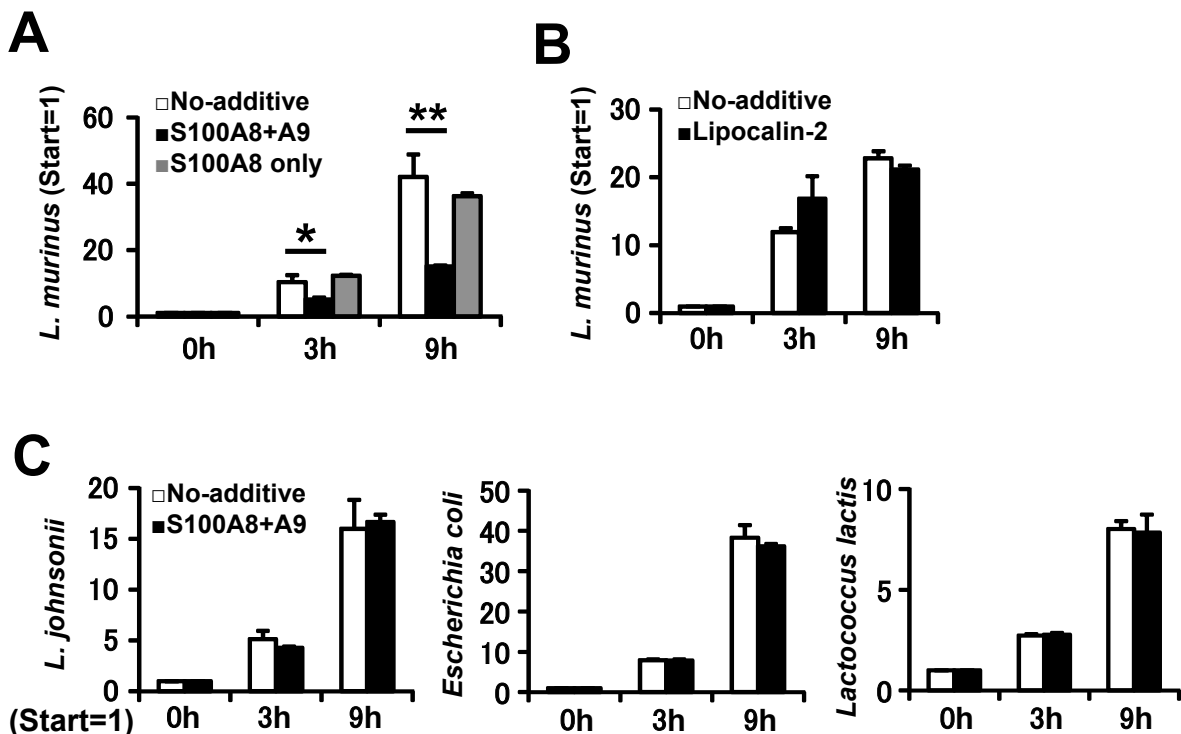


Figure 14. S100A8+A9 significantly suppresses the growth of *L. murinus* in vitro.

(A-C) *L. murinus*, *L. johnsonii*, *Escherichia coli*, or *Lactococcus lactis* were cultured in the presence of recombinant mouse S100A8 (5 µg/ml) (A), or S100A8+A9 (1:1 mixture, 5 µg/ml of each peptide) (A, C), or lipocalin-2 (5 µg/ml) (B), for 3 and 9 h, and bacterial growth was measured with a spectrophotometer (the OD value at the start of culture was set to 1). Data in A are representative of three independent experiments, and data in B and C are representative of two independent experiments. Data in A-C are expressed as means \pm SD. * $p < 0.05$, ** $p < 0.01$ vs control.

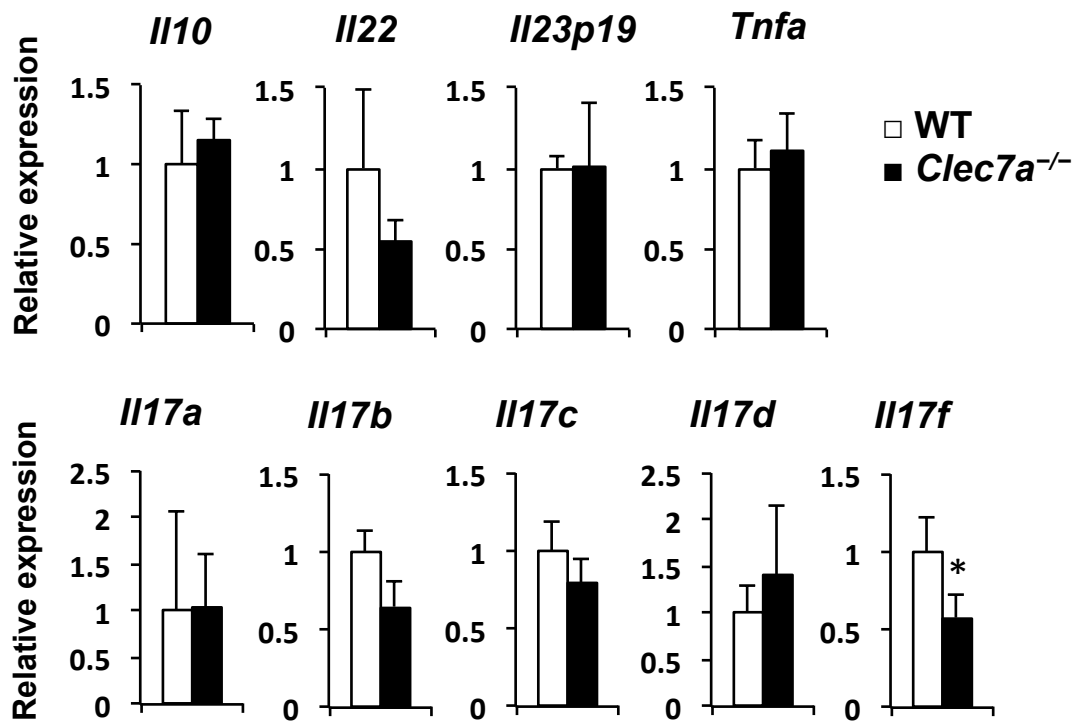


Figure 15. Gene expression of *Il17f*, but not other cytokine genes, is significantly lower in the cLP layer of *Clec7a*^{-/-} mice than in that of WT mice.

Cytokine gene expression in the cLP layer was examined by qRT-PCR, and expression levels in *Clec7a*^{-/-} mice were indicated relative to those in WT mice ($n = 3-8/\text{group}$). Data are representative of two independent experiments and expressed as means \pm SD. * $p < 0.05$ vs WT control.

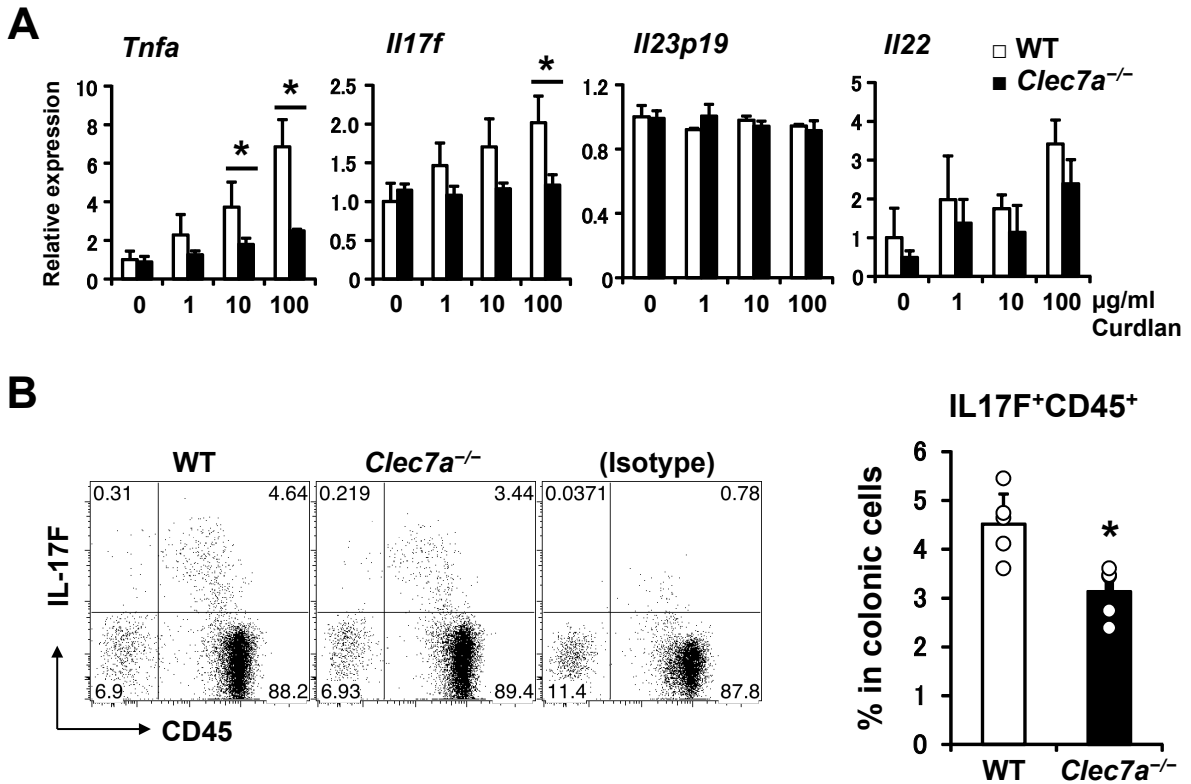


Figure 16. Dectin-1 signaling induces IL-17F in cLPs.

(A) CD11b⁺ and CD11c⁺ cLP cells from WT or *Clec7a*^{-/-} mice were cultured with curdlan at different concentrations for 6 h, and cytokine gene expression was measured by qRT-PCR. (B) IL-17F⁺CD45⁺ cell population in the cLP layer from WT or *Clec7a*^{-/-} mice was examined by flow cytometry ($n = 4-5/\text{group}$). Data are representative of two independent experiments, and in A and B are expressed as means \pm SD. * $p < 0.05$ vs WT control.

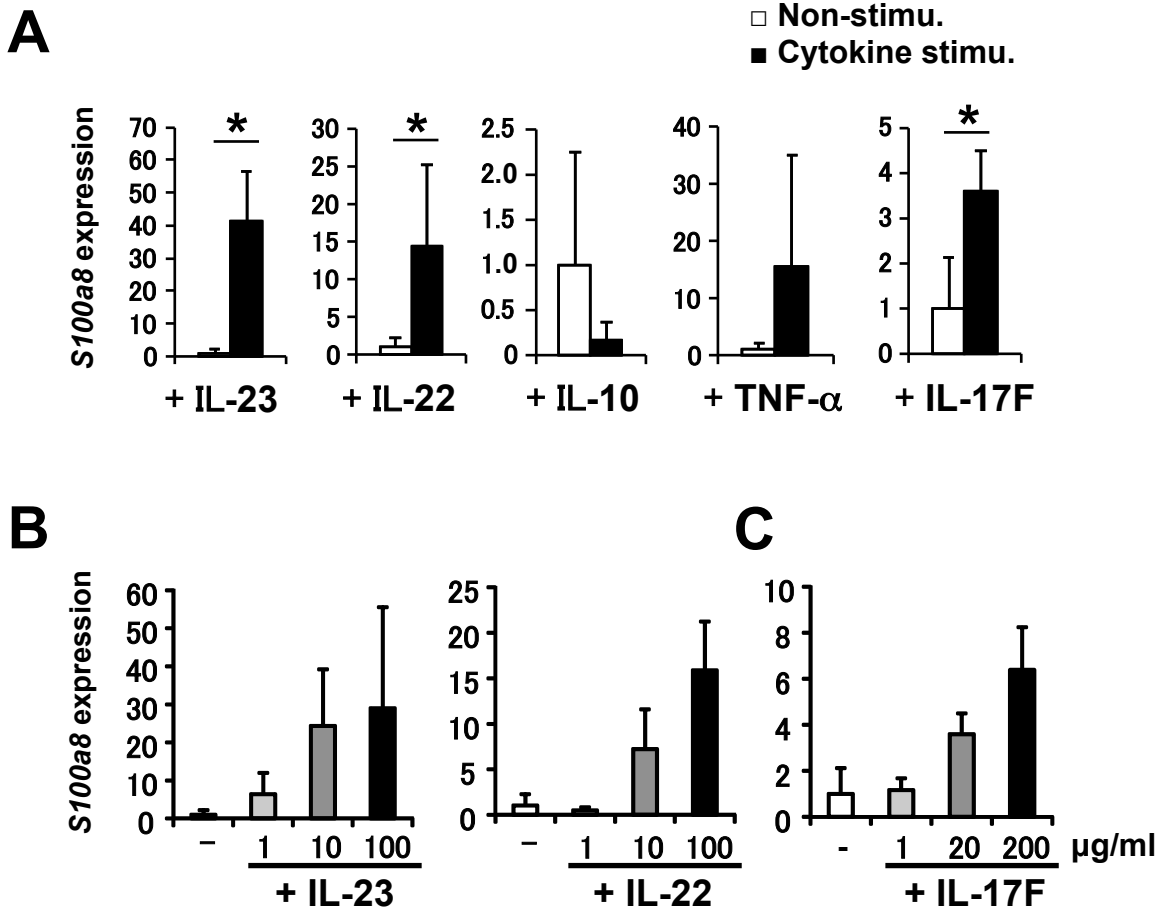


Figure 17. *S100a8* gene expression is induced by cytokines such as IL-17F, IL-23, and IL-22 in the colon cultured *ex vivo*.

(A-C) Colon pieces of WT mice were stimulated with indicated cytokines (20 ng/ml in A and 1-100, 1-200 ng/ml in B and C) for 6 h (A) or 12 h (B and C) *in vitro*, and *S100a8* expression was determined by qRT-PCR. Data in A and B are representative of two independent experiments and are expressed as means \pm SD. * $p < 0.05$ vs. control.

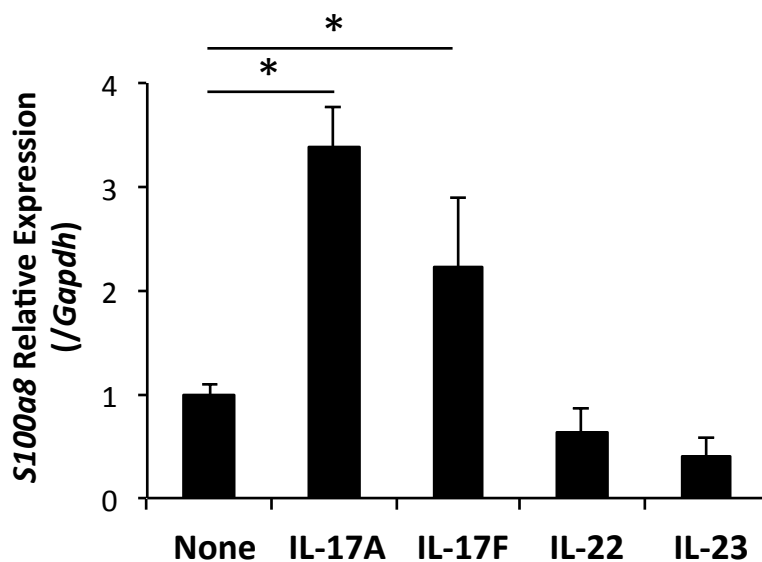


Figure 18. *S100a8* gene expression is induced by IL-17A/F in a colonic epithelial cell line.

CMT93 cells were stimulated with indicated cytokines (20 ng/ml) for 6 h, and gene expression was analyzed by qRT-PCR. Data are representative of two independent experiments and are expressed as means \pm SD. * $p < 0.05$ vs. control ("None").

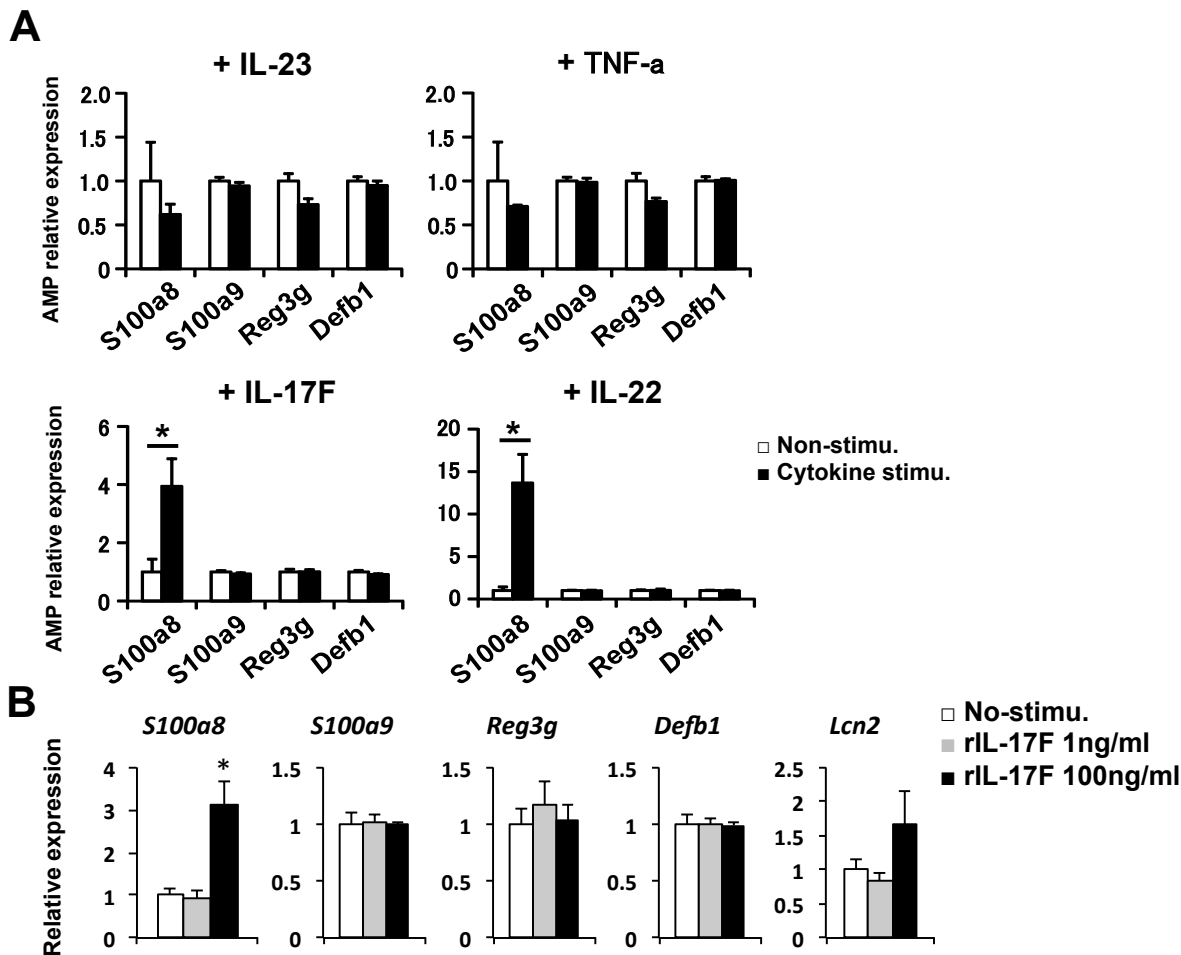


Figure 19. *S100a8* gene expression is induced by IL-17F and IL-22 in cEC primary culture.

(A) WT cECs were incubated with recombinant mouse IL-23, TNF, IL-17F, and IL-22 (100 ng/ml) for 6 h, and *S100a8*, *S100a9*, *Reg3g*, and *Defb1* gene expression was determined by qRT-PCR ($n = 3/\text{group}$). (B) Primary cECs from a WT mouse colon were stimulated with recombinant mouse IL-17F for 6 h, and AMP gene expression was determined by qRT-PCR. Data in A and B are representative of two independent experiments and are expressed as means \pm SD. * $p < 0.05$ vs. control.

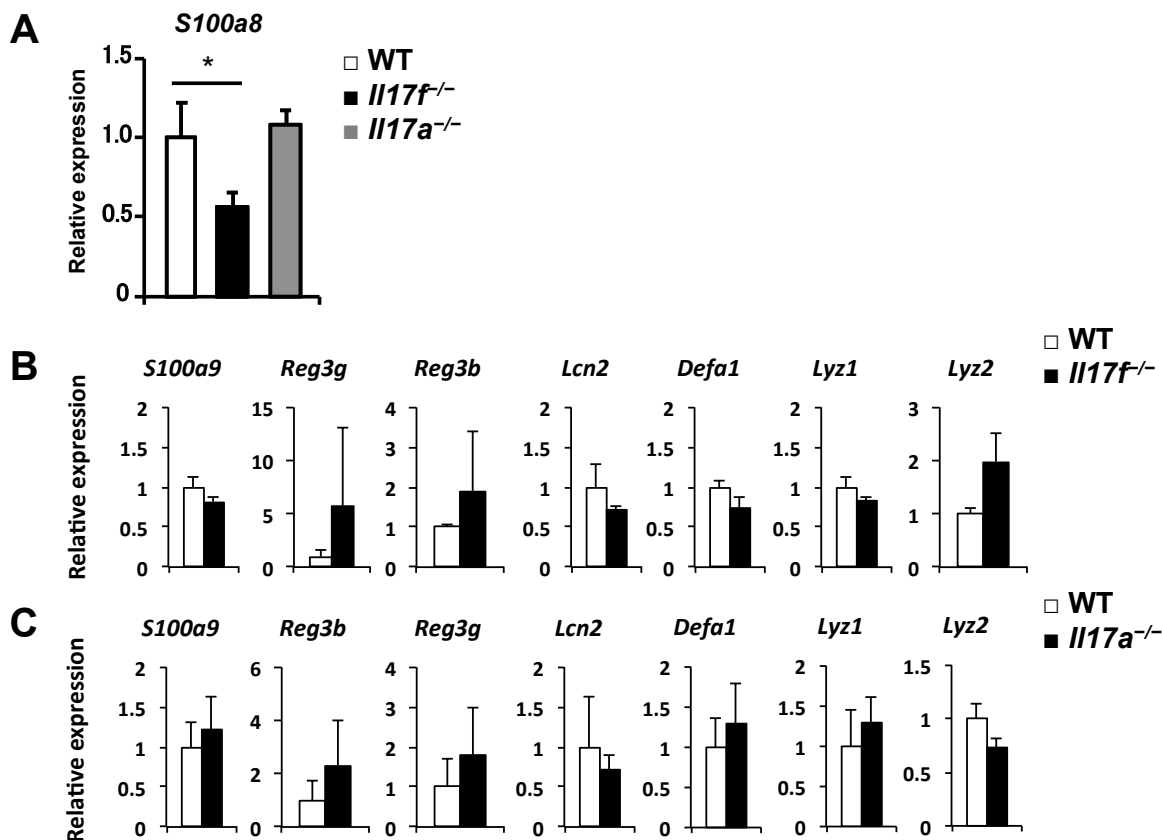


Figure 20. *S100a8* gene expression in the colon is reduced in *Il17f*^{-/-} mice. (A) *S100a8* expression in cECs from WT, *Il17f*^{-/-} mice or *Il17a*^{-/-} mice ($n = 3/\text{group}$) was determined by qRT-PCR. (B, C) Indicated AMP gene expression in cECs from WT and *Il17f*^{-/-} mice ($n = 3/\text{group}$) (B) and WT and *Il17a*^{-/-} mice ($n = 3/\text{group}$) (C) was determined by qRT-PCR. Data in A-C are representative of two independent experiments and are expressed as means \pm SD. * $p < 0.05$ vs. control.

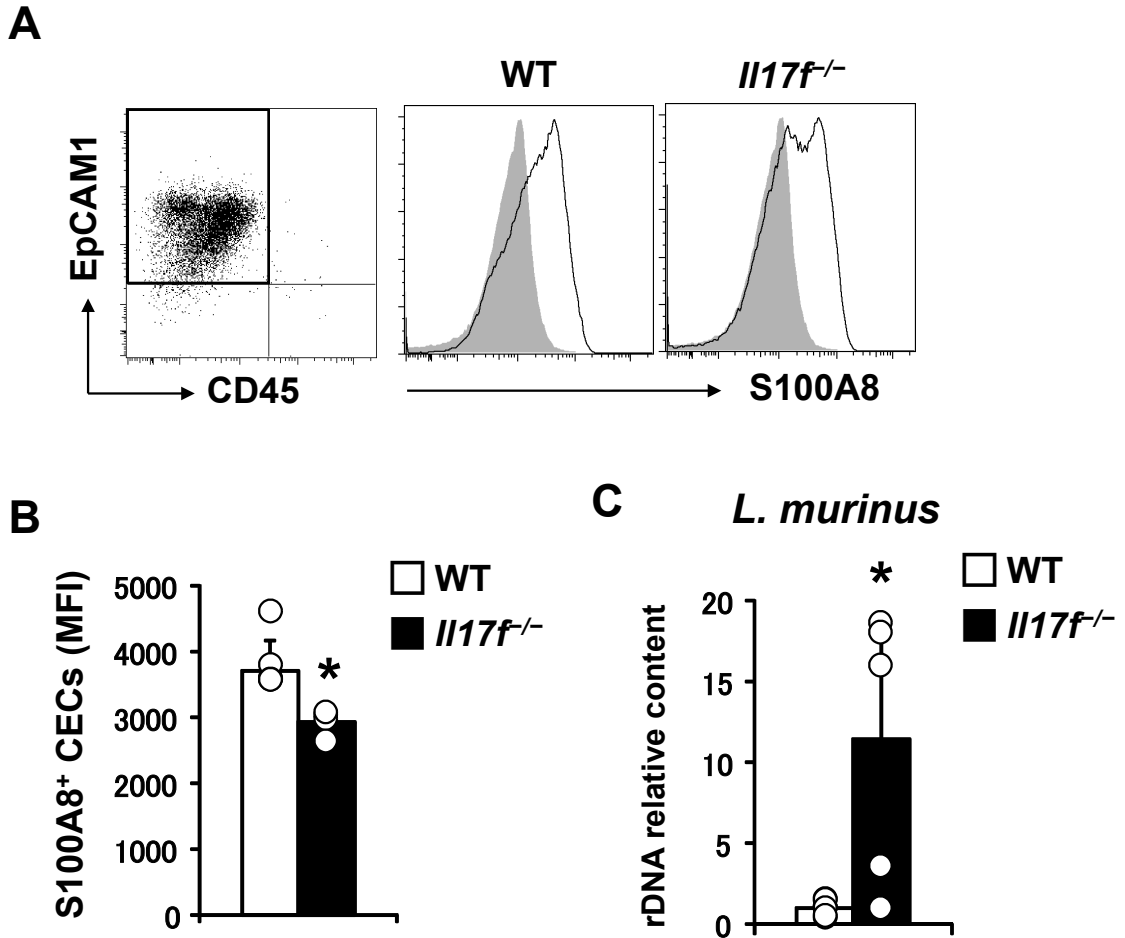


Figure 21. Percentage of S100A8⁺ epithelial cells is reduced and *L. murinus* population is increased in *Il17f*^{-/-} mice compared with WT mice. (A, B) S100A8 expression in CD45⁻ EpCAM-1⁺ cECs of WT or *Il17f*^{-/-} mice was examined by flow cytometry (A), and the mean fluorescent intensity (MFI) is shown in (B) ($n = 4-5/\text{group}$). (C) The content of *L. murinus* 16S rDNA in feces from WT or *Il17f*^{-/-} mice was determined by qRT-PCR, and the relative contents in *Il17f*^{-/-} mice to that in WT mice are shown ($n = 4-5/\text{group}$). Data in A-C are representative of two independent experiments and are expressed as means \pm SD. * $p < 0.05$ vs WT control.

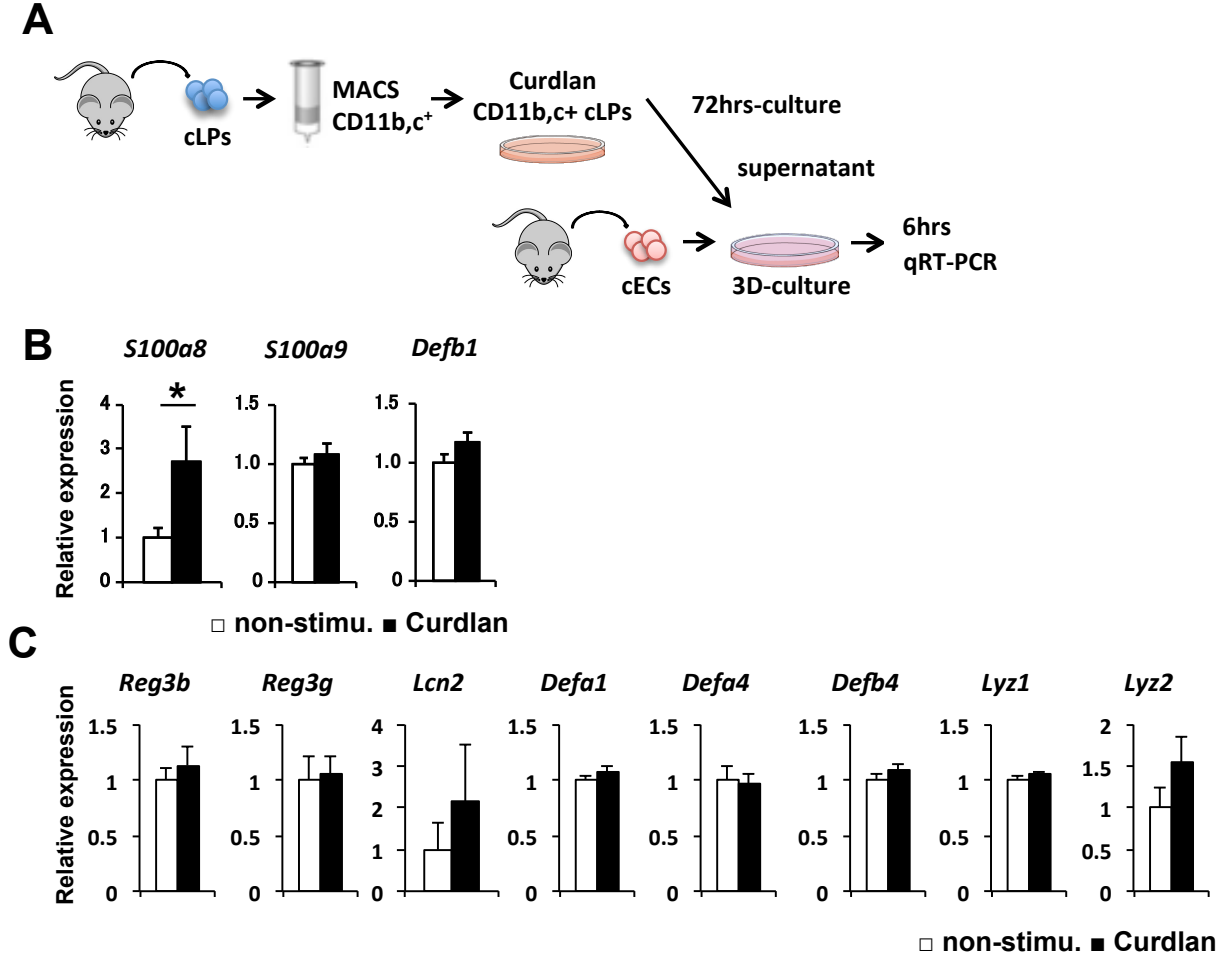


Figure 22. *S100a8* gene expression in cECs is induced by the conditioned medium of β -glucan-stimulated $CD11b^+$ myeloid cells.

(A-C) Colonic LP-derived $CD11b^+$ and $CD11c^+$ cells from WT mice purified by MACS were stimulated with curdlan for 72 h, and the culture supernatants were used to stimulate cECs of WT mice for 6 h. After the stimulation, *S100a8*, *S100a9*, and *Defb1* (B) or indicated AMPs (C) gene expression in cECs was determined by qRT-PCR. Data in B and C are representative of two independent experiments and are expressed as means \pm SD. * $p < 0.05$ vs. control.

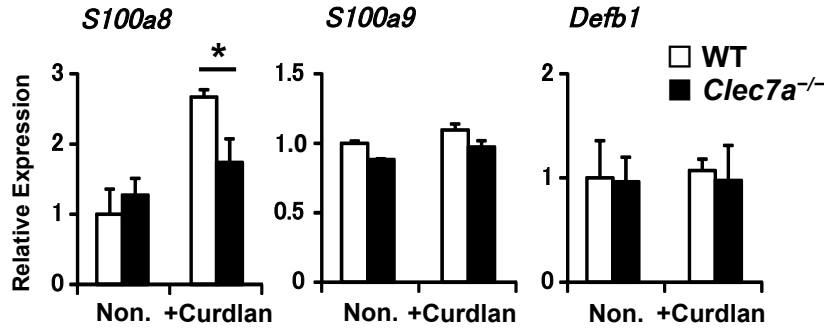
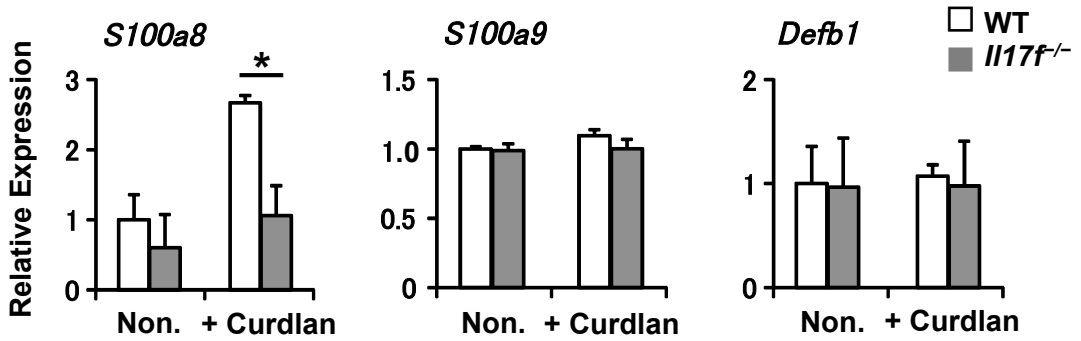
A**B**

Figure 23. Dectin-1 signaling promotes *S100a8* gene expression through induction of IL-17F in cLP CD11b⁺ myeloid cells.

(A, B) Colonic LP-derived CD11b⁺ and CD11c⁺ cells from WT and *Clec7a*^{-/-} (A) or in *Il17f*^{-/-} (B) mice purified by MACS were stimulated with curdlan for 72 h, and the culture supernatants were used to stimulate the cECs of WT mice for 6 h. After the stimulation, indicated AMP gene expression in cECs was determined by qRT-PCR. Data in A and B are representative of two independent experiments and are expressed as means \pm SD. * $p < 0.05$ vs. control.

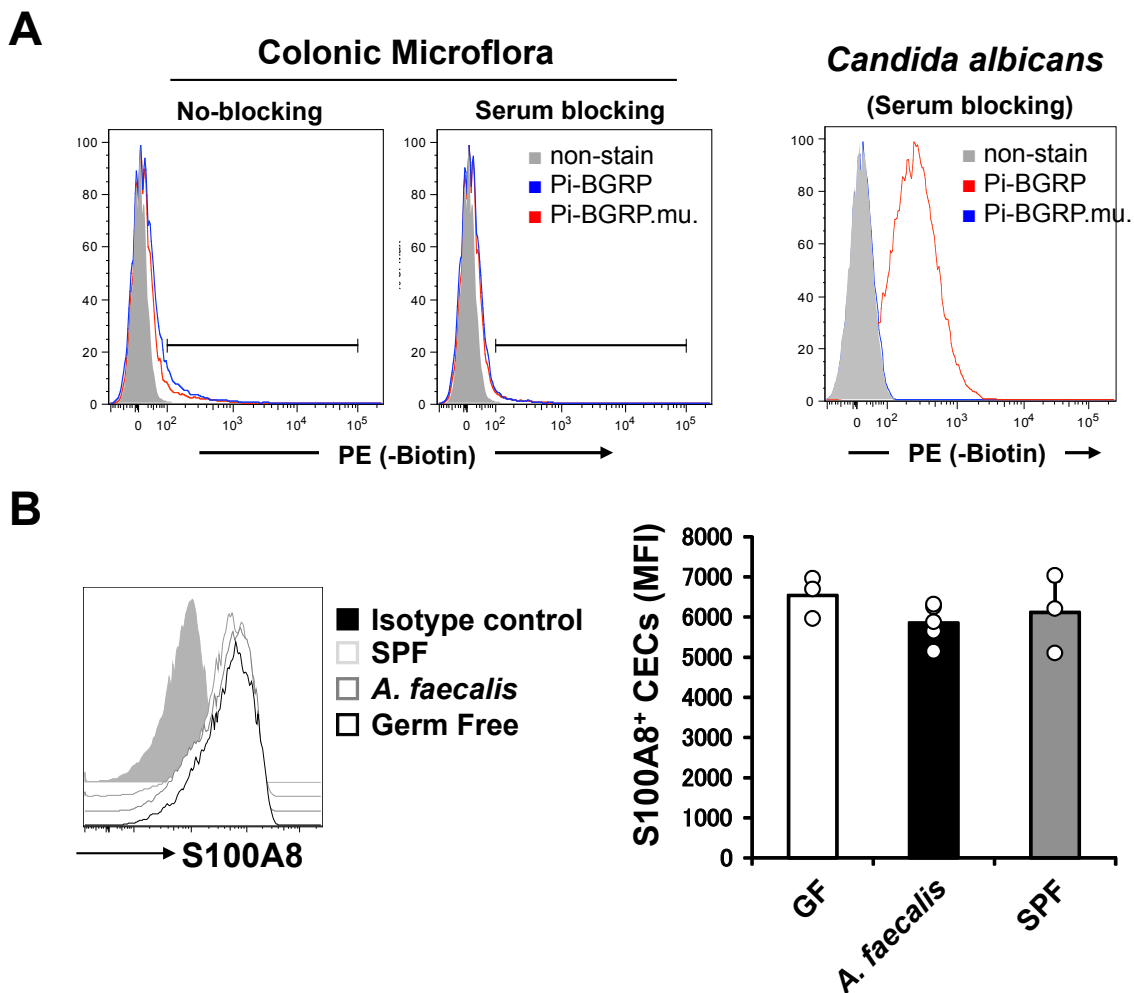


Figure 24. Dectin-1 ligands are not detected in intestinal commensal microbiota.

(A) WT mouse fecal microflora or *Candida albicans* (SC5314) were pretreated with or without goat serum to block non-specific binding. They were then treated with the β -glucan-recognizing protein (Pi-BGRP) or the mutant form of the β -glucan recognition site (Pi-BGRP.M), and the β -glucan⁺ population was determined by flow cytometry with or without 5% goat serum blocking. (B) S100A8 expression in cECs from *A. faecalis* mono-colonized (*A. faecalis*), germ-free (GF), or SPF mice was examined by flow cytometry ($n = 3-4/\text{group}$). Data in A and B are representative of two independent experiments and are expressed as means \pm SD.

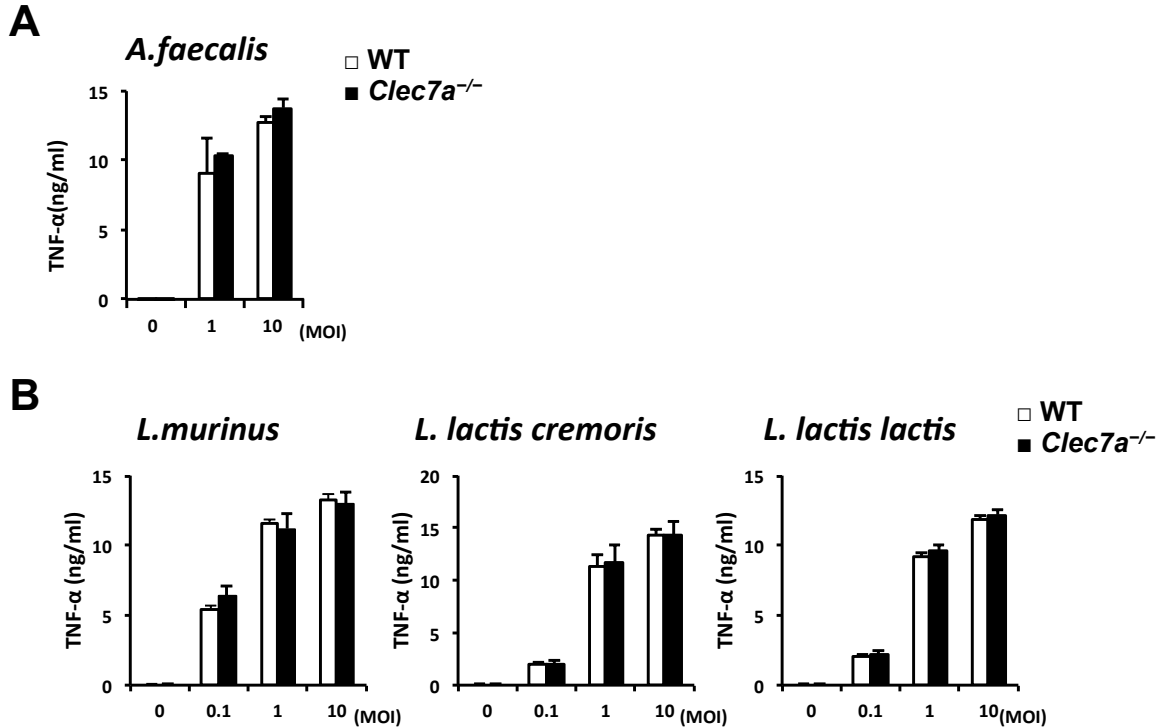


Figure 25. β -glucan synthase-expressing intestinal bacteria do not express Dectin-1 ligands.

(A) Thioglycollate-induced peritoneal M ϕ s from WT or *Clec7a*^{-/-} mice were co-cultured with live *A. faecalis* (MOI: 1 or 10) for 24 h, and TNF concentration in the culture supernatant was examined by ELISA ($n = 3$ /group). (B) Bone marrow-derived DCs from WT or *Clec7a*^{-/-} mice were co-cultured with live *L. murinus*, *L. lactis cremoris*, or *L. lactis* (MOI: 0.1, 1 or 10) for 24 h, and TNF concentration in the culture supernatant was examined by ELISA ($n = 3$ /group). Data in A and B are expressed as means \pm SD.

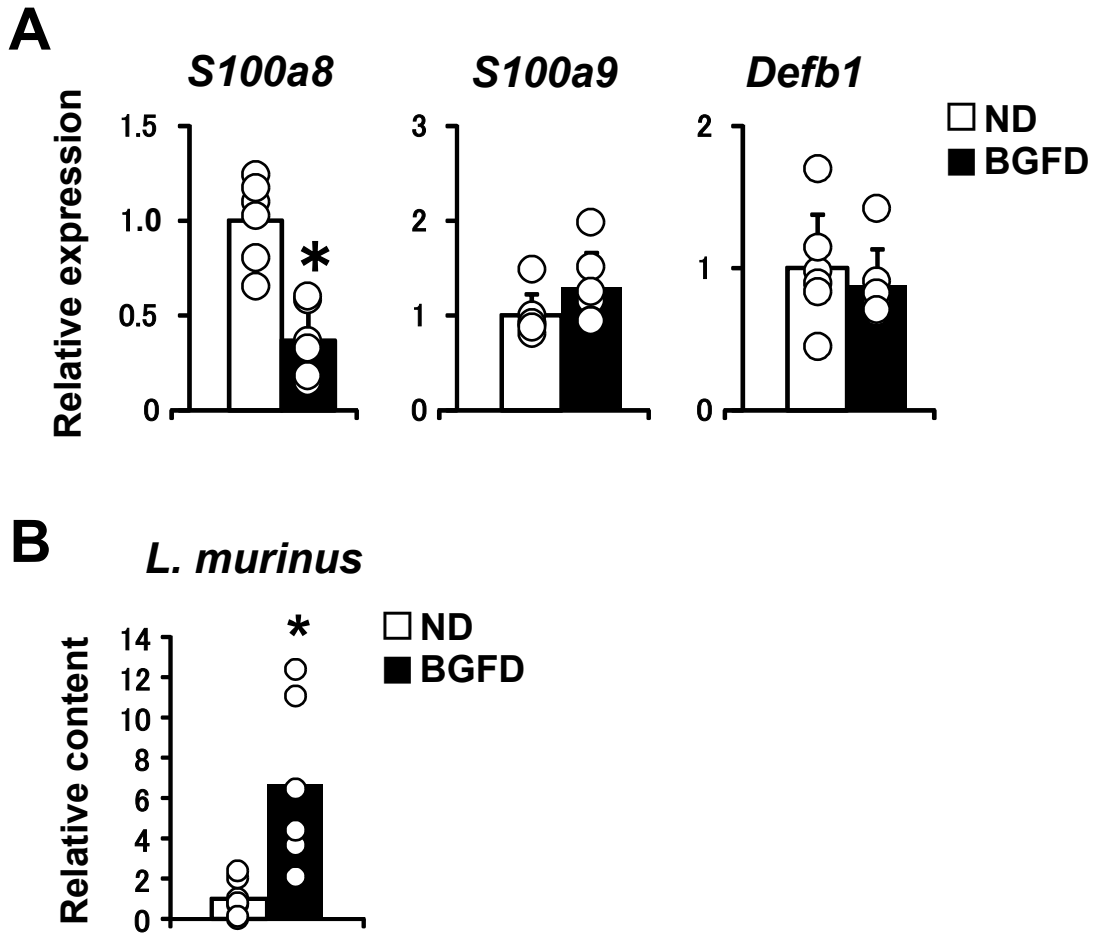


Figure 26. *S100a8* expression is significantly decreased in cECs, while *L. murinus* relative content is increased, in BGFD-fed mice compared with those of ND-fed mice.

(A, B) Mice were fed a ND or a BGFD for 3 weeks, and colonic AMP expression (A) and fecal *L. murinus* rDNA content (B) were analyzed by qRT-PCR ($n = 6/\text{group}$). Data in A and B are representative of two independent experiments and are expressed as means \pm SD. * $p < 0.05$, ** $p < 0.01$ vs. ND control.

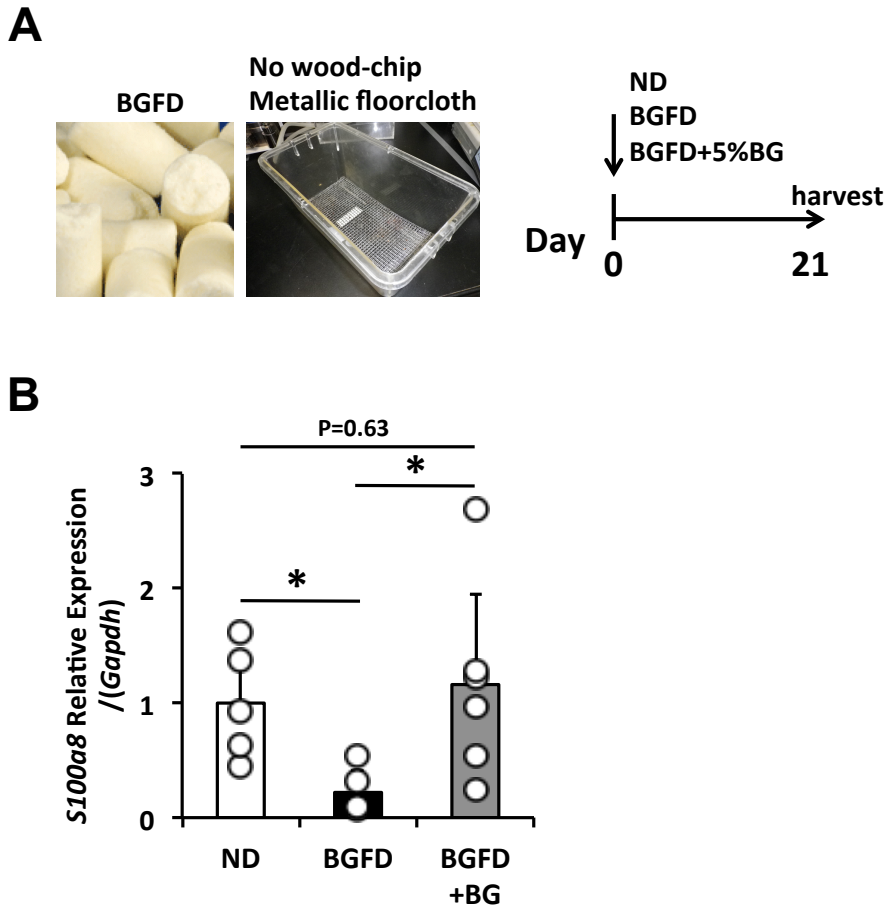


Figure 27. β -glucans in food induce *S100a8* gene expression. (A, B) Mice were fed an ND, BGFD, or 5% β -glucan-added BGFD (BG; curdlan) for 21 days (A), and colonic *S100a8* gene expression was analyzed by qRT-PCR ($n = 6/\text{group}$). Data in B are representative of two independent experiments and are expressed as means \pm SD. * $p < 0.05$.

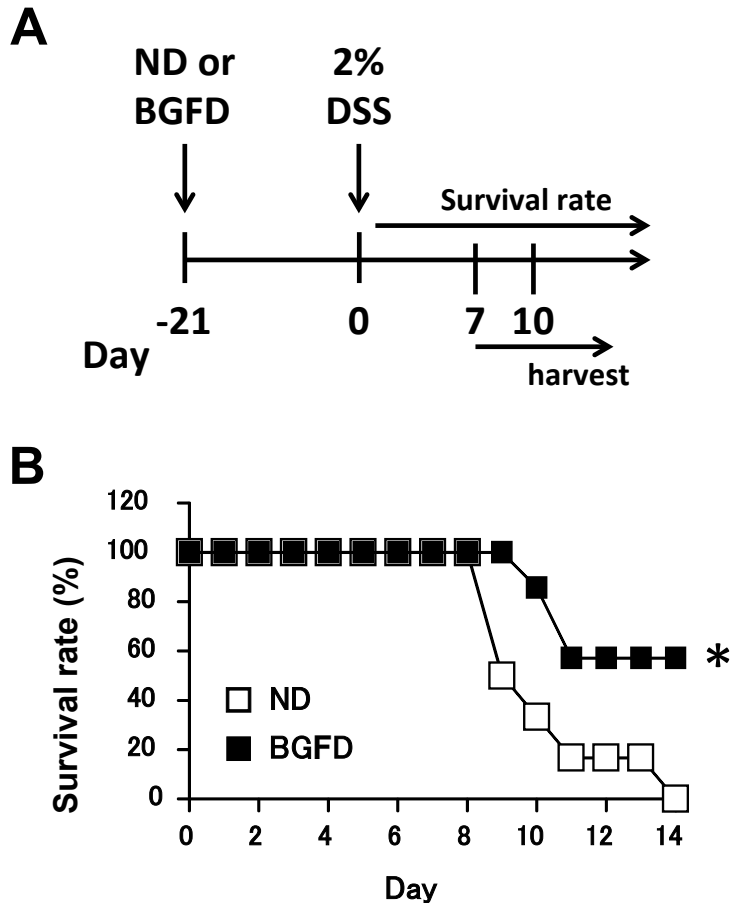


Figure 28. BGFD-fed mice are resistant to DSS-colitis.

(A) ND and BGFD feeding started 21 days before DSS administration. To induce colitis, mice were administered 2% DSS in their drinking water (weight/volume). The survival rate was observed daily, and mice were sacrificed at day 7 and 10 to analyze neutrophil infiltration and histological analysis. (B) ND- or BGFD-fed mice were administered 2% DSS in drinking water, and the survival rate was evaluated daily ($n = 6-7/\text{group}$). Data are representative of two independent experiments. $*p < 0.05$ vs. ND control.

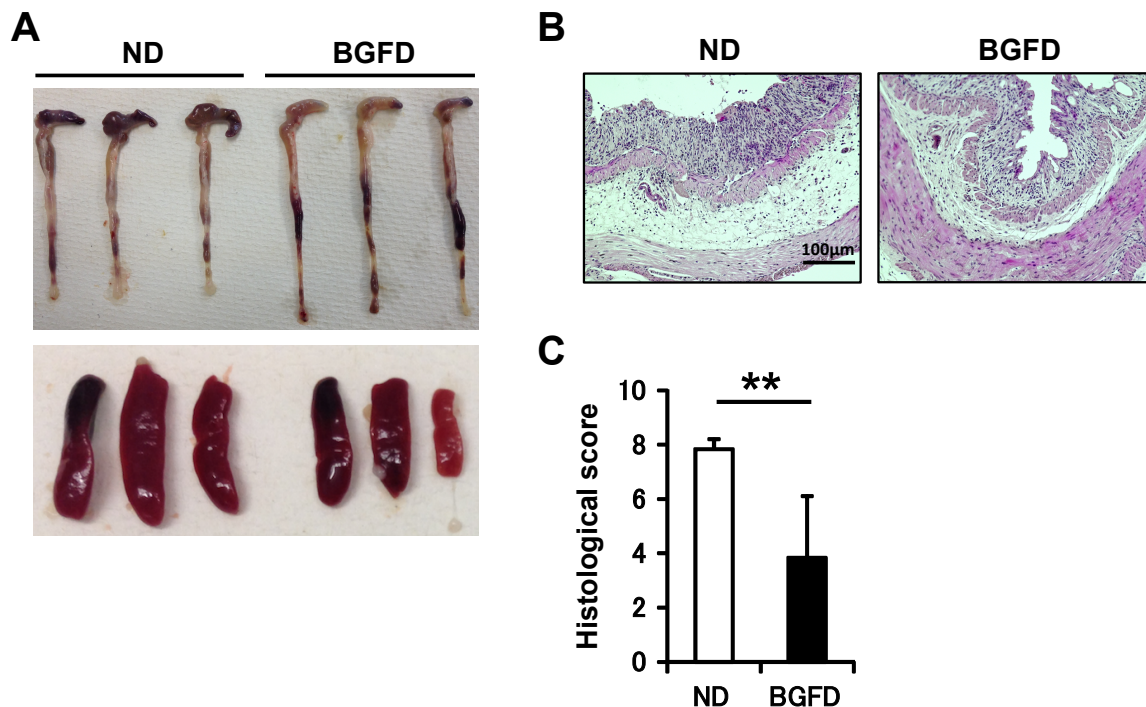


Figure 29. Histological analysis of DSS administration for ND- and BGFD-fed mice.

(A) Comparison of the colon and spleen from DSS-treated ND- and BGFD-fed mice. (B, C) Histological analysis (HE staining) of the distal colon sections from ND- or BGFD-fed mice was performed on day 10 after 2% DSS treatment. Data in A-C are representative of two independent experiments and are expressed as means \pm SD. ** $p < 0.01$ vs. ND control.

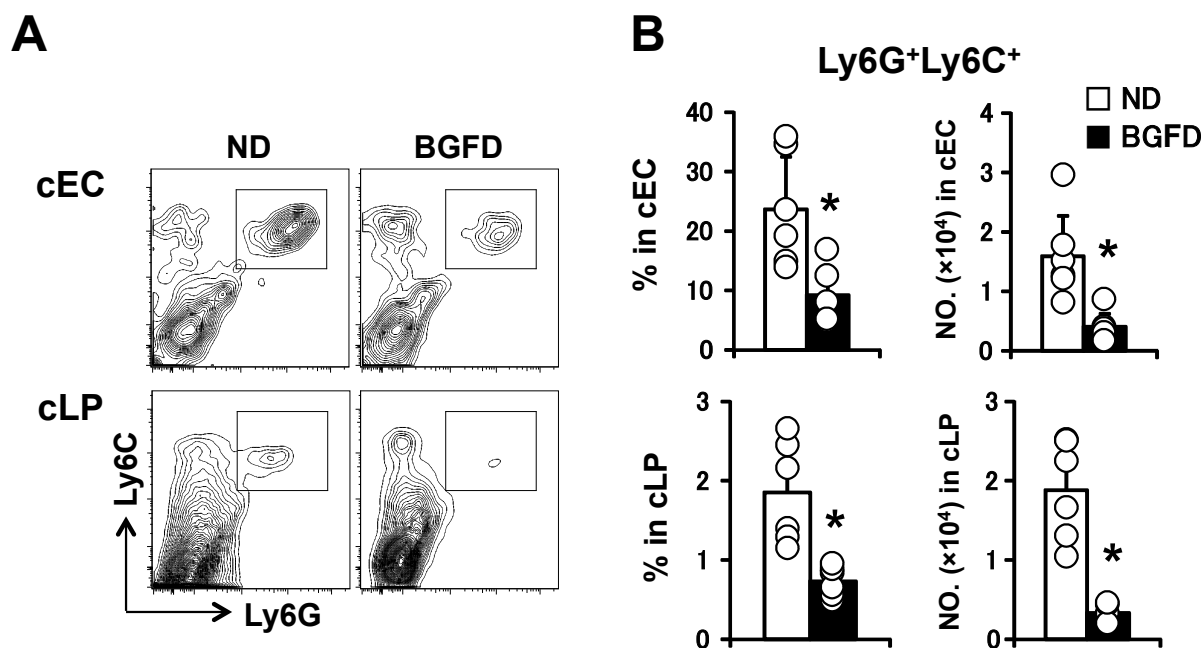


Figure 30. Neutrophil infiltration in both cEC and cLP layers is significantly suppressed in BGFD-fed mice.

(A, B) On day 7 after DSS treatment, ND- or BGFD-fed mice were sacrificed, and neutrophil infiltration in the cEC or cLP layer was examined by flow cytometry ($n = 6/\text{group}$). Data are representative of two independent experiments and are expressed as means \pm SD. * $p < 0.05$ vs. ND control.

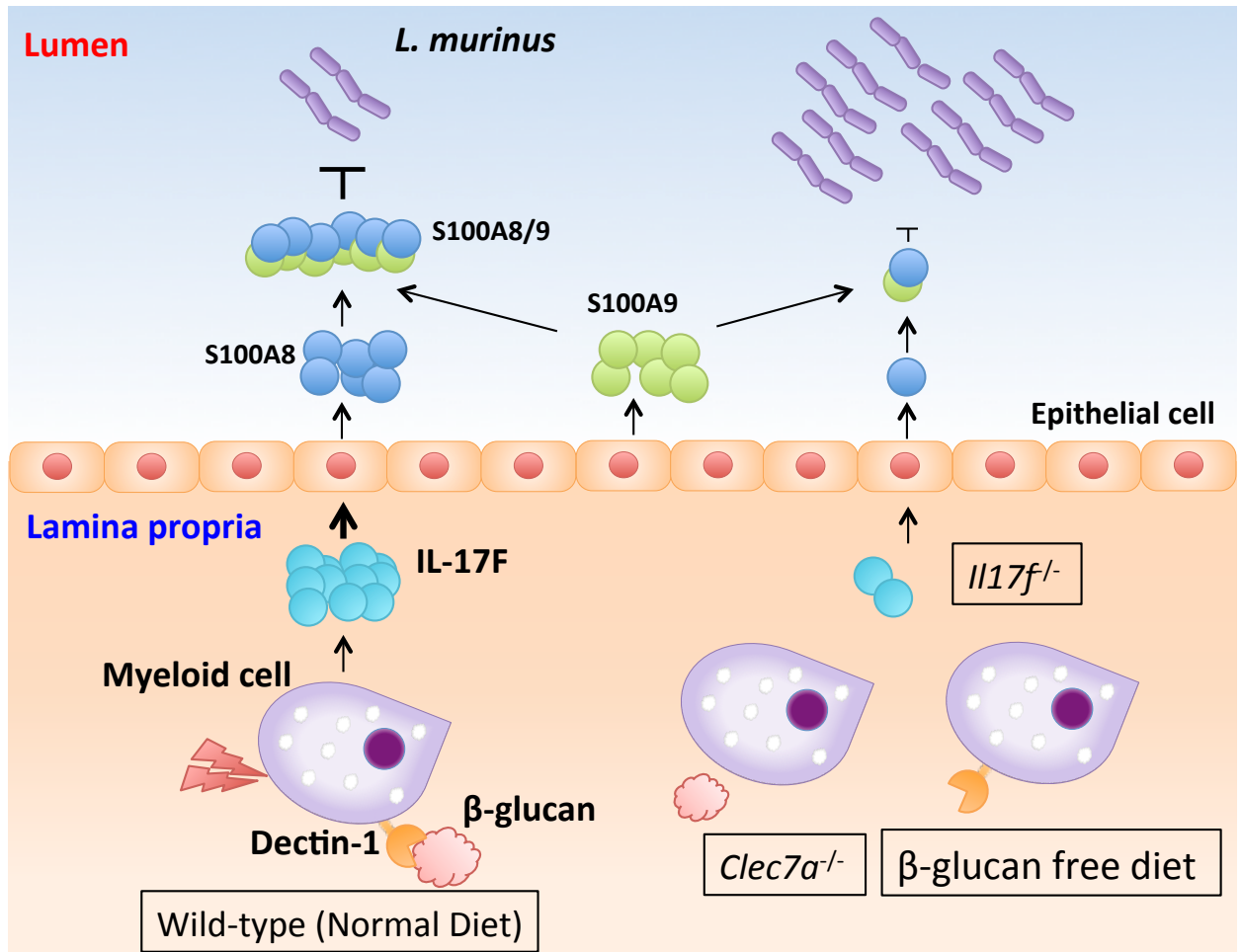


Figure 31. β -glucans indirectly regulate the growth of *L. murinus* via the Dectin1-IL-17F-S100A8 axis.

Table Real-time PCR Primers Used.

Gene name	F	R
<i>Tnfa</i>	GCCTCCCTCTCA TCAGTTCT	CACTTGGTGGTTTGCTACGA
<i>Il10</i>	GTGGAGCAGGTGAAGAGTGATT	TCCCTGGATCAGATTTAGAGAGC
<i>Il17a</i>	TTTAACTCCCTTGGCGCAAAA	CTTCCCTCCGCATTGACAC
<i>Il17f</i>	TGCTACTGTTGATGTTGGGAC	AA TGCCCTGGTTTTGGTTGAA
<i>Il22</i>	TGACGACCAGAACATCCAGA	AGCTTCTTCTCGCTCAGACG
<i>Il23p19</i>	TGGCTGTGCCTAGGAGTAGCA	CATCCTCTTCTTCTCTTAGTAG
<i>S100a8</i>	TCAAGACATCGTTTGAAAGGAAATC	GGTAGACATCAATGAGGTTGCTC
<i>S100a9</i>	AAAGGCTGTGGGAAGTAATTAAGAG	GCCATTGAGTAAGCCATTCCC
<i>Reg3b</i>	CTCTCCTGCCTGATGCTCTT	GTAGGAGCCATAAGCCTGGG
<i>Reg3g</i>	TCAGGTGCAAGGTGAAGTTG	GGCCACTGTTACCACTGCTT
<i>Lcn2</i>	CAGCTTTCAGATGTACAGCACC	CATGGCGAACTGGTTGTAGTC
<i>Defa1</i>	TCAAGAGGCTGCAAAGGAAGAGAAC	TGGTCTCCATGTTGAGCGACAGC
<i>Defa4</i>	CCAGGGGAAGATGACCAGGCTG	TGCAGCGACGATTTCTACAAAGGC
<i>Defb1</i>	AGGTGTTGGCATTCTCACAAG	GCTTATCTGGTTTACAGGTTCCC
<i>Defb4</i>	GCAGCCTTTACCCAAATTATC	ACAATTGCCAATCTGTGCGAA
<i>Lyz1</i>	GCCAAGGTCTACAATCGTTGTGAGTTG	CAGTCAGCCAGCTTGACACCACG
<i>Lyz2</i>	GGCTGGCTACTATGGAGTCAGCCTG	GCATTACAGCTCTTGGGGTTTTG
<i>Gapdh</i>	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA

8. Discussion

This study revealed that Dectin-1-expressing myeloid cells in the intestine produce IL-17F through stimulation by β -glucans. It was also shown that IL-17F promoted the production of S100A8 from cECs, thereby inhibiting the growth of *L. murinus*.

It was reported that Dectin-1 plays an important role in host defense against fungal infections [67], [74]. A previous report indicated that Dectin-1 is expressed on intestinal cells and promotes the production of cytokines and chemokines from epithelial cells of humans [100]. In this study, I analyzed the protein expression of Dectin-1 on cECs, taking advantage of using Dectin-1-deficient mice. As a result, I found that Dectin-1 is only expressed on myeloid cells but not on cECs in the intestine (Fig. 6). Expression of Dectin-1 on ILCs was not observed either (Fig. 7). Thus, the role of Dectin-1 in the intestine is considered to be attributed to the action on myeloid cells.

I found that the population of the genus *Lactobacillaceae*, especially strain *L. murinus* NBRC 14221, was significantly increased in feces of Dectin-1-

deficient mice (Fig. 10B). Interestingly, Treg cells in the cLPs were increased in *L. murinus* gnotobiotic mice, suggesting Treg differentiation inducing activity of this bacterium (Fig. 11). Consistent with this notion, the increase of Treg cells in the intestine was also observed in *Clec7a*^{-/-} mice (Fig. 9), in which *L. murinus* population is increased. Although other *Lactobacillaceae* than *L. murinus* NBRC14221 were also expanded in *Clec7a*^{-/-} mouse intestine, this bacterial strain produced higher levels of anti-inflammatory cytokines compared with other *Lactobacillaceae* [1]. Although several bacterial contents including *Lachnospiraceae* decreased in *Clec7a*^{-/-} mice compared with WT mice under steady-state conditions the roles of these bacterial families in intestinal inflammation have not been studied yet. It is worth noting that remission is achieved in patients with *Clostridium difficile* infectious colitis who received fecal microbiota transplantation [101]. Thus, these observations indicate that intestinal microbiota play crucial roles in maintaining intestinal immune homeostasis.

Intestinal microflora are mainly regulated by host antimicrobial molecules such as IgA and AMPs and environmental factors including foods. In

this study, I focused on the role of AMPs in regulating microbiota in *Clec7a*^{-/-} mice. I found that S100A8, a component of an AMP calprotectin which is composed of S100A8 and S100A9, was significantly decreased in cECs of *Clec7a*^{-/-} mice. The antimicrobial action of calprotectin for *Candida* and *Staphylococcus* has been previously reported [34], [102]. Although detailed killing mechanism remains unknown, antimicrobial activity is explained by the activity of calprotectin to inhibit intracellular protein transport or metabolism of target bacteria [102]. I showed that calprotectin specifically suppresses *L. murinus* growth, but not *E. coli*, or *L. Johnsonii*, or *L. lactis*, explaining why *L. murinus* population expands in *Clec7a*^{-/-} mice (Fig. 14).

Ca²⁺ is indispensable for the antimicrobial activity of calprotectin. Thus, LB and MRS culture media, which contained Ca²⁺, were used for the growth of these bacteria. Although a single bacterial culture *in vitro* is an effective method to examine antibacterial activity of AMPs, the effect may differ from that *in vivo*, because some intestinal bacteria grow in a 3 dimensional (3D) microenvironment in contact with intestinal epithelial cells. Thus, in this study, I took advantage of

using new 3D culture method which mimics in vivo intestinal conditions to analyze the effect of antimicrobial peptides [103]. Further improvement of the culture system is necessary to represent actual intestinal microenvironment in which epithelial cell-bacteria and bacteria-bacteria interaction are important for the regulation of bacterial growth.

The present results have first revealed a unique mechanism how Dectin-1 in the intestine regulates microflora. I found that Dectin-1-expressing myeloid cells in the large intestine produce IL-17F upon stimulation with β -glucans (Fig. 16), followed by the induction of S100A8 in epithelial cells of the intestine, using an organ culture of the colon. Although IL-22 and IL-23 are already known to induce *S100a8* gene expression [46] [50], I showed for the first time that IL-17F also induces this gene. I also confirmed that IL-23 is able to induce AMPs in the *ex vivo* culture system. However, the effect of IL-23 was not observed in intestinal epithelial cell lines and primary cEC cultures, suggesting that IL-23 probably activates cells other than epithelial cells, such as myeloid-derived cells, in the *ex vivo* culture of the colon, and induces AMP production indirectly. Although I

observed strong *S100a8*-inducing activity of IL-22 in cECs primary culture, I could not detect any such activity in a colonic epithelial cell line. The reason for this discrepancy is not known yet, but one possible explanation will be that the IL-22 receptor is defect particularly in this cell line. Although both IL-22 and IL-17A also promote the production of S100A8, the expression levels of these genes in cLPs of *Clec7a*^{-/-} mice did not change compared with WT mice. Therefore, it is clear that IL-17F, but not IL-22, IL-23, or IL-17A, induced by Dectin-1 signaling is involved in the induction of S100A8. In the experiment shown in Figure 23, The importance of the Dectin-1–IL-17F–S100A8 axis is also confirmed using cLP myeloid cells and cECs, providing a controlling mechanism of intestinal microbiota via Dectin-1.

β -glucans are contained in various foods such as mushrooms and breads made by fermenting yeasts and also sold as healthy foods. Mice are also exposed to β -glucans because most mouse foods contain yeast extract as a component. Moreover, some commensal bacteria in the intestine encode β -glucan synthase, which can synthesize β -glucans [104]. For instance, *Curdian*,

which is used as a typical β -glucan for *in vitro* and *in vivo* assay, is made from cultivated *A. faecalis* [97]. Some commensal fungi are also suggested to activate Dectin-1 in the intestine [94]. Thus, I examined the source of β -glucans in the mouse intestine. I showed that intestinal bacteria are not involved in the activation of Dectin-1, because these bacteria with β -glucan synthase gene did not induce cytokines in a Dectin-1-dependent manner. Neither intestinal commensal bacteria nor *A. faecalis*, which has β -glucan synthase, bind β -glucan-recognizing protein, which can bind *C. albicans* through the carbohydrate recognition site. Furthermore, S100A8 expression in cECs from *A. faecalis* mono-colonized mice was not increased compared to that in cECs from germ-free or SPF mice. Regarding commensal fungi, we previously showed that our SPF mouse colonies are free from fungal infection [1]. Thus, these results suggest that β -glucans derived from food are mainly responsible to the activation of Dectin-1 in the intestine.

Then, I next examined the effect of β -glucan-free diet (BGFD) on the intestinal microflora and the immune system. To exclude the effects of β -glucans

from wooden chips on the floors, cage floor was replaced with metal mats. As a result, I found that *S100a8* gene expression was greatly reduced and *L. murinus* population was significantly increased in mice fed with BGFD compared with mice fed with ND. Because BGFD-fed mice were also resistant to the DSS-colitis similarly to *Clec7a*^{-/-} mice, I conclude that food-derived β -glucans are mainly involved in the regulation of *L. murinus* and induction of Treg cells.

Previous studies reported the influence of nutrients in food on the immune system. Mice fed with a diet lacking vitamin A develop retinoic acid deficiency. Retinoic acid deficiency causes decreased expression of $\alpha 4\beta 7$, which results in abnormal T-cell trafficking and aberrant Treg cell differentiation [105]–[107]. Inflammation of the intestinal tract is aggravated in mice fed with a diet lacking vitamin A [107]. Mice given excess saturated fatty acid led to the increase of taurocholic acid, promoting the growth of *Bilophila wadsworthia* in the intestine. Spontaneous colitis in IL-10-deficient mice is further exacerbated by *Bilophila wadsworthia*, although the mechanism of this bacterial increase remains to be elucidated [108]. Thus, foods in addition to commensal bacteria are considered

to be an important environmental factor that controls intestinal immunity. As I have shown in this study, foods regulate intestinal immunity through modification of commensal microbiota. Thus, understanding the functional roles of various food components in the regulation of intestinal microbiota as well as the immune system should provide us with a clue to treat or prevent intestinal diseases.

9. References

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