Video Article

The *Ex Vivo* Colon Organ Culture and Its Use in Antimicrobial Host Defense Studies

S. M. Nashir Udden[1], Sumyya Waliullah[1], Melanie Harris[1], Hasan Zaki[1]

[1]Department of Pathology, UT Southwestern Medical Center

Correspondence to: Hasan Zaki at Hasan.Zaki@utsouthwestern.edu

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Abstract

The intestine displays an architecture of repetitive crypt structures consisting of different types of epithelial cells, lamina propia containing immune cells, and stroma. All of these heterogeneous cells contribute to intestinal homeostasis and participate in antimicrobial host defense. Therefore, identifying a surrogate model for studying immune response and antimicrobial activity of the intestine in an *in vitro* setting is extremely challenging. *In vitro* studies using immortalized intestinal epithelial cell lines or even primary crypt organoid culture do not represent the exact physiology of normal intestine and its microenvironment. Here, we discuss a method of culturing mouse colon tissue in a culture dish and how this *ex vivo* organ culture system can be implemented in studies related to antimicrobial host defense responses. In representative experiments, we showed that colons in organ culture express antimicrobial peptides in response to exogenous IL-1β and IL-18. Further, the antimicrobial effector molecules produced by the colon tissues in the organ culture efficiently kill *Escherichia coli in vitro*. This approach, therefore, can be utilized to dissect the role of pathogen- and danger-associated molecular patterns and their cellular receptors in regulating intestinal innate immune responses and antimicrobial host defense responses.

Video Link

The video component of this article can be found at https://www.jove.com/video/55347/

Introduction

The intestine represents a dynamic system that acts as a barrier for commensal microorganisms, fights against invading pathogens, and regulates the microbial composition[1]. The intestinal epithelial cells, consisting of enterocytes, goblet cells, Paneth cells and enteroendocrine cells, are the major cell populations that provide host defense responses against intestinal microbiota. The goblet cells produce mucins that create a demilitarized zone on the top of the epithelial layer[2]. The Paneth cells and enterocytes produce antimicrobial peptides, cytokines, and reactive oxygen and nitrogen species that constitute antimicrobial host defense responses and contribute to shaping the intestinal microbial composition[3,4]. In addition to epithelial cells, the immune cells including macrophages, dendritic cells, neutrophils, natural killer cells, lymphocytes, and innate lymphoid cells in the lamina propria and submucosa play a critical role in intestinal antimicrobial host defense responses by producing cytokines, chemokines, and other mediators[5-7]. In order to understand how the mucosal immune system regulates microbiota and provides protection against microbial infection, it is important to consider the complex interaction of the heterogeneous cell populations of the gut. However, an *in vitro* model that encompasses all of the features of the intestine is not available. Therefore, molecular studies on host-pathogen interaction in the intestine are highly challenging.

Over the past few years, several model systems that mimic aspects of the intestinal mucosa have been developed for investigating the pathophysiological processes involved in inflammatory bowel diseases (IBD) and other gastrointestinal disorders[8-14]. Immortalized intestinal epithelial cell lines are often used to study epithelial cell specific responses. However, because of differential gene expression and function in immortalized cells, the data obtained from using those cells do not often match with those observed in *in vivo* studies. Intestinal crypt organoid culture has recently emerged as a potential tool for assessing the response of the intestinal epithelium to different stimuli[13]. In this system, crypt stem cells are allowed to grow and develop a 3D organoid structure. While the organoid culture system is very useful for studying many aspects of the intestinal epithelium, it does not mimic the complex interaction of immune cells, epithelial cells and microbial products. The *ex vivo* culture of the intestinal tissue offers a better representation of *in vivo* host defense responses. In this method, a part of the intestine is cultured in a cell culture plate with appropriate media allowing the different types of cell populations in the intestine to be metabolically active for at least 48 h. Thus, an *ex vivo* culture of the organ can be used to measure the expression of antimicrobial genes and the host defense responses of the intestine to a particular stimulus.

Investigators have been using the *ex vivo* organ culture system to study host defense responses against microbial infection in the intestine[15-21]. We recently adopted the organ culture system to study the role of the inflammasome in antimicrobial host defense responses in mouse colons[22]. The inflammasome is a molecular platform for the activation of caspase-1, which is required for the production of matured IL-1β and IL-18. We showed that IL-1β and IL-18 induce antimicrobial peptides which effectively kill commensal pathobionts such as *E. coli*. This observation

was consistent with increased *E. coli* burden in inflammasome-defective mouse colons[22]. This system therefore can be used to study the role of pattern recognition receptors (PRRs) and other innate immune molecules in intestinal antimicrobial host defense responses as well as pathogenesis of intestinal disorders such as inflammatory bowel disease (IBD) and colorectal cancer (CRC). There are more than 200 IBD susceptibility genes, and mutations in many of these genes are associated with altered microbial composition in the gut. It is of great clinical significance to determine the precise mechanism through which the IBD-susceptibility genes regulate gut microbiota. The overall goal of this method is to introduce a basic protocol of *ex vivo* colon organ culture and demonstrate how this culture method can be used to study antimicrobial host defense responses of the intestine.

Protocol

All experiments described here were performed using 6-8 weeks old male wild-type (C57BL6/J) mice maintained in a specific pathogen free (SPF) facility at the Animal Resource Center (ARC), UT Southwestern Medical Center. All studies were approved by the Institutional Animal Care and Use Committee (IACUC) and were conducted in accordance with the IACUC guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

1. Collection and Preparation of the Colon

1. Euthanize mice with CO_2 asphyxiation followed by cervical dislocation.
2. Spray mice with 70% ethanol and pin limbs to a pinning board keeping the mouse dorsal side down on the board.
3. Using pre-sterilized dissecting scissors and forceps, make a mid-line incision in the peritoneum of the abdomen. Open the abdomen by folding the peritoneum with forceps.
4. Remove the intestine from the abdominal cavity with dissecting forceps and scissors. Separate the colon from the small intestine by cutting at the bottom of the cecum and the other end at the rectum.
5. Place the colon in a sterile petri dish containing ice-cold PBS. Flush the contents of the lumen of the colon with ice-cold PBS using a 20 mL syringe holding a 20 G needle or an oral gavage needle until the stool within the lumen of the colon is completely removed.
6. Cut the colon with scissors longitudinally. Wash the colon by shaking vigorously in ice-cold PBS in a sterile Petri dish.
7. Cut the colon tissue into pieces approximately 1 cm long using a sterile scalpel or scissors.
8. Record the weight of the colon pieces.
   NOTE: All the steps in the Section 1 can be performed either inside or outside of a biological safety cabinet. Once colons are ready for culture, all the steps must be performed inside a biological safety cabinet to maintain sterility.

2. Colon Organ Culture

1. Place a cell strainer (100 µm) on a 6-well cell culture plate. Transfer all of the colon pieces collected from one mouse into the cell strainer.
2. Add 5 mL DMEM/F12 medium containing 5% FBS, Penicillin-Streptomycin (1x), and Gentamycin (20 µg/ml). Completely cover the colon pieces with the media.
3. Incubate for 2 hr at 37 °C in an incubator with 5% CO_2 and 95% air.
4. Lift the cell strainer and aspirate the media.
5. Place the cell strainer back on the well and add 5 ml fresh media without any antibiotics. Lift the cell strainer and aspirate the media.
6. Repeat the above washing step (Step 2.5) twice more (total 3x) to remove all of the residual antibiotics.
7. Transfer the colon pieces from a single cell strainer into a single well of a sterile 12-well cell culture plate.
8. Add DMEM/F12 medium containing 5% FBS (without any antibiotics). Adjust the volume of the medium with the weight of the colon pieces, *e.g.*, 1 ml medium for 100 mg of tissue. Incubate for 12 h at 37 °C in an incubator injecting 5% CO_2 and 95% air.
9. Collect the culture supernatant in a sterile 1.5 ml tube.
10. Centrifuge at 12,000 x g at 4 °C for 5 min. Separate the supernatant into a new 1.5 mL tube for use in the bacteria killing assay and/or other immune assays. The supernatant can be stored at -80 °C until the assay.
11. Collect the colon pieces in a tube for RNA isolation.

3. *E. coli* Killing Assay

1. Inoculate *E. coli* in 5 mL Luria-Bertani (LB) broth in a 15 ml tube and incubate at 37 °C with shaking at 200 rpm overnight. Keep the cap of the culture tube slightly loose.
2. Centrifuge the bacterial culture tube at 1,200 x g for 10 min at 4 °C. Remove the supernatant and resuspend the bacterial pellet into 5 ml ice-cold PBS.
3. Transfer 1 mL of the bacterial suspension into a cuvette and measure OD at 600 nm. Use PBS as a blank.
4. Calculate the colony forming unit (cfu) using a predetermined standard curve. Here, assume that 1 OD = 2 x 10^9 cfu/ml (approximately).
5. Dilute the bacterial suspension to make the stock suspension 1 x 10^5 cfu/mL.
6. Transfer the colon organ culture supernatant (500 µl/well) collected in Section 2.10 into duplicate wells of a 24-well cell culture plate.
7. Add 10 µL *E. coli* culture (1,000 cfu) into one well containing 500 µL colon organ culture supernatant. Leave the other well without any *E. coli* inoculation to confirm that the colon organ culture supernatant does not contain any contamination.
8. Incubate the same number of bacteria (1,000 cfu) in the culture media (DMEM/F12 plus 5% FBS) without any antibiotics as a control.
9. Incubate at 37 °C for 1 h.
10. Place 50 µL of each sample drop-wise on MacConkey agar plates. Incubate the MacConkey agar plates at 37 °C overnight.
11. Count the number of colonies and calculate the cfu/ml.

4. The Effect of Extrinsic and Intrinsic Factors on Colonic Antimicrobial Host Defense Responses

NOTE: The antimicrobial killing assay as described here can be adopted to examine the effect of pathogen associated molecular patterns (PAMPs) and cytokines on bactericidal activity of organ culture supernatant. An example of such experiment using IL-1β and IL-18 is described below.

1. Collect colons from mice as described in Section 1.
2. Place the colon on a sterile paper towel. Cut the colon longitudinally into three parts using scissors (**Figure 2**).
3. Wash each part of the colon in ice cold PBS as described in Section 1.
4. Cut each part of the colon into small pieces and weigh aseptically. Transfer the pieces of each part of the colon into three separate cell strainers (100 µm) placed on three wells of a 6-well cell culture plate (**Figure 2**).
5. Add 2 ml DMEM/F12 medium containing 5% FBS, Penicillin-Streptomycin (1x), and Gentamycin (20 µg/ml).
6. Incubate for 2 h at 37 °C in an incubator injecting 5% CO_2 and 95% air.
7. Wash the colon pieces as described in 2.4-2.6. Transfer the colon pieces of a single cell strainer into a single well of a sterile 12-well cell culture plate. The three wells containing three parts of the colon from a single mouse should be designated as untreated, IL-1β, and IL-18 (**Figure 2**).
8. Add DMEM/F12 medium containing 5% FBS (without any antibiotics). Adjust the volume of the medium with the weight of the colon pieces, *e.g.*, 1 mL medium for 100 mg of tissue.
9. Stimulate the colon organ culture with IL-1β (20 ng/mL) or IL-18 (20 ng/mL) for 12 h. The untreated colon organ culture serves as control.
10. After 12 h incubation at 37 °C in an incubator injecting 5% CO_2 and 95% air, collect the culture supernatant in a sterile 1.5 mL tube.
11. Transfer 500 µL culture supernatant into duplicate wells of a 24-well plate.
12. Inoculate *E. coli* (1,000 cfu) in colon organ culture supernatant as described in Section 3. For each condition, inoculate *E. coli* in a single well. The other well containing same organ culture supernatant without *E. coli* will serve as a control for bacterial contamination.
13. Incubate the same amount of bacteria in culture media without any antibiotics as a control.
14. Incubate at 37 °C for 1 h.
15. Place 50 µL of each sample drop-wise on MacConkey agar plates. Incubate the MacConkey agar plates for overnight at 37 °C.
16. Count the number of colonies and calculate the cfu/mL (**Figure 4**).

5. Measurement of the Expression of Antimicrobial Genes

1. After overnight incubation (12 h) of colonic organ culture as described in Sections 2 and 4, wash the colon pieces with 2 ml ice-cold PBS (2x).
2. Collect the colon pieces into a 2 mL RNase/DNase free screw-cap tube. Place the tubes on ice.
3. Add 1 mL commercial Trizol reagent and lysing matrix beads into the tube.
4. Lyse the tissue using an automated tissue homogenizer.
5. Collect the tissue lysate into a new microcentrifuge tube.
6. Isolate RNA using standard protocol.
7. Measure RNA concentration.
8. Dilute RNA appropriately with deionized water and use 500 ng RNA to synthesize cDNA.
9. Use the cDNA for real-time RT-PCR analysis of targeted antimicrobial genes, cytokines, chemokines, and other genes of interest.

Representative Results

A representative picture of colons in organ culture is shown in **Figure 1**. The colon pieces in the culture remain metabolically and physiologically active. They respond efficiently to exogenous stimuli added to the culture media. A schematic work flow of the preparation of the colon tissue for *ex vivo* culture and stimulation with exogenous stimuli, *e.g.* IL-1β and IL-18, is shown in **Figure 2**. The representative data in **Figure 3** show that colons in organ culture express antimicrobial peptides such as β-defensin 2 (BD2), Reg3γ, S100a8, S100a9, and iNOS in response to IL-1β and IL-18.

The mediators released by the colon implants exhibit an antimicrobial killing effect as demonstrated by the significantly reduced growth of *E. coli* incubated with the organ culture supernatant (**Figures 4A** and **4B**). Such *E. coli* killing activity is further potentiated by the stimulation of IL-1β and IL-18 (**Figures 4A** and **4B**). Colon organ culture supernatants incubated without *E. coli* show no bacterial growth following culture on MacConkey agar (**Figures 4C** and **4D**). These results further suggest that the inflammasome plays an important role in the intestinal antimicrobial host defense.

Overall, the data presented here suggest that *ex vivo* colonic organ culture is a very useful technique to study intestinal antimicrobial immune responses *in vitro*.

GAPDH_F	TGGCAAAGTGGAGATTGTTGCC
GAPDH_R	AAGATGGTGATGGGCTTCCCG
β-defensin 2 (BD2)_F	TGACCACTGCCACACCAATG
β-defensin 2 (BD2) _R	CCTGGCAGAAGGAGGACAAA
Reg3γ_F	CAAGGTGAAGTTGCCAAGAA
Reg3γ_R	CCTCTGTTGGGTTCATAGCC
S100a8_F	TGTCCTCAGTTTGTGCAGAATATAAA
S100a8_R	TCACCATCGCAAGGAACTCC
S100a9_F	GGTGGAAGCACAGTTGGCA
S100a9_R	GTGTCCAGGTCCTCCATGATG
iNOS_F	TGTGACACACAGCGCTACAACA
iNOS_R	GAAACTATGGAGCACAGCCACAT

Table 1: List of mouse primers for real-time PCR.

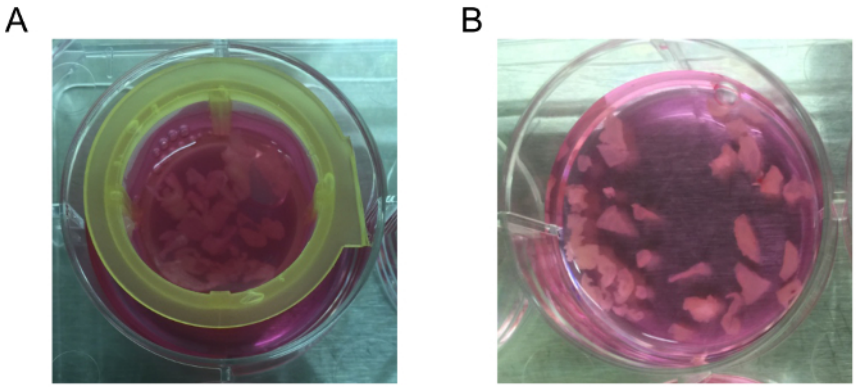

Figure 1: Representative picture of mouse colon pieces in the culture. (**A**) Incubation of colon pieces on a cell strainer (100 µm) placed on a 6-well plate. Colon pieces are submerged in 2 mL culture media supplemented with antibiotics. (**B**) Following 2 h incubation, colon pieces are transferred into the well of a new 12-well cell culture plate containing antibiotics-free medium. Please click here to view a larger version of this figure.

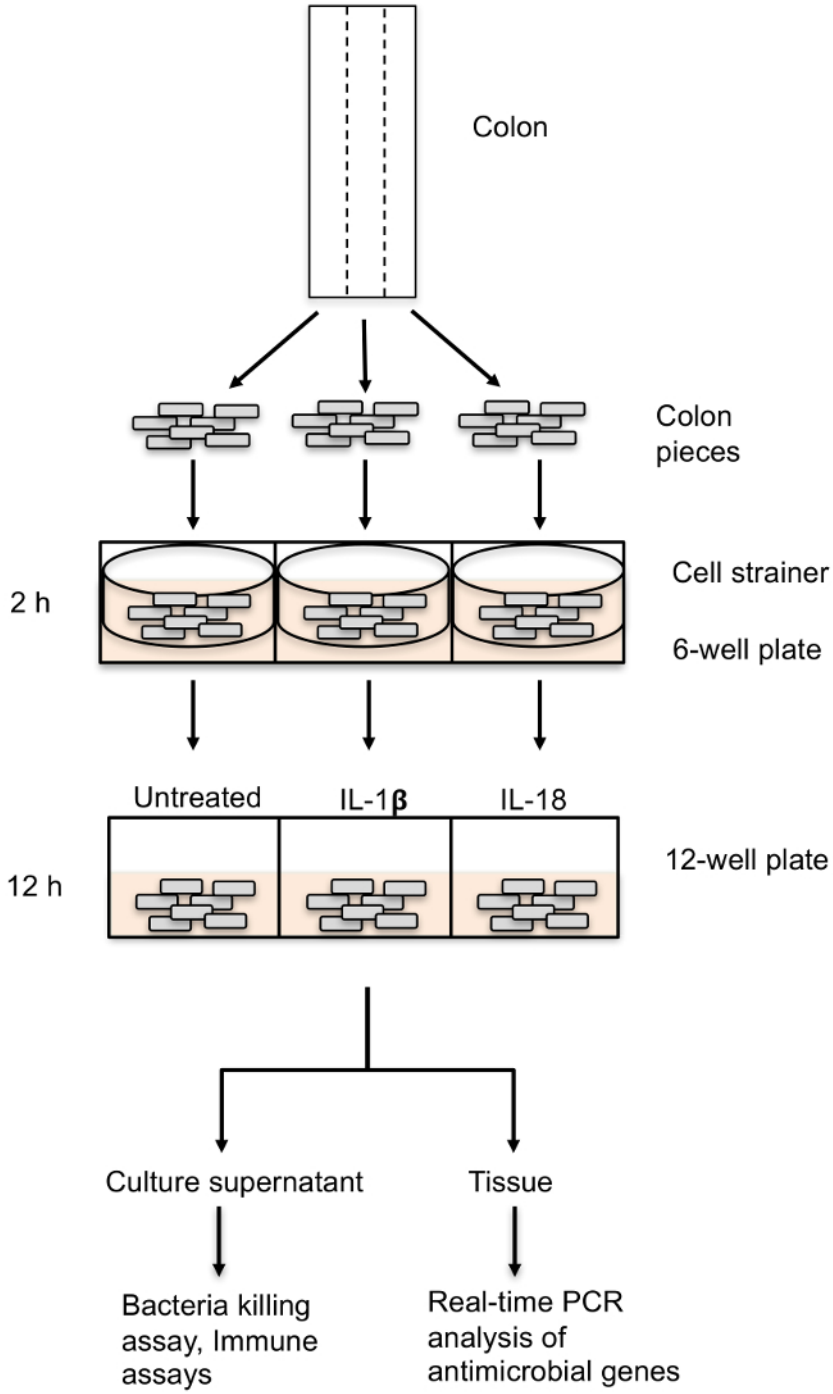

Figure 2: Schematic presentation of the steps for the preparation of colon tissues and *ex vivo* stimulation with IL-1β and IL-18. The colon from one mouse is longitudinally dissected into three parts. Each part was cut into small pieces and weighed. The colon pieces from each section of the colon are transferred onto cell strainers placed on wells of a 6-well plate containing 2 ml DMEM/F12 medium plus 5% FBS and antibiotics. Following a 2 h incubation, colon pieces from a single cell strainer are transferred into a single well of a 12-well cell culture plate. DMEM/F12 plus 5% FBS (no antibiotics) is added into each well and the volume of the medium is adjusted according to the weight of the tissue (1 mL media for 100 mg tissue). Colon tissues are stimulated with IL-1β (20 ng/ml) and IL-18 (20 ng/ml) for 12 h. Culture supernatants are centrifuged and used for *E. coli* killing assay and/or other immune assays. The colon tissues are collected into commercial trypsin reagent for RNA isolation and subsequent analyses of antimicrobial gene expression by real-time PCR. Please click here to view a larger version of this figure.

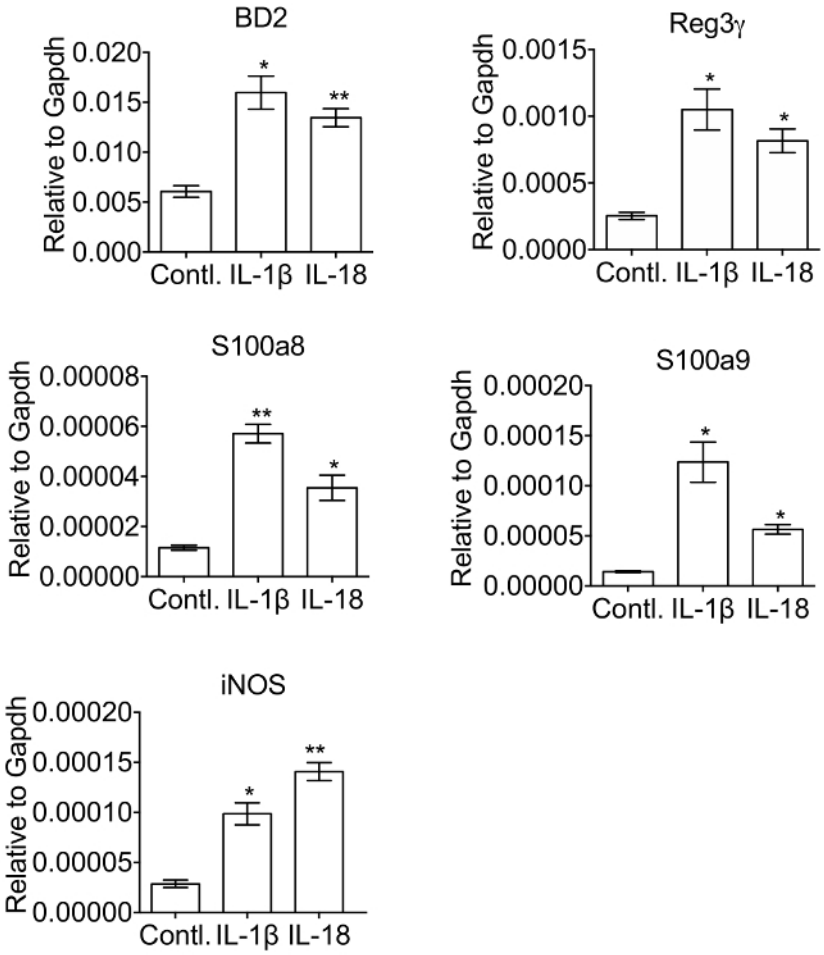

Figure 3: Mouse colon tissues express cytokines and antimicrobial peptides in response to IL-1β or IL-18 during *ex vivo* culture. Colon organ cultures were stimulated with IL-1β (20 ng/ml) or IL-18 (20 ng/ml) for 12 h. The expression of BD2, Reg3γ, S100a8, S100a9, and iNOS was measured by real-time RT-PCR (**Table 1**). Data represent means ± SD; *p <0.05, **p <0.01. Please click here to view a larger version of this figure.

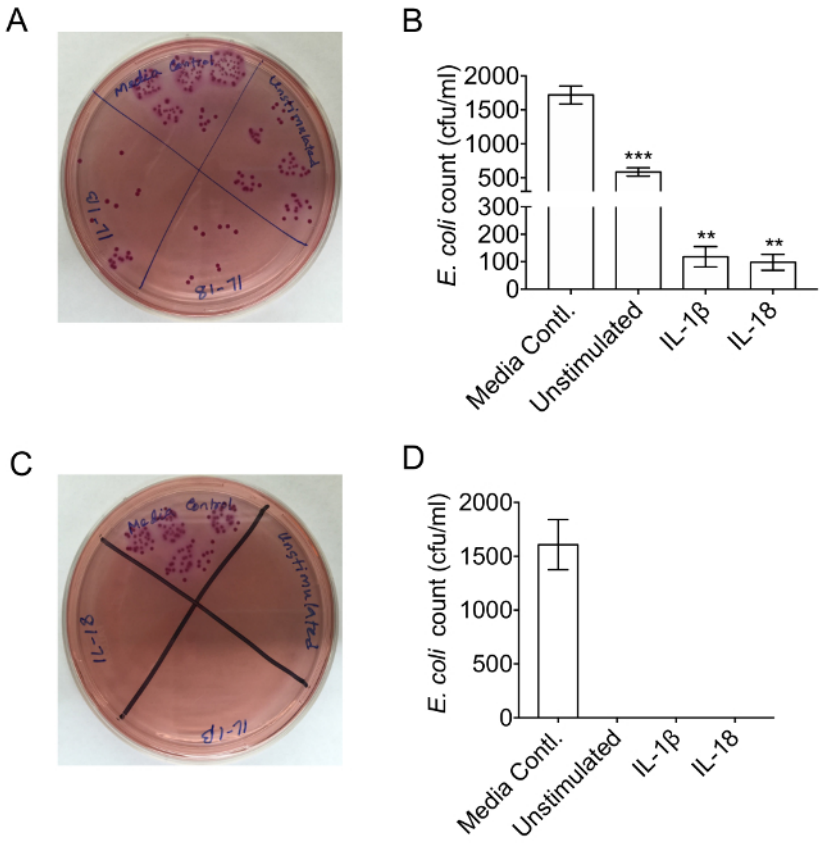

Figure 4: Supernatant of mouse colon organ culture exhibits bactericidal activity. Colon pieces were cultured *ex vivo* in the presence or absence of IL-1β (20 ng/mL) or IL-18 (20 ng/mL) for 12 h. 500 µl of the culture supernatants were incubated with *E. coli* (1,000 cfu) for 1 hr at 37 °C. Colon organ culture supernatant without *E. coli* was also incubated as a control. Following 1 h incubation, 50 µL of each sample was placed dropwise on MacConkey agar plates and incubated at 37 °C overnight. (**A**) Picture of *E. coli* colonies on MacConkey agar showing antimicrobial activity of colon organ culture. (**B**) *E. coli* count showing killing efficiency of IL-1β- and IL-18-treated colon organ culture supernatant. (**C**) Picture of MacConkey agar plate showing no bacterial growth in colon organ culture incubated without *E. coli*. (**D**) No *E. coli* colonies were identified in colon organ culture incubated without *E. coli*, indicating the absence of any contaminating bacteria in the sample. Data represent means ± SD; **p <0.01, ***p <0.001. Please click here to view a larger version of this figure.

Discussion

The intestinal epithelial cells are very sensitive in terms of their growth requirements and therefore difficult to culture. The epithelial cells isolated by EDTA treatment do not survive in conventional cell culture media such as DMEM[8]. Therefore, host-pathogen interaction studies using isolated crypt or primary epithelial cells are very challenging. Recently, Sato et al. described a crypt organoid culture system which is very promising and useful for studies related to intestinal pathophysiology[13]. While organoids represent many features of intestinal crypt, there are concerns as to whether the immune responses of the organoid cells, which are being grown in a sterile environment, recapitulate the responses of intestinal epithelial cells *in vivo*. The precise techniques required for isolation and culture of epithelial stem cells and requirement of expensive reagents for the culture of organoids prevent many laboratories from taking advantage of this system. While the use of *ex vivo* organ culture is not an alternative to organoid culture, this system offers an easy and inexpensive method to study the antimicrobial responses of intestinal epithelium.

The major advantage of this technique is that the tissue architecture of the intestine is maintained while culturing in the dishes. We observed that the colon tissues are physiologically active for at least 24 h after their culture. During the culture of the colon, the colonic epithelial cells respond to exogenous stimuli as measured by expression of cytokines and antimicrobial peptides. The viability of the epithelial cells of the intact tissue can be further enhanced by the addition of appropriate growth factors and inhibitors of cell death in the culture medium. Previous reports suggest that the normal human colonic tissue organ could be maintained *ex vivo* for several days[8,23,24].

The protocol for the *ex vivo* culture of mouse colonic organ may have many applications in studies related to antimicrobial immune responses of the intestinal epithelium. The pathogens and their PAMPs are recognized by PRRs such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) present in epithelial and immune cells. Defective expression and function of many TLRs and NLRs are associated with susceptibility to IBD, colorectal tumorigenesis, and bacterial infections. Since immortalized epithelial cell lines do not represent all the features of the intestinal niche, the *ex vivo* colon organ culture is a very useful experimental tool. Using this system, we can examine the effect of various PAMPs or stimuli on the induction of antimicrobial effector molecules in the intestinal epithelial cells. In the representative experiment, we showed that cytokines like IL-1β and IL-18 trigger the expression of antimicrobial peptides (**Figure 3**). We also show here that antimicrobial peptides

produced by colon tissue can effectively kill *E. coli* (**Figure 4**). These techniques can be applied to test the influence of lipopolysaccharide (LPS), peptidoglycan (PGN), muramyldipeptide (MDP), and other PAMPs in antimicrobial host defense responses in the gut.

We have slightly modified the colon organ culture protocol as described previously[12,16,20,25]. We cultured the colon in two phases. At first, we incubated the colon tissue in antibiotics containing medium for 2 h. We then removed the antibiotics with repeated washing of the colon pieces followed by incubation with antibiotics free media. Thus, the culture supernatant obtained from overnight culture of the colon exhibits only the effect of secreted antimicrobial peptides when incubated with *E. coli*. This approach can be utilized to see the bactericidal effect of colon or intestine derived antimicrobial peptides on any bacteria of interest. An appropriate selective agar media should be used to culture the bacteria.

Like most techniques, there are limitations of the *ex vivo* organ culture system. First, unlike cell culture or organoid culture, the organs do not grow and proliferate and therefore cannot be expanded. Second, intestinal epithelial cells are very sensitive and they die relatively quickly without additional growth factors and apoptosis inhibitors, which are used for crypt organoid culture. Third, the immune response observed from the organ culture is a collective response of different kinds of cell populations. Therefore, the role of individual cell type in the expression of antimicrobial and effector genes cannot be assessed. Additionally, unlike in cell lines and organoid culture, the expression of antimicrobial peptides and other effector molecules in the colons may vary from mouse to mouse of same genetic background.

In summary, we describe here a simple protocol for *ex vivo* colonic organ culture that is very useful for studying intestinal antimicrobial immune responses. This approach can be employed to test the role of diverse innate immune genes in the expression of antimicrobial peptides by culturing colons from genetic mutant mice of interest. Further, this protocol can be adapted for use in clinical studies to examine the intestinal antimicrobial responses of IBD patients by conducting organ culture of colonic biopsy samples.

Disclosures

The authors have no competing financial interests.

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