

Toll-Like Receptor 5 Engagement Induces Interleukin-17C Expression in Intestinal Epithelial Cells

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The family of Interleukin-17 (IL-17) cytokine is the essential inflammatory mediator that influences the pathophysiology of various inflammatory diseases. Many studies focused on investigating the expression, signaling, and biological impacts of IL-17A and IL-17F, and the neutralization of these cytokines exhibited some promising results in clinical trials. In contrast, the expression resources and physiological relevance of IL-17C remained to be studied. In this study, through a microarray approach conducted with nontransformed human colonic epithelial cells (NCM460), we found that bacterial flagellin stimulation elicited potent IL-17C mRNA expression. We also confirmed that IL-17C protein production was strongly induced by flagellin in these cells. Flagellin-induced IL-17C expression was also observed in human colon adenocarcinoma cells such as DLD-1 and HT-29, indicating that IL-17C could be a signature inflammatory cytokine from intestinal epithelial cells in response to flagellin. Since inhibited in TLR5-, or MyD88- or TRIF-silenced cells, flagellin-induced IL-17C expression was specifically mediated by TLR5 and, subsequently, MyD88 and TRIF adaptor molecules. Furthermore, in line with inflammatory nature of IL-17, we found that IL-17C expression was substantially enhanced in the intestinal tissues from Ulcerative colitis patients. Given the facts that TLR5 is a key pattern recognition receptor which mediates microbial recognition in the intestinal epithelium and IL-17C turned out to be a unique member of the IL-17 family expressed in intestinal epithelial cells on TLR5 activation, our study may provide an important clue on understanding how intestinal microbes would contribute to an inflammatory program in the gut.

Introduction

THE FAMILY OF INTERLEUKIN-17 (IL-17) cytokines, including IL-17A, B, C, D, E (known as IL-25), and F, is emerging as a critical element that induces the pro-inflammatory program. As a signature cytokine, IL-17 is primarily produced by Th-17 cells, which play a key role in inflammatory diseases (Pappu and others 2011). Although IL-17 is also expressed in subsets of $\gamma\delta$ T cells, invariant natural killer T (iNKT) cells, NKT cells, and macrophages (Gaffen 2011), IL-17 expression still remains to be studied in intestinal epithelial cells. Meanwhile, epithelial cells turned out to be responsive to IL-17 stimulation, resulting in the expression of antimicrobial peptides such as β -defensin (Kao and others 2008) or cathelicidin (Peric and others 2008). Thus, IL-17 responsiveness in epithelial cells appears to play an important role in anti-microbial defense. Indeed, IL-17A deficient or its receptor IL-17RA deficient mice exhibited increased susceptibility to *Candida albicans* (Huang and others 2004; Saijo and others 2010). Impaired IL-17F or IL-17RA resulted in high susceptibility to

chronic mucocutaneous candidiasis in human beings (Puel and others 2011).

Although the biochemical features and biological impacts of each IL-17 family member still remain to be intensively studied, IL-17A and F have mesmerized many to study their intracellular signaling and inflammatory impacts for years. Intriguingly, IL-17A and F were known to collaborate with inflammatory cytokines such as tumor necrosis factor- α (TNF- α) or interferon- γ or IL-1 β to synergistically derive potent inflammatory responses, although the molecular mechanism of the synergy is not yet clear (Pappu and others 2011). In line with the pro-inflammatory propensity of IL-17, indeed, elevated IL-17A and F were observed in various autoimmune diseases (Pappu and others 2011).

As for IL-17B, C, D, and E, their expression resources, targeting cells, cellular signaling, and biological impacts have been poorly studied. Very recent studies, however, showed that IL-17C mRNA is expressed in human colon adenocarcinoma cells SW480 and HCT-15 stimulated with bacterial products (peptidoglycan, lipopolysaccharide, or

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flagellin) or inflammatory cytokines (IL-1 β , TNF- α) (Ramirez-Carrozzi and others 2011; Song and others 2011). These studies also suggested that IL-17C utilizes a heterodimeric receptor complex of IL-17RA and IL-17RE with a higher affinity to IL-17RE than IL-17RA. Since IL-17RE and IL-17RA are preferentially expressed in epithelial cells, IL-17C appears to play an essential role in host mucosal defense against microbial infection and inflammation in the intestine (Ramirez-Carrozzi and others 2011; Song and others 2011).

TLR5 is a pattern recognition receptor that specifically recognizes bacterial flagellin at the plasma membrane and is abundantly present in many epithelial cell types from various organs (Rhee and others 2004; Schaefer and others 2004; Blohmke and others 2008). We demonstrated that TLR5 utilizes the adaptor molecules MyD88 and TRIF, but not TRAM, to mediate flagellin-induced NF- κ B and AP-1 transcription factor activation and corresponding cytokine expression in intestinal epithelial cells (Choi and others 2010a). Given the facts that intestinal epithelial cells are at the front line of microbial recognition and these cells are strongly responsive to flagellin via TLR5 to induce potent inflammatory and innate immune responses, it would be of interest to study whether TLR5 engagement elicits the expression of IL-17 family members in intestinal epithelial cells.

In this study, using the microarray approach, we found that TLR5 activation by flagellin elicited both IL-17C protein production and IL-17C mRNA expression in nontransformed human colonic epithelial cells (NCM460). We also showed that the adaptor molecule MyD88 and TRIF mediated TLR5-induced IL-17C expression. In light of the inflammatory impact of IL-17, we examined the expression of IL-17 family members in the intestinal tissues from inflammatory bowel disease (IBD) patients represented by Crohn's disease (CD) and Ulcerative colitis (UC), and observed that IL-17C expression was significantly augmented in UC patients' tissues compared with normal tissues. Thus, our data suggest that IL-17C expression by flagellin-TLR5 engagement at the surface of epithelial cells may confer a crucial impact on the inflammatory pathophysiology of intestinal mucosa.

Materials and Methods

Materials

Nontransformed human colonic epithelial (NCM460) cells developed from normal human colon (Moyer and others 1996; Kumar and others 1997) were cultivated as previously described (Rhee and others 2004). MyD88-knock down NCM460 (NCM460-MyD88-KD), NCM460-TRIF-KD, and its control NCM460-control cells were previously described (Choi and others 2010a). NCM460-TLR5-KD and its control cells were previously described (Rhee and others 2006). Human colon carcinoma DLD-1 cells, DLD-1-TLR5-KD, and DLD-1-control cells were previously described (Rhee and others 2008). Human colon carcinoma HT-29 cells were obtained from ATCC (Manassas, VA) and maintained in McCoy's 5A complete media containing fetal bovine serum to a final concentration of 10%. Flagellin purified from *Salmonella typhimurium* (<0.1 Endotoxin Unit/ μ g purified protein determined by Limulus Amebocyte Lysate test) was purchased from "Enzo Life Sciences" (Farmingdale, NY) and dissolved in endotoxin-free water. Human IL-17C and IL-8 ELISA were from "MyBioSource" (San Diego, CA) and "Invitrogen" (Grand Island, NY), respectively.

Microarray analysis

NCM460-control, NCM460-MyD88-KD, and NCM460-TRIF-KD cells were cultivated in 100 mm dishes in quadruplicate and treated with or without flagellin (100 ng/mL) for 3 h. Total RNA was isolated using the miRNeasy Mini kit (Qiagen, Valencia, CA). Microarray analysis was performed by the Clinical Microarray Core at UCLA. Briefly, an equal amount of total RNA (200 ng) was processed using the Low Input Quick Amp Labeling Kit One-Color (Agilent Technologies, Santa Clara, CA), and Cyanine-3-labeled cRNA was purified by RNeasy Mini Kit (Qiagen). Cy-3-labeled cRNA (600 ng) was then fragmented for 30 min at 60°C before loading onto Agilent Whole Human Genome arrays (Agilent Technologies). The samples were hybridized for 17 h at 65°C with constant rotation. Arrays were washed, dried, and scanned on an Agilent DNA Microarray Scanner at a resolution at 3 μ m by following the manufacturer's protocol. Microarray images were analyzed using the Agilent's Feature Extraction software.

Array normalization, comparison analysis, and hierarchical clustering were performed with the dChip software (Li and Wong 2001) (www.dchip.org). Differentially expressed genes were selected by the following criteria: There should be at least a 2-fold difference in expression between controls and experiments; the *P*-value of the 2-tailed, 2-sample unpaired *t*-test should be <0.05.

Quantitative real-time PCR

As previously described (Im and others 2009; Choi and others 2010b), total RNA was prepared from cultivated cells, using the "RNeasy Plus Mini Kit" (QIAGEN, Valencia, CA), and an equal amount of RNA (2 μ g) was transcribed into cDNA, using "High Capacity Reverse Transcription Kit" obtained from "Applied Biosystems" (Foster City, CA) by following the manufacturer's instruction. To evaluate the cytokine gene expression, quantitative real-time PCR (qPCR) was performed on "Applied Biosystems 7500 Fast Real-Time PCR System" with the *TaqMan* Universal Master Mix, using the standard condition from "Applied Biosystems". After the sample had been incubated for 2 min at 50°C followed by *AmpliTaq* Gold activation for 10 min at 95°C, 40 cycles were run with a denaturing temperature of 95°C (15 s) and an annealing/extension temperature 60°C (1 min). The primer pairs and FAM™ dye-labeled *TaqMan*® MGB (minor groove binding) probes for human TRIF, MyD88, IL-8, IL-17(A, B, C, D, and F), IL-1 β , and the internal control GAPDH gene were purchased from "Applied Biosystems." The level of expression was calculated based on the PCR cycle number (C_T) at which the exponential growth in fluorescence from the probe passes a certain threshold value (C_T). Relative gene expression was determined by the difference in the C_T values of the target genes after normalization to the RNA input level, using a C_T value of GAPDH. Relative quantification was represented by standard $2^{-\Delta C_T}$ calculations. $\Delta C_T = (C_{T\text{-target gene}} - C_{T\text{-GAPDH}})$. Each reaction was performed in triplicate.

Measuring IL-17C protein production

An enzyme-linked immunosorbent assay (ELISA) was performed to measure the level of human IL-17C protein production using the appropriate kits from "MyBioSource" (San Diego, CA) by following the manufacturer's

instructions. All assays were performed in triplicate, and data were shown as mean \pm SD.

IPA network analysis

Excel files exhibiting fold change values were obtained from Significance Analysis of Microarrays analyses using a 10% false discovery rate, followed by including the gene name and symbol. The microarray data were analyzed by the Ingenuity Pathway Analysis (IPA) 7.5 software (Ingenuity Systems, Inc., Mountain View, CA) (Li and others 2005; Dorsam and others 2010). Network scores and *p*-values were assigned by IPA software; thus, according to the degree of interconnectedness between the molecules, IPA software determined a higher or lower IPA network score. The most significant networks of each dataset were imported into the path designer, and networks were graphically displayed to describe the relationships between genes and their neighboring genes.

Quantitative real-time PCR with human UC CD and normal intestinal tissues

We used a commercially available cDNA panel (TissueScan™ qPCR Arrays, Origene Technologies, Inc., Rockville, MD) made from tissues of CD and UC patients and normal controls, which were selected from mixed ages, gender, and ethnic groups. The panels are of normalized cDNA prepared from pathologist-verified human CD and UC tissues. qPCR was performed as described earlier (Im and others 2009; Choi and others 2010b).

Statistical analysis

Statistical analyses were conducted with GraphPad Prism (GraphPad Software, La Jolla, CA), and detailed information of statistical analysis was presented in the corresponding figure legends.

Results

Gene array analysis reveals IL-17C expression in flagellin-treated human colonic epithelial (NCM460) cells

Intestinal epithelial cells are in continuous contact with microbial factors released from commensal and pathogenic microbes in the gut. Therefore, the gene expression in the intestinal epithelial cells is significantly influenced by these microbial factors that are recognized by pattern recognition receptors such as TLRs. Bacterial flagellin is an abundant microbial pattern molecule that is released by microbes in the gut, and the level of serum antibody against flagellin is elevated in IBD patients. Therefore, flagellin is considered one of the biomarkers for IBD (Lodes and others 2004; Yu and others 2011). Accordingly, it is reasonable to speculate that an array of gene expression elicited by flagellin in intestinal epithelial cells would be related to the development and progress of IBD. Thus, using a microarray analysis, we examined the flagellin-induced gene expression profile in nontransformed human colonic epithelial cells (NCM460). In order to perform a microarray analysis, we used MyD88-knock-down NCM460 (NCM460-MyD88-KD) cells and TRIF-knock-down NCM460 (NCM460-TRIF-KD) cells, which

were stably transfected with MyD88 and TRIF shRNA encoding constructs, respectively, as previously described (Choi and others 2010a). In parallel, we also used NCM460-control cells, which were also stably transfected with scramble shRNA constructs (Choi and others 2010a).

To conduct a microarray analysis, NCM460-control, NCM460-MyD88-KD, and NCM460-TRIF-KD cells were stimulated with flagellin or a vehicle, and total RNA was isolated. To confirm whether the knock-down cells have genuinely silenced MyD88 or TRIF gene expression, we first checked MyD88 and TRIF expression. Our qPCR data clearly showed that MyD88 expression was dramatically silenced in NCM460-MyD88-KD cells, compared with the intact MyD88 expression in both vehicle- and flagellin-treated NCM460-control cells (Fig. 1A). We also confirmed that MyD88 expression was preserved in flagellin-treated NCM460-TRIF-KD cells. In addition, we examined TRIF expression in these cells, and as expected, TRIF expression was significantly diminished in NCM460-TRIF-KD cells (Fig. 1B). It is of interest to note that flagellin stimulation resulted in increased TRIF expression in NCM460-control cells, compared with vehicle-treated NCM460-control cells and flagellin-treated NCM460-MyD88-KD cells. As previously shown, inflammatory stimulation such as TNF- α enhanced TRIF expression in NCM460 cells (Choi and others 2010b), and flagellin stimulation appeared to up-regulate TRIF expression. Nonetheless, these data (Fig. 1A, B) demonstrated that MyD88 and TRIF gene expressions were successfully silenced in NCM460-MyD88-KD and NCM460-TRIF-KD cells, respectively, and NCM460-control cells have intact MyD88 and TRIF gene expression.

Given that these cells were stimulated with flagellin, before proceeding with a microarray analysis, we checked whether these cells were properly stimulated with flagellin. To confirm this, we tested whether these cells exhibited differential IL-8 expression in response to flagellin, because a previous study had already shown that IL-8 expression was induced by flagellin, and blocking MyD88 or TRIF expression reduced IL-8 expression in intestinal epithelial cells (Rhee and others 2006; Choi and others 2010a). Indeed, flagellin stimulation dramatically induced IL-8 expression in NCM460-control cells, compared with vehicle-treated NCM460-control cells (Fig. 1C). In contrast, flagellin-induced IL-8 expression was significantly reduced in NCM460-MyD88-KD and NCM460-TRIF-KD cells, although MyD88 knock down resulted in more potent inhibition than TRIF knock down. Collectively, these data assured that MyD88 or TRIF gene expression was successfully silenced, and flagellin stimulation in these cells were successfully achieved.

From total RNA isolated from these cells, we subsequently performed a microarray analysis. Among the various genes whose expressions were differentially altered in flagellin-treated NCM460 cells, intriguingly, IL-17C expression was dramatically elevated in flagellin-treated NCM460-control cells compared with vehicle-treated NCM460-control cells (Fig. 1D). Moreover, our microarray analysis also showed that flagellin-induced IL-17C expression was dramatically diminished in both NCM460-MyD88-KD and NCM460-TRIF-KD cells (Fig. 1D). These data imply that flagellin stimulation in nontransformed human colonic epithelial cells elicits IL-17C gene expression, for which the TLR-associated adaptor molecules, MyD88 and TRIF, are required as a regulatory adaptor molecule.

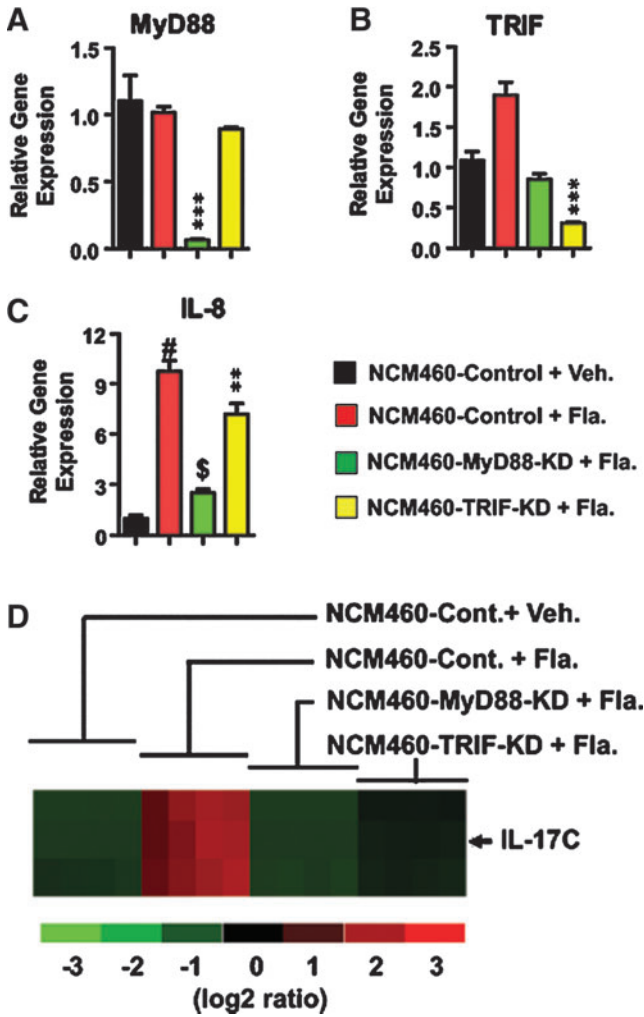


FIG. 1. Microarray analysis reveals that flagellin stimulation elicits IL-17C expression, mediated by adaptor molecules MyD88 and TRIF. MyD88-knock down (NCM460-MyD88-KD), TRIF-knock down (NCK460-TRIF-KD), and its control (NCM460-control) cells were stimulated with vehicle or flagellin (100 ng/mL) for 3 h. (A, B) The expression of MyD88 and TRIF in these cells was measured by qPCR. $***P < 0.0001$, NCM460-control+Veh. versus NCM460-MyD88-KD+Fla. (A); NCM460-control+Veh. versus NCM460-TRIF-KD+Fla. (B). (C) From these cells, IL-8 expression was evaluated by qPCR. $^{\#}P < 0.0001$, NCM460-control+Veh. versus NCM460-control+Fla.; $^{\$}P < 0.0001$, NCM460control+Fla. versus NCM460-MyD88-KD+Fla.; $**P < 0.01$, NCM460-control+Fla. versus NCM460-TRIF-KD+Fla. $n=4$ /group. (D) Using the total RNA from these cells, we carried out a microarray analysis, and 2-dimensional hierarchical clustering of IL-17C expression was presented as changes in expression (\log_2). Each column corresponds to an experimental sample ($n=4$ /group). Statistical significance was tested by a 2-tailed t test. Error bars indicate \pm SD. All qPCR data are representative of at least 3 independent experiments. Veh., vehicle; Fla., flagellin; qPCR, quantitative PCR.

Silencing TLR5 expression inhibits flagellin-induced IL-17C expression

Having found that flagellin stimulation elicited IL-17C expression in NCM460 cells determined by a microarray analysis, we subsequently carried out independent qPCR

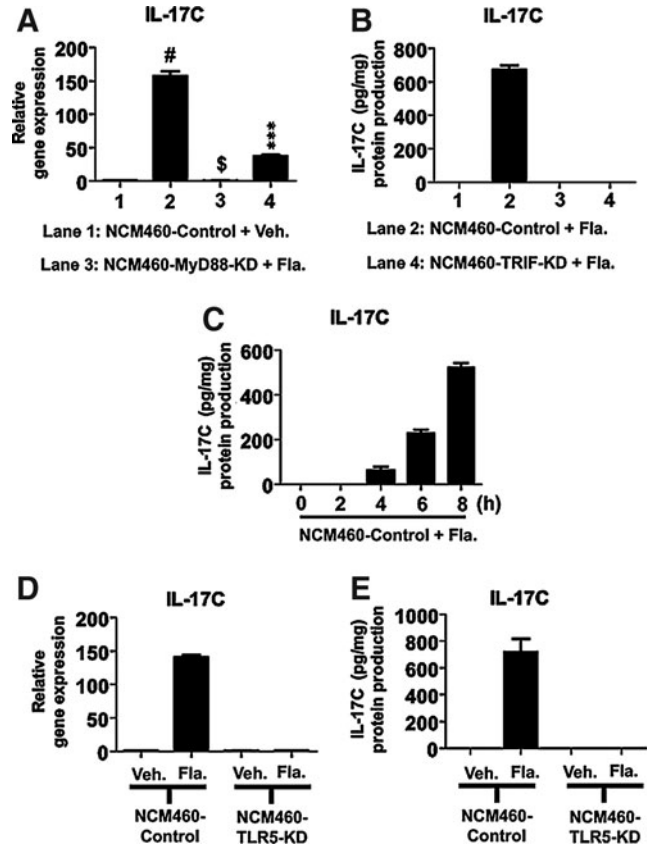


FIG. 2. Flagellin-induced IL-17C expression is specifically mediated by TLR5. (A) To substantiate flagellin-induced IL-17C expression determined by microarray analysis, NCM460-MyD88-KD, NCM460-TRIF-KD, and NCM460-control cells were stimulated with flagellin, as in Fig. 1, followed by measuring IL-17C expression by qPCR. $^{\#}P < 0.0001$, NCM460-control+Veh. versus NCM460-control+Fla.); $^{\$}P < 0.0001$, NCM460-control+Fla. versus NCM460-MyD88-KD+Fla.; $***P < 0.01$, NCM460-control+Fla. versus NCM460-TRIF-KD+Fla. Statistical significance was tested by a 2-tailed t test. (B) These cells were stimulated with flagellin for 8 h. IL-17C protein production was evaluated by ELISA using the supernatant and normalized by the total protein concentration of cell extracts. (C) NCM460-control cells were stimulated with flagellin for the indicated time points. IL-17C protein production was evaluated by ELISA. (D, E) TLR5 knock-down NCM460 (NCM460-TLR5-KD) and its control (NCM460-control) cells were stimulated by flagellin, followed by measuring the level of IL-17C mRNA (D) by qPCR and protein production (E) by ELISA. Error bars indicate \pm SD. All data are the representative of at least 3 independent experiments. $n=3$ /group. ELISA, enzyme-linked immunosorbent assay.

experiments to confirm whether flagellin stimulation could induce IL-17C expression. Indeed, we observed that IL-17C expression was robustly up-regulated by flagellin stimulation in NCM460-control cells relative to vehicle-treated NCM460-control cells. In contrast, flagellin-induced IL-17C expression was dramatically inhibited in both NCM460-MyD88-KD and NCM460-TRIF-KD cells (Fig. 2A). In addition to evaluating IL-17C mRNA expression, we furthermore measured IL-17C protein production in these cells by ELISA. Similarly, IL-17C protein production was strongly induced by flagellin stimulation in NCM460-control cells (Fig. 2B, C),

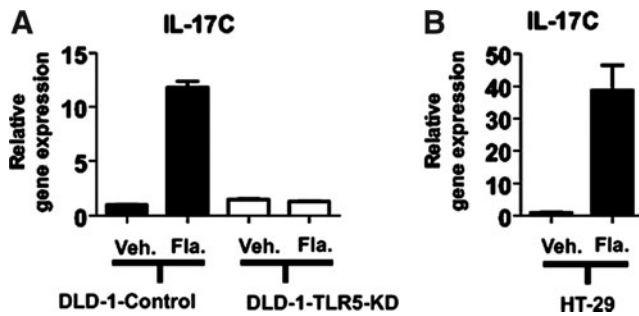


FIG. 3. Flagellin stimulation elicits IL-17C expression in DLD-1 and HT-29 cells. (A, B) TLR5 knock-down DLD-1 (DLD-1-TLR5-KD) and its control (DLD-1-control) cells (A) or HT-29 cells (B) were stimulated with flagellin, as in Fig. 1. IL-17C expression was evaluated by qPCR. Error bars indicate \pm SD ($n=3$ /group). All data are representative of at least 3 independent experiments.

compared with vehicle-treated NCM460-control cells. Again, both NCM460-MyD88-KD and NCM460-TRIF-KD cells exhibited almost completely inhibited IL-17C protein production in response to flagellin (Fig. 2B). Together, these data clearly showed that flagellin stimulation induced both IL-17C mRNA expression and IL-17C protein production in nontransformed human colonic epithelial cells.

Despite the fact that TLR5 is a specific pattern recognition receptor to bacterial flagellin at the plasma membrane, NLR4 (known as Ipaf) is also known to recognize flagellin in the cytosolic region of myeloid cells (e.g., macrophages) (Franchi and others 2006; Miao and others 2007). Although we believe that TLR5 is an exclusive receptor that specifically recognizes flagellin in NCM460 cells, it may be necessary to show that flagellin-elicited IL-17C expression is specifically mediated by TLR5 in these cells. To address this, TLR5-knock-down NCM460 (NCM460-TLR5-KD) (Rhee and others 2006) and its control cells were stimulated with flagellin, followed by evaluating IL-17C expression. Notably, silencing TLR5 completely blocked flagellin-induced IL-17C mRNA and protein expression in NCM460-TLR5-KD cells, while control cells strongly induced IL-17C gene expression on flagellin (Fig. 2D, E).

Taken together, these data demonstrate that flagellin stimulation elicits IL-17C expression in a TLR5-specific manner, and moreover, TLR5-interacting adaptor molecules, MyD88 and TRIF, participate in mediating TLR5-elicited IL-17C expression.

Flagellin-induced IL-17C expression is extended to other intestinal epithelial cells, DLD-1 and HT-29

Given that robust IL-17C expression by TLR5 activation was observed in NCM460 cells, which originated from normal human colon (Moyer and others 1996; Kumar and others 1997), we next tested whether TLR5-induced IL-17C expression could be observed in other types of human intestinal epithelial cells. Since it was recently suggested that IL-17C mRNA expression was enhanced in human colon adenocarcinoma SW480 and HCT-15 cells stimulated with flagellin, we tested DLD-1 cells which are human colon adenocarcinoma cells, and TLR5-knock-down DLD-1 (DLD-1-TLR5-KD) and its control (DLD-1-control) cells (Rhee and others 2008) were stimulated with or without flagellin. Notably, TLR5 activation

by flagellin strongly induced IL-17C expression in DLD-1-control cells relative to vehicle-treated DLD-1-control cells. However, flagellin-induced IL-17C expression was completely inhibited in DLD-1-TLR5-KD cells (Fig. 3A).

Furthermore, we tested whether another type of human colon adenocarcinoma cell line, HT-29 cells, could also express IL-17C in response to flagellin. Similar to DLD-1 and NCM460 cells, strong IL-17C expression was measured in HT-29 cells on flagellin stimulation compared with vehicle-treated HT-29 cells (Fig. 3B).

Together, these data show that, in addition to NCM460 cells, TLR5 activation induces IL-17C expression in other types of human intestinal epithelial cells, DLD-1 and HT-29. Thus, TLR5-induced IL-17C expression appears to be one of the general outcomes of TLR5 activation at least in human intestinal epithelial cells. It should be noted that TLR5-silenced DLD-1 cells failed to induce IL-17C expression on flagellin. Together with the data (Fig. 2C), this result further demonstrates that flagellin-induced IL-17C expression is specifically mediated by TLR5.

IL-17C expression is elevated in human IBD patients

A family of IL-17 consists of crucial inflammatory cytokines that regulate the pathophysiology of various inflammatory diseases. IBD represented by UC and CD is a representative inflammatory disease in a human bowel system in which intestinal epithelial cells are continuously in contact with gut microbes. Our data showed that flagellin recognition by TLR5 elicited robust IL-17C expression in intestinal epithelial cells. In this context, we hypothesized that IL-17C expression would be augmented in IBD patients' tissues. To test this, we evaluated the expression of IL-17 family members in the intestinal tissues from human IBD patients characterized into UC and CD. We found that IL-17C expression was remarkably enhanced in UC patients relative to normal control subjects (Fig. 4A). However, the expression level of IL-17C in CD patients was not significantly changed compared with normal subjects. In addition, the expression of IL-17A and IL-17F were substantially elevated in both UC and CD patients compared with control subjects (Fig. 4B, E), while the expression levels of IL-17B and IL-17D were similar in the tissues from IBD patients and normal subjects (Fig. 4C, D). In light of enhanced inflammatory responses in inflamed tissues, CD and UC tissues were featured with an augmented pro-inflammatory cytokine, IL-1 β , expression, compared with normal tissues (Fig. 4F).

These data suggest that the expression of IL-17 family members could be associated with the development and progress of the intestinal inflammation. Given the facts that IL-17C expression is up-regulated by flagellin-TLR5 engagement in intestinal epithelial cells and the intestinal tissues from UC patients is characterized by enhanced IL-17C expression, our study suggests that, by enhancing IL-17C expression in intestinal epithelial cells, TLR5-mediated microbial recognition could play an important role in regulating the pathophysiology of intestinal inflammatory diseases.

Discussion

Through microarray analysis, we found that TLR5 activation by flagellin elicited potent IL-17C protein production

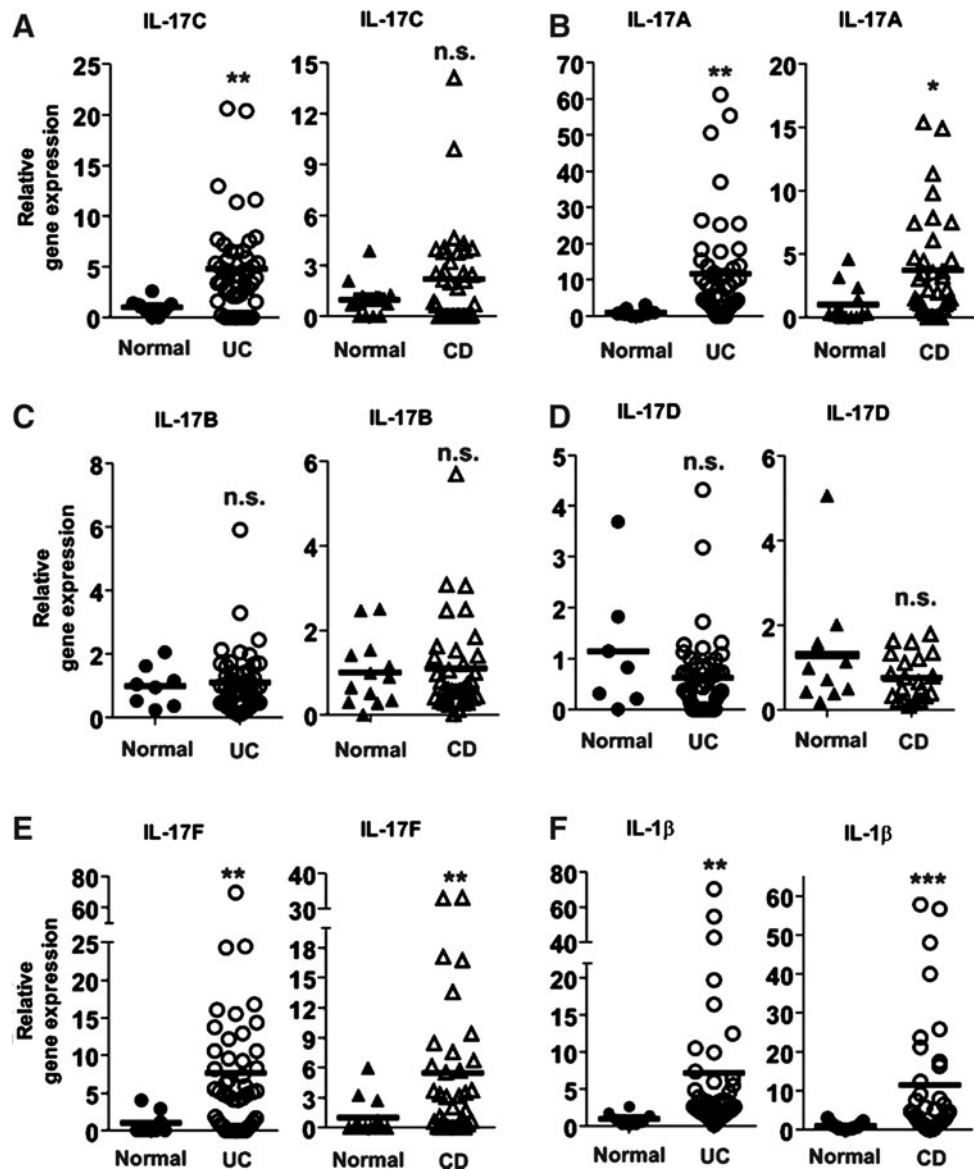


FIG. 4. IL-17C expression is augmented in ulcerative colitis patients. (A–F) We performed qPCR to evaluate the expression of IL-17 family members (A–E) and IL-1 β (F) in human IBD (including UC and CD) patients' tissues. The horizontal bar represents the mean values for each group. Statistical significance was tested by the Mann–Whitney *U* test with normal ($n=8$) versus UC ($n=46$) and normal ($n=13$) versus CD ($n=34$). * $P<0.05$; ** $P<0.01$; *** $P<0.001$; n.s., not significant. UC, ulcerative colitis; CD, Crohn's disease; IBD, inflammatory bowel disease.

and IL-17C mRNA expression in nontransformed human intestinal epithelial cells. Moreover, flagellin-induced IL-17C expression was also observed in other intestinal epithelial cells such as DLD-1 and HT-29 cells. It is noticeable that flagellin stimulation induced more potent IL-17C mRNA expression in NCM460 (fold change ≥ 150) cells than DLD-1 (fold change ≥ 11) or HT-29 (fold change ≥ 38) cells, compared with corresponding vehicle-treated control cells (Figs. 2 and 3). Similarly, while flagellin stimulation elicited robust IL-17C protein production in NCM460 cells, we could not measure IL-17C protein production in flagellin-treated DLD-1 and HT-29 cells. These data indicate that with regard to flagellin-induced IL-17C expression, NCM460 cells are more sensitive to flagellin stimulation than DLD-1 and HT-29 cells. Although originally developed from human colon tissues, DLD-1 and HT-29 cells are adenocarcinoma cells from terminal colon cancer patients and, therefore, harbor various oncogenic mutations such as K-Ras (Rhee and others 2008), p53 (Rodrigues and others 1990; Ray and others 2001), and APC mutation (Rowan and others 2000), which may compound TLR5-induced responses. In contrast, NCM460 cells

are developed from normal human colon (Moyer and others 1996; Kumar and others 1997), and, therefore, NCM460 cells have many advantages compared with adenocarcinoma cell lines in response to flagellin, resulting in potent cytokine gene expression. This might be the reason that we were able to measure TLR5-induced IL-17C protein production in NCM460 cells, but not in DLD-1 and HT-29 cells, while IL-17C mRNA expression by TLR5 activation was observed in all these cells.

The list of genes whose expression levels were altered (fold change ≥ 2 , $P<0.05$) by flagellin stimulation was obtained by analyzing the microarray data after making a comparison of vehicle- and flagellin-treated NCM460-control cells. Then, to obtain IL-17C interactome profiling in the mammalian system, we performed IPA Core Analysis to interpret the microarray data in the context of biological functions, pathways, and networks. The most significant IPA-generated gene networks were presented in Fig. 5. The networks suggested NF κ B and IRF1 as the transcription factors that would be possibly involved in regulating the expression of IL-17C by TLR5 activation. Moreover,

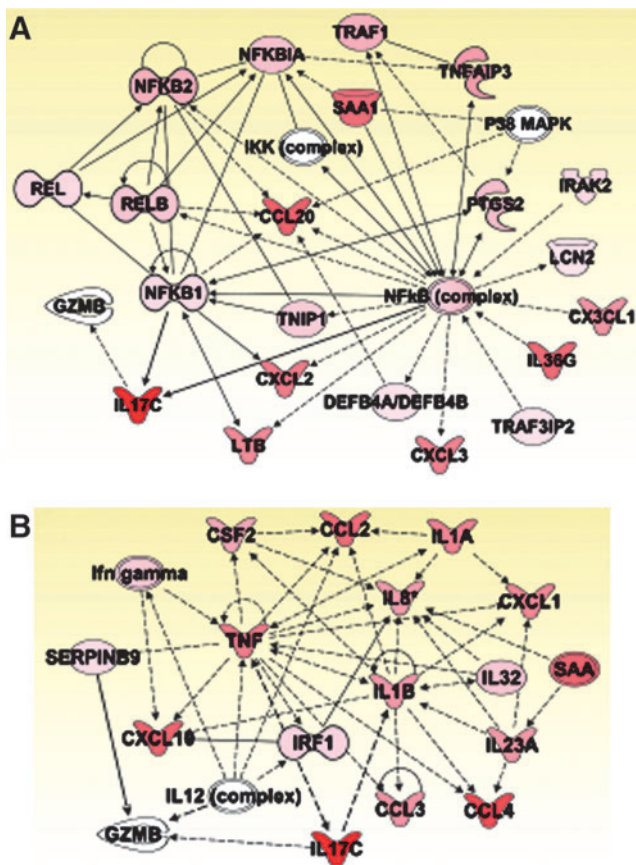


FIG. 5. IPA generates IL-17C interactome. (A, B) A list of differentially expressed genes was obtained by comparing the full microarray data between NCM460-control+Veh. and NCM460-control+Fla. With the dataset, IL-17C regulatory interaction network mapping in the mammalian system was carried out with IPA, providing 2 potential networks linked with IL-17C. Solid and dashed lines represent direct interactions and indirect interactions, respectively. IPA, Ingenuity Pathway Analysis CCL2, chemokine (C-C motif) ligand 2; CCL3, chemokine (C-C motif) ligand 3; CCL4, chemokine (C-C motif) ligand 4; CCL20, chemokine (C-C motif) ligand 20; CSF2, colony stimulating factor 2; CX3CL1, chemokine (C-X3-C motif) ligand 1; CXCL1, chemokine (C-X-C motif) ligand 1; CXCL2, chemokine (C-X-C motif) ligand 2; CXCL3, chemokine (C-X-C motif) ligand 3; DEFBA4, defensin beta 4A; DEFBA4B, defensin beta 4B; IFN gamma, interferon gamma; IKK, inhibitor kappa B kinase; IL1A, interleukin-1 alpha; IL1B, interleukin-1 beta; IL8, interleukin 8; IL12, interleukin 12; IL23A, interleukin 23, alpha, subunit p19; IL32, interleukin 32; IL36G, interleukin 36 gamma; IRAK2, interleukin-1 receptor-associated kinase-like 2; IRF1, interferon regulatory factor 1; GZMB, granzyme B; LCN2, lipocalin-2; LTB, lymphotoxin-beta; MAPK, mitogen-activated protein kinases; NF- κ B, nuclear factor kappa B; NFKB1, nuclear factor kappa B (p105/p50); NFKB1A, inhibitor kappa B alpha (I κ B α); PTGS2, prostaglandin-endoperoxide synthase 2; REL, nuclear factor kappa B (c-Rel); RELB, nuclear factor kappa B (RelB); SAA, serum amyloid A; SERPINB9, serpin peptidase inhibitor, clade B, member 9; TNF, tumor necrosis factors; TNFAIP3, tumor necrosis factor, alpha-induced protein 3; TRAF1, tumor necrosis factor receptor-associated factor 1; TRAF3IP2, tumor necrosis factor receptor-associated factor 3 interacting protein 2.

biological pathways algorithmically based on their connectivity implied that granzyme B (GZMB) and an inflammatory cytokine IL-1 β may have a significant biological relationship with IL-17C. Since recent studies suggested that IL-17C binds to a receptor complex of IL-1RA and IL17-RE (Ramirez-Carrozzi and others 2011; Song and others 2011), it would be worthwhile studying whether IL-17C stimulation alters the expression of GZMB or IL-1 β . Thus, the network analysis confers a clue to speculate significant biological relationships between IL-17C and related molecules.

Many studies indicated that a family of IL-17 cytokine plays an essential role in host defense and various inflammatory diseases, including psoriasis, RA, asthma, or IBD (Pappu and others 2011). While IL-17C expression in IBD remained to be examined, in agreement with our data (Fig. 4B, E), elevated IL-17A and F expressions were indeed previously shown in IBD patients (Fujino and others 2003; Seiderer and others 2008). Since clinical trials conducted with humanized IL-17A antibody showed that the neutralization of IL-17A could provide therapeutic benefits to psoriasis or RA or uveitis patients (Genovese and others 2010; Hueber and others 2010), investigating the expression of IL-17 in IBD would provide important information on a pro-inflammatory cytokine profile of the disease.

Since our study demonstrated that TLR5 activation by flagellin elicited strong IL-17C expression, which is a crucial cytokine in inflammatory disease activity, it would be reasonable to consider TLR5-induced events in pro-inflammatory responses. However, the impacts of TLR5-induced responses in the intestine appear to be controversial. Dr. Gewirtz's group reported that 10% of TLR5^{-/-} mice (generated by Dr. Akira) exhibited spontaneous colitis in a TLR4-dependent manner (Vijay-Kumar and others 2010; Vijay-Kumar and others 2007), suggesting that TLR5 may protect intestinal mucosa from inflammation. However, spontaneous colitis in TLR5^{-/-} mice was not observed in rederived TLR5^{-/-} mice (Vijay-Kumar and others 2010), implying that the incidence of spontaneous colitis in TLR5^{-/-} mice is caused by unique commensal microflora, which may be dependent on a housing environment. They also suggested that TLR5^{-/-} mice had a metabolic syndrome, making the mice obese (Vijay-Kumar and others 2010). On the other hand, Dr. McSorley's group recently demonstrated that TLR5^{-/-} mice developed neither spontaneous colitis nor metabolic defects (Letran and others 2011). In the study, TLR5^{-/-} mice were simultaneously maintained in 2 independent animal facilities, and they concluded that TLR5^{-/-} mice developed neither spontaneous colitis, nor metabolic defects. Moreover, the other line of TLR5^{-/-} mice independently generated by Dr. Flavell's group has a normal phenotype without any obvious abnormalities (Feuillet and others 2006). Collectively, these studies demonstrate that spontaneous colitis and metabolic deficiency are not an obligatory phenotype of TLR5^{-/-} mice (Feuillet and others 2006; Letran and others 2011). Meanwhile, several studies suggested that flagellin-TLR5 engagement were able to promote intestinal inflammation (Rhee and others 2005; Sitaraman and others 2005; Targan and others 2005; Gewirtz and others 2006; Carvalho and others 2008; Ivison and others 2009), and the level of serum antibody against flagellin is elevated in IBD patients (Lodes and others 2004; Yu and others 2011), while a few studies indicate that flagellin may protect mucosal epithelium against mucosal insults (e.g., radiation)

(Burdelya and others 2008; Vijay-Kumar and others 2008). Therefore, it still remains to be studied whether TLR5-induced responses provoke inflammatory impacts in the intestine. Since our study shows that flagellin-TLR5 engagement elicits potent IL-17C expression in intestinal epithelial cells, however, our finding may suggest a possibility that TLR5 activation could participate in evoking inflammatory impacts in the gut. Thus, our study may provide an extensive insight into understanding an impact of microbial recognition by TLR5 in the gut.

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Author Disclosure Statement

No competing financial interests exist.

References

- Blohmke CJ, Victor RE, Hirschfeld AF, Elias IM, Hancock DG, Lane CR, Davidson AG, Wilcox PG, Smith KD, Overhage J, Hancock RE, Turvey SE. 2008. Innate immunity mediated by TLR5 as a novel antiinflammatory target for cystic fibrosis lung disease. *J Immunol* 180(11):7764–7773.
- Burdelya LG, Krivokrysenko VI, Tallant TC, Strom E, Gleiberman AS, Gupta D, Kurnasov OV, Fort FL, Osterman AL, Didonato JA, Feinstein E, Gudkov AV. 2008. An agonist of toll-like receptor 5 has radioprotective activity in mouse and primate models. *Science* 320(5873):226–230.
- Carvalho FA, Barnich N, Sauvanet P, Darcha C, Gelot A, Darfeuille-Michaud A. 2008. Crohn's disease-associated *Escherichia coli* LF82 aggravates colitis in injured mouse colon via signaling by flagellin. *Inflamm Bowel Dis* 14(8):1051–1060.
- Choi YJ, Im E, Chung HK, Pothoulakis C, Rhee SH. 2010a. TRIF Mediates Toll-like Receptor 5-induced Signaling in Intestinal Epithelial Cells. *J Biol Chem* 285(48):37570–37578.
- Choi YJ, Im E, Pothoulakis C, Rhee SH. 2010b. TRIF modulates TLR5-dependent responses by inducing proteolytic degradation of TLR5. *J Biol Chem* 285(28):21382–21390.
- Dorsam ST, Vomhof-Dekrey E, Hermann RJ, Haring JS, Van der Steen T, Wilkerson E, Boskovic G, Denvir J, Dementieva Y, Primerano D, Dorsam GP. 2010. Identification of the early VIP-regulated transcriptome and its associated, interactome in resting and activated murine CD4 T cells. *Mol Immunol* 47(6):1181–1194.
- Feuillet V, Medjane S, Mondor I, Demaria O, Pagni PP, Galan JE, Flavell RA, Alexopoulou L. 2006. Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria. *Proc Natl Acad Sci U S A* 103(33):12487–12492.
- Franchi L, Amer A, Body-Malapel M, Kanneganti TD, Ozoren N, Jagirdar R, Inohara N, Vandenabeele P, Bertin J, Coyle A, Grant EP, Nunez G. 2006. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nat Immunol* 7(6):576–582.
- Fujino S, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y, Bamba T, Fujiyama Y. 2003. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* 52(1):65–70.
- Gaffen SL. 2011. Recent advances in the IL-17 cytokine family. *Curr Opin Immunol* 23(5):613–619.
- Genovese MC, Van den Bosch F, Roberson SA, Bojin S, Biagini IM, Ryan P, Sloan-Lancaster J. 2010. LY2439821, a humanized anti-interleukin-17 monoclonal antibody, in the treatment of patients with rheumatoid arthritis: a phase I randomized, double-blind, placebo-controlled, proof-of-concept study. *Arthritis Rheum* 62(4):929–939.
- Gewirtz AT, Vijay-Kumar M, Brant SR, Duerr RH, Nicolae DL, Cho JH. 2006. Dominant-negative TLR5 polymorphism reduces adaptive immune response to flagellin and negatively associates with Crohn's disease. *Am J Physiol Gastrointest Liver Physiol* 290(6):G1157–G1163.
- Huang W, Na L, Fidel PL, Schwarzenberger P. 2004. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. *J Infect Dis* 190(3):624–631.
- Hueber W, Patel DD, Dryja T, Wright AM, Koroleva I, Bruin G, Antoni C, Draelos Z, Gold MH, Durez P, Tak PP, Gomez-Reino JJ, Foster CS, Kim RY, Samson CM, Falk NS, Chu DS, Callanan D, Nguyen QD, Rose K, Haider A, Di Padova F. 2010. Effects of AIN457, a fully human antibody to interleukin-17A, on psoriasis, rheumatoid arthritis, and uveitis. *Sci Transl Med* 2(52):52ra72.
- Im E, Choi YJ, Pothoulakis C, Rhee SH. 2009. Bacillus polyfermenticus ameliorates colonic inflammation by promoting cytoprotective effects in colitic mice. *J Nutr* 139(10):1848–1854.
- Iverson SM, Himmel ME, Hardenberg G, Wark PA, Kifayet A, Levings MK, Steiner TS. 2010. TLR5 is not required for flagellin-mediated exacerbation of DSS colitis. *Inflamm Bowel Dis* 16(3):401–409.
- Kao CY, Kim C, Huang F, Wu R. 2008. Requirements for two proximal NF-kappaB binding sites and IkappaB-zeta in IL-17A-induced human beta-defensin 2 expression by conducting airway epithelium. *J Biol Chem* 283(22):15309–15318.
- Kumar CK, Moyer MP, Dudeja PK, Said HM. 1997. A protein-tyrosine kinase-regulated, pH-dependent, carrier-mediated uptake system for folate in human normal colonic epithelial cell line NCM460. *J Biol Chem* 272(10):6226–6231.
- Letran SE, Lee SJ, Atif SM, Flores-Langarica A, Uematsu S, Akira S, Cunningham AF, McSorley SJ. 2011. TLR5-deficient mice lack basal inflammatory and metabolic defects but exhibit impaired CD4 T cell responses to a flagellated pathogen. *J Immunol* 186(9):5406–5412.
- Li C, Wong WH. 2001. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci U S A* 98(1):31–36.
- Li X, Hansen PA, Xi L, Chandraratna RA, Burant CF. 2005. Distinct mechanisms of glucose lowering by specific agonists for peroxisomal proliferator activated receptor gamma and retinoic acid X receptors. *J Biol Chem* 280(46):38317–38327.
- Lodes MJ, Cong Y, Elson CO, Mohamath R, Landers CJ, Targan SR, Fort M, Hershberg RM. 2004. Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest* 113(9):1296–1306.
- Miao EA, Andersen-Nissen E, Warren SE, Aderem A. 2007. TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system. *Semin Immunopathol* 29(3):275–288.
- Moyer MP, Manzano LA, Merriman RL, Stauffer JS, Tanzer LR. 1996. NCM460, a normal human colon mucosal epithelial cell line. *In Vitro Cell Dev Biol Anim* 32(6):315–317.
- Pappu R, Ramirez-Carrozzi V, Sambandam A. 2011. The interleukin-17 cytokine family: critical players in host defence and inflammatory diseases. *Immunology* 134(1):8–16.
- Peric M, Koglin S, Kim SM, Morizane S, Besch R, Prinz JC, Ruzicka T, Gallo RL, Schaubert J. 2008. IL-17A enhances vitamin D3-induced expression of cathelicidin antimicrobial peptide in human keratinocytes. *J Immunol* 181(12):8504–8512.
- Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Lim HK, Migaud M, Israel L, Chrabieh M, Audry M, Gumbleton M, Toulon A, Bodemer C, El-Baghdadi J, Whitters M, Paradis T,

- Brooks J, Collins M, Wolfman NM, Al-Muhsen S, Galicchio M, Abel L, Picard C, Casanova JL. 2011. Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science* 332(6025):65–68.
- Ramirez-Carrozzi V, Sambandam A, Luis E, Lin Z, Jeet S, Lesch J, Hackney J, Kim J, Zhou M, Lai J, Modrusan Z, Sai T, Lee W, Xu M, Caplazi P, Diehl L, de Voss J, Balazs M, Gonzalez L, Jr., Singh H, Ouyang W, Pappu R. 2011. IL-17C regulates the innate immune function of epithelial cells in an autocrine manner. *Nat Immunol* 12(12):1159–1166.
- Ray RM, McCormack SA, Johnson LR. 2001. Polyamine depletion arrests growth of IEC-6 and Caco-2 cells by different mechanisms. *Am J Physiol Gastrointest Liver Physiol* 281(1):G37–G43.
- Rhee SH, Im E, Pothoulakis C. 2008. Toll-like receptor 5 engagement modulates tumor development and growth in a mouse xenograft model of human colon cancer. *Gastroenterology* 135(2):518–528.
- Rhee SH, Im E, Riegler M, Kokkotou E, O'Brien M, Pothoulakis C. 2005. Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. *Proc Natl Acad Sci U S A* 102(38):13610–13615.
- Rhee SH, Keates AC, Moyer MP, Pothoulakis C. 2004. MEK is a key modulator for TLR5-induced interleukin-8 and MIP3 α gene expression in non-transformed human colonic epithelial cells. *J Biol Chem* 279(24):25179–25188.
- Rhee SH, Kim H, Moyer MP, Pothoulakis C. 2006. Role of MyD88 in phosphatidylinositol 3-kinase activation by flagellin/toll-like receptor 5 engagement in colonic epithelial cells. *J Biol Chem* 281(27):18560–18568.
- Rodrigues NR, Rowan A, Smith ME, Kerr IB, Bodmer WF, Gannon JV, Lane DP. 1990. p53 mutations in colorectal cancer. *Proc Natl Acad Sci U S A* 87(19):7555–7559.
- Rowan AJ, Lamlum H, Ilyas M, Wheeler J, Straub J, Papadopoulou A, Bicknell D, Bodmer WF, Tomlinson IP. 2000. APC mutations in sporadic colorectal tumors: A mutational “hot-spot” and interdependence of the “two hits”. *Proc Natl Acad Sci U S A* 97(7):3352–3357.
- Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, Fujikado N, Kusaka T, Kubo S, Chung SH, Komatsu R, Miura N, Adachi Y, Ohno N, Shibuya K, Yamamoto N, Kawakami K, Yamasaki S, Saito T, Akira S, Iwakura Y. 2010. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. *Immunity* 32(5):681–691.
- Schaefer TM, Desouza K, Fahey JV, Beagley KW, Wira CR. 2004. Toll-like receptor (TLR) expression and TLR-mediated cytokine/chemokine production by human uterine epithelial cells. *Immunology* 112(3):428–436.
- Seiderer J, Elben I, Diegelmann J, Glas J, Stallhofer J, Tillack C, Pfennig S, Jurgens M, Schmechel S, Konrad A, Goke B, Ochsenkuhn T, Muller-Myhsok B, Lohse P, Brand S. 2008. Role of the novel Th17 cytokine IL-17F in inflammatory bowel disease (IBD): upregulated colonic IL-17F expression in active Crohn's disease and analysis of the IL17F p.His161Arg polymorphism in IBD. *Inflamm Bowel Dis* 14(4):437–445.
- Sitaraman SV, Klapproth JM, Moore DA, 3rd, Landers C, Targan S, Williams IR, Gewirtz AT. 2005. Elevated flagellin-specific immunoglobulins in Crohn's disease. *Am J Physiol Gastrointest Liver Physiol* 288(2):G403–G406.
- Song X, Zhu S, Shi P, Liu Y, Shi Y, Levin SD, Qian Y. 2011. IL-17RE is the functional receptor for IL-17C and mediates mucosal immunity to infection with intestinal pathogens. *Nat Immunol* 12(12):1151–1158.
- Targan SR, Landers CJ, Yang H, Lodes MJ, Cong Y, Papadakis KA, Vasiliauskas E, Elson CO, Hershberg RM. 2005. Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. *Gastroenterology* 128(7):2020–2028.
- Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, Sitaraman SV, Knight R, Ley RE, Gewirtz AT. 2010. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. *Science* 328(5975):228–231.
- Vijay-Kumar M, Aitken JD, Sanders CJ, Frias A, Sloane VM, Xu J, Neish AS, Rojas M, Gewirtz AT. 2008. Flagellin treatment protects against chemicals, bacteria, viruses, and radiation. *J Immunol* 180(12):8280–8285.
- Vijay-Kumar M, Sanders CJ, Taylor RT, Kumar A, Aitken JD, Sitaraman SV, Neish AS, Uematsu S, Akira S, Williams IR, Gewirtz AT. 2007. Deletion of TLR5 results in spontaneous colitis in mice. *J Clin Invest* 117(12):3909–3921.
- Yu JE, De Ravin SS, Uzel G, Landers C, Targan S, Malech HL, Holland SM, Cao W, Harpaz N, Mayer L, Cunningham-Rundles C. 2011. High levels of Crohn's disease-associated anti-microbial antibodies are present and independent of colitis in chronic granulomatous disease. *Clin Immunol* 138(1):14–22.

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