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Establishment of a New Culture Model of Intestinal Inflammation: Autonomous Cure of Damaged Human Colon Epithelial FPCK-1-1 Cells

Akira Tominaga[1*], Yuko Konishi[2] and Takahiro Taguchi[1]
[1]Laboratory of Human Health and Medical Science, Graduate School of Kuroshio Science, and Department of Molecular and Cellular Biology, Kochi Medical School, Kochi University,
[2]Life and Functional Material Section, Science Research Center, Kochi University, Kohasu, Okoh-cho, Nankoku, Kochi 783-8505, Japan,

Abstracts

We have established a new *in vitro* co-culture model of intestinal inflammation, in which the monolayer of human colon epithelial FPCK-1-1 cells showed the decrease of transepithelial electrical resistance (TER) and the increase in FITC-dextran permeability by co-culturing with THP-1 cells stimulated with peptidoglycans (PGN) of *Staphylococcus aureus* (*S. aureus*). In the previous model, Caco-2 cells were co-cultured with THP-1 cells stimulated with phorbol myristate acetate (PMA). The combination and condition of this co-culture system are different from actual events occurring *in vivo* in many ways. Caco-2 cells are adenocarcinoma cells with tetraploid nuclei. In contrast, FPCK-1-1 cells, which have neither a point mutation on codon 12 of K-ras gene nor gene amplification of myc, c-H-ras, and/or c-K-ras genes. It is reported that PGN from *S. aureus* causes inflammation and organ injury by inducing TNF-α, IL-6, and IL-10 Using a newly established model, we found Spirulina complex polysaccharides not only inhibit the injuring of epithelial cells but also recover the decreased TER of FPCK-1-1 monolayer cells after they were damaged by co-culturing with PGN-stimulated THP-1 cells. These results suggest that Spirulina complex polysaccharides have both defensive and therapeutic potency. Recovery of the expression level of claudin-2 is a sign of the recovery from the early stage of injury of the tight junction, but not from the late phase injured status in terms of TER. In contrast, the mucosa reconstitution may be involved in the recovery from the late phase damage. Furthermore, we found that the curative effect of Spirulina complex polysaccharides on the early and the late phase damage of human colon epithelial cells was abolished by anti-IL-22 antibodies, suggesting that IL-22 is necessary for the recovery from these damages. Signals of Spirulina complex polysaccharides through both TLR2 and TLR4 are indispensable for the production of IL-22. We found that IL-22 was produced from FPCK-1-1 cells in response to Spirulina complex polysaccharides in the absence of THP-1 cells. Furthermore, we found that the stimulation with Spirulina complex polysaccharides can be replaced with the addition of IL-1 + IL-6 + IL-23. In conclusion, our results suggest that damage to human colon epithelial FPCK-1-1 cells caused by PGN-stimulated THP-1 cells are cured, at least in part, by their own production of IL-22.

Key words: human colon epithelial cells, FPCK-1-1, IL-22, TLR2, TLR4

*corresponding author : e-mail tominaga@kochi-u.ac.jp

1. #Introduction

Inflammation of the intestine is caused by infectious, genetic, immunological, and environmental factors. Although the inflamed, injured area heals and is replaced by the regenerated tissue, excess mucosal inflammation of the gut has been reported to induce ulcerative colitis or Crohn's disease (Baumagart and Carding, 2007; Kaser et al., 2010). Continuous inflammation is associated with an increased risk for colon cancer. It also promotes the propagation, metastasis, and infiltration of cancer. Therefore, it is important to regulate inflammation to maintain human health.

In this study, we focused on the establishment of a new inflammation model using an intestinal epithelial cell line with quasi-normal chromosomes, FPCK-1-1 cells from a tubular adenoma developing in a male familial polyposis coli patient (Kawaguchi et al., 1991).

Although the *in vitro* model of inflammatory bowel disease has been reported on, it is using human colon carcinoma Caco-2 co-culturing with THP-1 cells stimulated with phorbol myristate acetate (PMA) (Watanabe et al., 2004) and the condition of this combination is different from actual events occurring in vivo in many ways. Caco-2 cells activated with PMA resulted in the disassembly of the adherence junction (Barbosa et al., 2003). Caco-2 cell are adenocarcinoma cells with tetraploid nuclei (Fogh et al., 1977). In contrast, FPCK-1-1 cells, which have neither a point mutation on codon 12 of the K-ras gene nor gene amplification of myc, c-H-ras, and/or c-K-ras genes (Kawaguchi et al., 1991). PGN from *Staphylococcus aureus (S. aureus)* causes inflammation and organ injury by inducing TNF-α, IL-6, and IL-10 (Wang et al., 2004). Intramural injection of peptidoglycan-polysaccharide (PG-PS) from *Streptococcus pyogenes (S. pyogenes)* into the distal colon produces a chronic granulomatous colitis in rats (Yamada et al., 1993), and PGN is also known to exist in gut epithelial cells. Compared to the responses in remissive Crohn's disease, responses of peripheral blood mononuclear cells and in particular, lymph node mononuclear cells in active Crohn's disease to the eubacterial cell wall and streptococcal cell wall antigen from *Streptococcus pyogenes* were significantly higher (Klasen et al., 1994).

Here, a new culture model of intestinal inflammation using human colon epithelial FPCK-1-1 cells was established by co-culturing with PGN-stimulated monocytic leukemia THP-1 cells. This co-culture system shows the decrease of TER and the increase in FITC-dextran permeability and is useful to elucidate the mechanism of injury to gut epithelial cells. In addition, using this model, we explored the new substances that have anti-inflammatory effects without having toxicity against FPCK-1-1 cells.

Recently, the effects of anti-allergic substances from marine algae have been studied (Tominaga et al., 2010). Many species of marine algae have been taken as part of the daily diet by cultures around the world. Of the four different algae tested, phenol extracts of Spirulina CPS have an anti-inflammatory effect without showing cytotoxicity.

Spirulina is a traditional food of some Mexican and African peoples. It is a planktonic blue-green algae found in warm water alkaline volcanic lakes. It has a highly unusual nutritional profile and has been used as a nutrient for decades (Belay, 2008)..

IL-22 belongs to the IL-10 family of cytokines and has recently been shown to be preferentially expressed by the Th17 subset (Dumoutier et al., 2000; Pestka et al., 2004). IL-22 targets immune pathways due to the restricted expression of IL-22 receptors on innate cells, such as epithelial cells, keratinocytes, and hepatocytes but not acquired immune cells, including T or B cells (Pestka et al., 2004; Sonnenberg et al., 2011). IL-22 is expressed at barrier surfaces and is engaged in the border patrol in regulating immune responses, inflammation and tissue homeostasis (Sonnenberg et al., 2011). It promotes epithelial cell proliferation, survival and repair in the skin, airway or intestine (Sonnenberg et al., 2011).

Interestingly, recent studies have demonstrated that colonic IL-22 expression is induced in inflammatory bowel disease (IBD)(Andoh et al., 2005). Although a pathogenic role of IL-22 in Crohn's disease has been proposed (Ando et al., 2005; Brand et al., 2006), a regulatory role of IL-22 in IBD has also recently been proposed due to the ability of IL-22 to dampen systemic inflammatory response (Wolk et al., 2007; Sugimoto et al., 2008; Zenewicz et al., 2008) through the

induction of lipopolysaccharide-binding protein. The role of IL-22 in IBD is unclear and still remains to be established.

In this report, we established a new model of epithelial inflammation of the gut consisting of human colon epithelial FPCK-1-1 cells and PGN-stimulated monocytic THP-1 cells; signals through TLR2 and TLR4 in response to Spirulina complex polysaccharides have curative effects on FPCK-1-1 cells damaged by PGN-activated THP-1 cells. This curative effect of Spirulina complex polysaccharides was blocked by anti-IL-22 antibodies, suggesting IL-22 is engaged in the wound healing process of intestinal epithelial cells in an autocrine manner. Even in the absence of THP-1 cells, FPCK-1-1 cells produce IL-22 in response to Spirulina complex polysaccharides and this stimulus was replaced by the addition of IL-1β + IL-6 + IL-23.

2. Materials and Methods

1) Cell lines

Caco-2 cells: human colon carcinoma was purchased from Riken BioResource Center, Tsukuba, Japan (RCB0988). FPCK-1-1 cells are non-tumorigenic and derived from a colonic polyp in a patient with familial adenomatous polyposis (Kawaguchi et al, 1991). Human monocytic leukemia THP-1 cells were purchased from Health Science Research Resources Bank, Japan Health Science Foundation, Osaka, Japan (JCRB0112, Fogh et al., 1977). Caco-2 cells, FPCK-1-1 cells and THP-1 cells were maintained at 37℃ in 5% CO_2 in Dulbecco's-modified Eagle medium (DMEM) supplemented with 8% FCS, 20 U/ml penicillin, 50 μg/ml kanamycin and subcultured on 1.1 cm^2, 0.4 μm pore Transwell permeable supports (Transwell) precoated with equimolar mixture of types I and III collagen (3493, Corning, Ithaca, NY).

2) Reagents

Spirulina pacifica was generously given by Dr. G. Cysewski, Cyanotech Coop. (Kailua-Kona, Hawaii). *Spirulina pacifica* has been selected from a strain of edible *Spirulina (Arthrospira) platensis* since 1984 and expresses different enzymes from the parental strain. Complex polysaccharides (CPS) Westphal fraction (lipopolysaccharide (LPS) fraction) was prepared from *Spirulina pacifica* freeze-dried cells according to the method described (Westphal et al., 1965) by Dr. Satoshi Fukuoka, Health Research Institute, National Institute of Advanced Industrial Scienc, and fucoidanalginic acide and Technology (Takamatsu. Japan). Molecular Mass (MS) of Spirulina CPS is 1284 ~ 28315. Peptidoglycan (PGN) from *Staphylococcus aureus,* alginic acid, fucoidan were purchased Sigma-Aldlich (St Louis, MO). Petalonia polusaccharides (Petalonia PS) were 66% ethanol precipitates from hot water extracts of *Petalonia binghamiae* (Tominaga et al., 2011).

3) Co-culture system and the treatment of intestinal epithelial cells

Early phase damage model: Caco-2 cells or FPCK-1-1 cells were cultured at a density of 2 x 10^5cells /insert in 12 well cell culture inserts described above to form a monolayer. THP-1 cells at a density of 1x10^5/well were cultured in 12 well culture plates (Corning 3513) in the presence of phrobol myristate acetate (PMA 200 nM) or PGN (100 μg/ml) for one day. The cell culture inserts in which the Caco-2 or FPCK-1-1 cells had been cultured for five days were placed in the wells in which the THP-1 cells were cultured together with PGN. After co-culturing for three days, the cell-culture inserts including Caco-2 or FPCK-1-1 cells were removed and served for the assay(Fig. 1). Spirulina CPS, E. coli LPS, anti-TNF-α antibodies were added to the apical side of the co-culture system one day before starting the co-culture (Fig. 1).

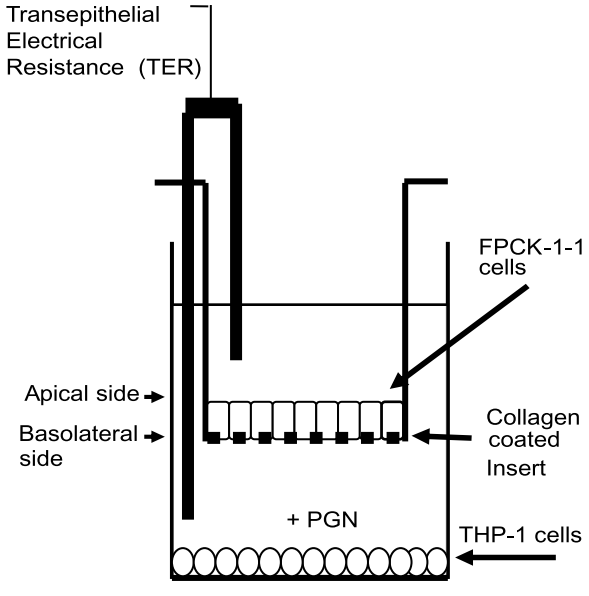


Fig. 1. In vitro co-culture system of FPCK-1-1 cells with PGN-stimulated THP-1 cells. Collagen-coated membrane is made of polytetrafluoroehtylene with pores of 0.4μm. Coated collagen is a mixture of bovine type I and type III collagen. PGN: peptidoglycan prepared from *Staphylococcus aureus*. Co-culture condition is described in Materials and methods.

Late phase damage model: Three days after starting the co-culture of FPCK-1-1 cells with PGN-stimulated THP-1 cells as described above, we confirmed the decrease of transepithelial electrical resistance. After that, various reagents such as Spirulina CPS, fucoidan, alginic acid, Petalonia PS, and E. coli LPS, or IL-22 or anti-IL-22 antibodies were added to the apical side of the co-culture system to observe the effects of these reagents three days after the treatments (Fig. 1).

4) Measurement of Transepithelial Electrical Resistance

The medium in the Transwell was changed 2 hours before measuring transepithelial electrical resistance (TER). The electrode was soaked in 70% ethanol and rinsed with sterile DMEM prior to use. The electrical resistance between the lower compartment (well) and the upper compartment (filter inserts) was measured using an voltmeter Millicell-ERS and an electrode MERSSTX01 (Millipore, Bedford, MA). Triplicate measurements were taken, ensuring that the temperature remained close to 37℃ to avoid alterations in resistance due to temperature changes and the meaning was calculated. TER of the medium alone was 50 - 80 x cm^2,while TER of the FPCK-1-1 monolayers were 600 - 900 x cm^2.

5) Cytokines, antibodies, and ELISA

Recombinant human IL-6 and IL-22 were purchased from PeproTech Inc. (Rocky Hill, NJ). Recombinant human IL-1β and IL-23 were purchased from Human Zyme Inc. (Chicago, IL). IL-22 was measured by ELISA kit from R&D Systems, Inc. (Minneapolis, MN).

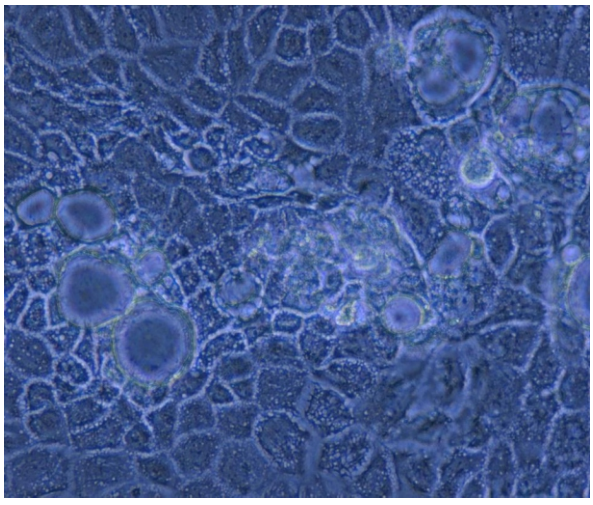
Recombinant human TNF-α was purchased from e-Bioscience (San Diego, CA). TNF-αwas measured by ELISA kit from e-Bioscience (San Diego, CA). To examine the expression of claudins -2, -4, and -8, Western blotting was performed using rabbit anti-human claudins-2, -4, and -8 antibodies (Ab) kindly provided by Dr. Mikio Furuse. (Kobe University, Kobe, Japan). Anti-human beta-actin, TLR2, and TLR4 antibodies were purchased (Biolegend, San Diego, CA). Anti-human TNF-α and anti-human IL-22 antibodies were obtained from R&D Systems, Inc. (Minneapolis, MN).

3. Results and Discussion

Caco-2 cells are adenocarcinoma cells established from a primary colonic tumor of a 72 year old male with chromosome numbers varying from 91 to 107. Ten common markers were detected i.e., t(1q;?), 10q-, t(11q17q) and seven others. The t(1q17q) and M11 were found in a portion of cells. The ins(2), 10 q-, and t(15q;?) were generally paired, and t(11q;17q) and t(21q;?) were mostly three-copied. Normal N9 was absent, and N21 was lost in some cells. One to four small acrocentric chromosomes were detected. No Y chromosome with bright distal q-band was detected by Q-observation (Fogh et al., 1977, ATCC Cell Lines and Hybridomas, 1994). The doubling time of Caco-2 was reported to be 30 hours (Rousset, et al., 1997).

FPCK-1-1 cells were established from a tubular adenoma developing in a 34-year-old male familial polyposis coli (FPC) patient (Kawaguchi, et al., 1991). FPCK-1-1 has been maintained at a splitting ratio of 1:2 and then 2:3 every three months by detaching adhered cells with dispase. Alcian blue-staining reveals abundant production of mucus. No chromosomal abnormalities were detectable at the 6th passage. No loss of heterozygosity was observed on chromosomes 5, 17p, 18p, and 22q. They could not detect either gene rearrangement or gene amplicication in H-ras, K-ras, myc loci, nor any point mutation in codon 12 of the c-K-ras gene. Although FPCK-1-1 cells grew very slowly (22 passages in 3 years), we maintained these cells once a week by splitting them 1:10 in 8% FCS DMEM high glucose medium. Therefore, we concluded that the FPCK-1-1 cell line was more representative than the Caco-2 cell line in retaining features of adenoma cells at a very early phase.

The microscopic observation revealed that FPCK -1-1 cells form a monolayer consisting of similar size-cells, while Caco-2 cells pile up on a monolayer with irregular cells in size and shape (Fig. 2).



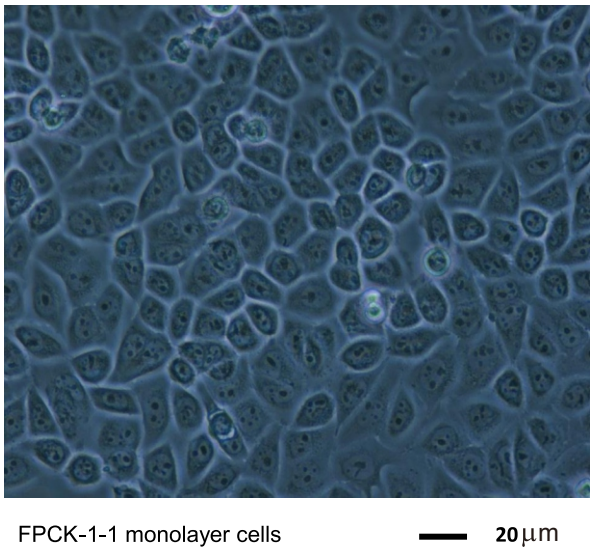


Fig. 2. Microscopic observation of monolayer Caco-2 and FPCK-1-1 cells.

Based on the co-culture model proposed by Watanabe et al. (Watanabe et al., 2004), we used FPCK-1-1 cells instead of Caco-2 cells. We confirmed that TER of a monolayer of Caco-2 cells declined sharply, when THP-1 cells (basolateral side) were stimulated with 200 nM PMA (Fig. 3). However, we could not found any decline of TER of Caco-2, when PGN from *S. aureus* was added instead of PMA (Fig. 3). It was much better using PGN as a stimulant to THP-1 cells, as bacterial PGN is a natural product that causes intestinal inflammation (Klasen et al., 1994).

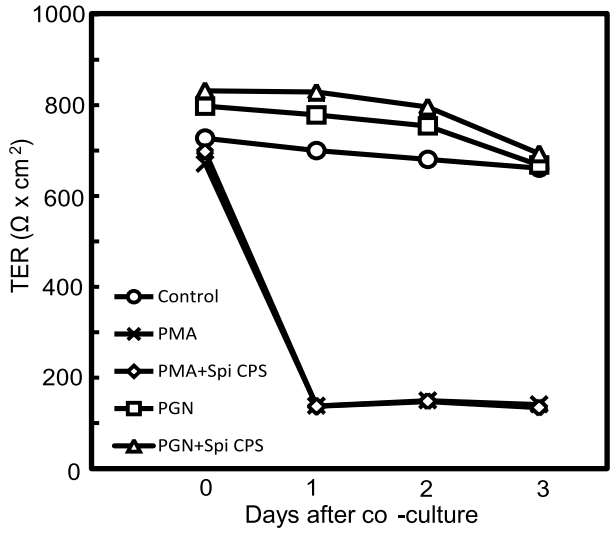


Fig. 3. PMA-stimulated THP-1 cells but not PGN-stimulated THP-1 cells cause damages of Caco-2 monolayer cells.
PMA: phrobol myristate acetate (200 nM), **PGN:** peptidoglycan (100 μg/ml) prepared from *Staphylococcus aureus.* ***Spi*** **CPS:** Complex polysaccharides from *Spirulina pacifica* prepared as described in Materials and methods.

Following that, we used FPCK-1-1 monolayer cells instead of Caco-2 monolayer cells and co-cultured with PGN-stimulated THP-1 cells. PGN-stimulated THP-1 cells reduced the TER of FPCK-1-1 cells to two thirds of that before the co-culture (Fig. 4). Spirulina CPS recovered this reduced TER to the control level (Fig. 4), lower pcmel though it could not recover the damage by PMA (Fig. 4 upper panel).

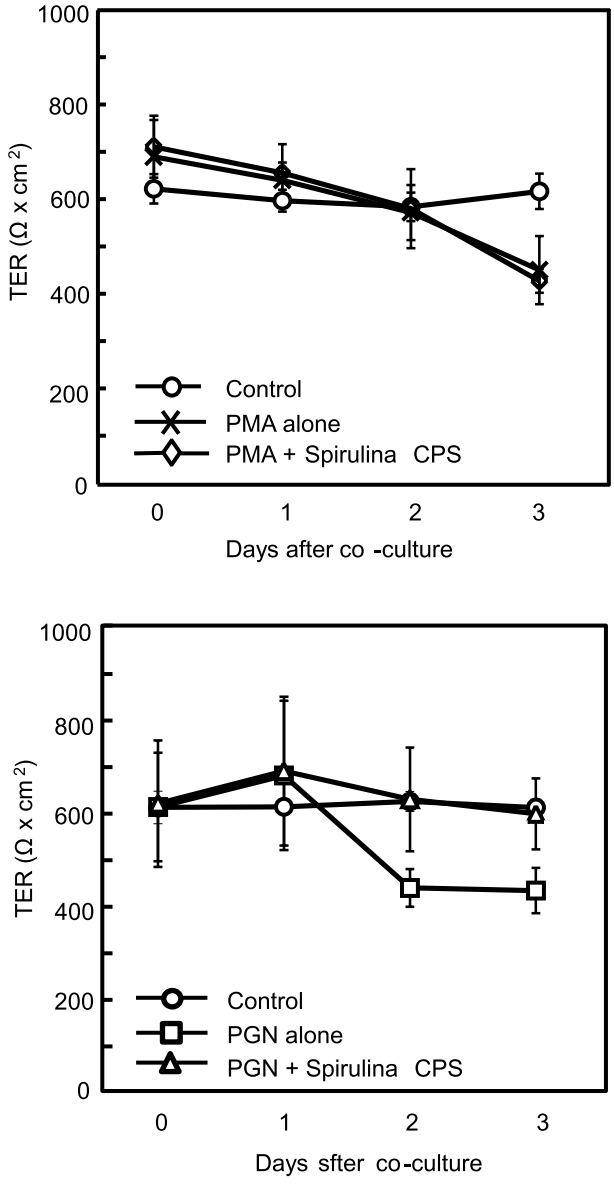


Fig. 4. PGN-stimulated THP-1 cells cause damage of FPCK-1-1 monolayer cells and this damage can be recovered by the addition of *Spirulina pacifica* complex polysaccharides. PGN: peptidoglycan (100 μg/ml) prepared from *Staphylococcus aureus.* ***Spi* CPS:** Complex polysaccharides from *Spirulina pacifica* prepared as described in Materials and methods.

It is known that the intestinal barrier is made of an intact layer of epithelial cells, which is connected by a structure constituted of tight junction. It is reported that pore-forming claudin 2 is up-regulated and sealing claudins 5 and 8 were down-regulated in mild to moderately active Crohn's disease (Zeissig et al., 2007). In our early phase damage model, claudin-2 was decreased and recovered by the addition with Spirulina CPS. Expression levels of claudins 4 and 8 were not changed. This meant that FPCK-1-1 cells had the ability to minimize the damage by decreasing pore-forming claudin 2. In the late phase damage model, however, the level of claudin 2 was never recovered. Instead, our data suggests that FPCK-1-1 cells protect the barrier function by secreting polysaccharides (data not shown). Indeed, it is reported that mice housed under germ-free conditions had an extremely thin adherent colonic mucus layer, but when exposed to bacterial products (PGN or LPS), the thickness of the adherent mucus layer was quickly restored to levels observed in conventionally housed mice (Petersson et al., 2011).

Next, we examined whether IL-22 was effective in recovering the decreased TER of FPCK-1-1 in the present model, because IL-22 ameliorates the intestinal inflammation (Sugimoto et al., 2008), and it has already been reported that IL-22 is engaged in the mucosal wound healing of the intestine (Pickert et al., 2009, Sonnenberg et al., 2011). Indeed, IL-22 could recover the reduced TER of FPCK-1-1 cells caused by co-culturing with PGN-stimulated THP-1 cells (Table 1), and anti-IL-22 antibodies could block the curative effects of Spirulina CPS in this inflammation system. These results suggested that IL-22 was the major factor involved in recovering the TER.

Then, we checked what kind of receptors were responsible for the induction of IL-22 in response to Spirulina CPS. A significant increase in TLR2 expression in the terminal ileum of patients with inactive and active ulcerative colitis against controls has been reported (Frolova et al., 2008), as well as a significant upregulation of TLR4 expression relative to controls in the terminal ileum and rectum of ulcerative colitis patients in remission, and in the terminal ileum of Crohn's disease patients with active disease (Frolova et al., 2008). Netea et al. reported that a mutation in the NOD2 gene associated with Crohn's disease results in defective release of IL-10 from blood mononuclear cells after stimulation with the TLR2 ligands, pepetidoglycan and Pam3Cys-KKKK, but not with bacterial LPS, a TLR4 ligand (Netea et al.,

2004). Expression levels of TLR2 and TLR4 in FPCK-1-1 were low (data not shown). We examined the effects of anti-TLR2 and anti-TLR4 antibodies on the production of IL-22, because Spirulina CPS respond to TLR2 and TLR4 (Tominaga et al., 2010). We found that either anti-TLR2 antibodies or anti-TLR4 antibodies could block the production of IL-22 (Table 2). Next, we examined whether IL-22 was produced from FPCK-1-1 cells. Although IL-22 has been reported to be produced from Th17 cells, NK cells, and dendritic cells (Pickert et al., 2009), we found that intestinal epithelial FPCK-1-1 cells produce IL-22 in response to Spirulina CPS in the absence of PGN-stimulated THP-1 cells. It is reported that IL-23 is essential for the production of IL-22 and that induction of IL-22 was delayed in IL-6 knockout mice in the colon (Zheng et al., 2008). We found that IL-6 and IL-23 inhibited the production of IL-22 by FPCK-1-1 cells in response to Spirulina CPS. Furthermore, we found that IL-1 β + IL-6 + IL-23 induced FPCK-1-1 cells to produce IL-22 in the absence of Spirulina CPS (Table 3).

In conclusion, our results suggest that IL-22 is, at least in part, involved in the wound healing of intestinal epithelial cells by an autocrine system.

Table 1. ***Spirulina*** **CPS recovered decreased TER by PGN-stimulated THP-1 and IL-22 can replace the curative effects of** ***Spirulina*** **CPS in the late phase damage model.**

FPCK-1-1 cells with	**No treat**	**PGN-THP-1**	**PGN-THP-1 +** ***Spi*** **CPS**	**PGN-THP-1 +** ***Spi*** **CPS**	**PGN-THP-1 +** ***Spi*** **CPS**	**PGN-THP-1 + IL-22**
Antibodies				**Control IgG antibodies**	**Anti-IL-22 antibodies**	
TER	+++++ +++++	+++++ +	+++++ +++++	+++++ ++++	+++++ +	+++++ ++++

TER values were expressed as a relative resistance. Although these are the results in the late phase damage model, similar results were obtained in the early phase damage model.

Table 2. **Either anti-TLR2 Ab or anti-TLR4 Ab inhibited the production of IL-22 from FPCK 1_1_cells co cultured with PGN_THP_1 cells.**

FPCK-1-1 cells with	**No**	**PGN-THP-1**	**PGN-THP-1 +** ***Spi*** **CPS**	**PGN-THP-1 +** ***Spi*** **CPS**	**PGN-THP-1 +** ***Spi*** **CPS**	**PGN-THP-1 +** ***Spi*** **CPS**
Antibodies	**No**	**No**	**No**	**Control IgG antibodies**	**Anti-TLR-2 antibodies**	**Anti-TLR4 antibodies**
IL-22 production	+	±	+++++	++++	±	±

Either anti-TLR2 antibodies (Ab) or anti-TLR4 Ab was added in the apical side of the co-culture of FPCK-1-1 cells co-cultured with PGN-THP-1 cells. The relative amount of IL-22 in the apical side was expressed relatively. The average amount of IL-22 in the apical side of the co-culture was 50pg/ml. Without co-culture, the average amount of IL-22 produced from FPCK-1-1 cells was 5 pg/ml.

Table 3. IL-1β+IL-6 + IL-23 induced the production of IL-22 from FPCK-1-1 cells.

	Cytokines	Production of IL22
Medium		+
+ PGN		+
+ *Spi* CPS		+++
+ *Spi* CPS	IL-1β + IL-6 + IL23	+++
+ *Spi* CPS	IL-1β + IL-6	+
+ *Spi* CPS	IL-1β + IL-23	+
+ *Spi* CPS	IL-6 + IL23	+
Medium	IL-1β + IL-6 + IL23	+++

FPCK-1-1 cells were stimulated either with PGN or *Spi* CPS (*Spirulina* complex polysaccharides) together with cytokines described. The final concentration of each cytokine was 4 ng/ml. Concentration of IL-22 in the culture supernatants was measured and expressed as relative amounts. *Spirulina* CPS induce FPCK-1-1 cells to produce about 15 pg/ml IL-22, while background production of IL-22 was about 5 pg/ml.

Acknowledgments

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