

Use of a collagen membrane to enhance the survival of primary intestinal epithelial cells[†]

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ABSTRACT

Intestinal epithelial cell culture is important for biological, functional and immunological studies. Since enterocytes have a short *in vivo* life span due to anoikis, we aimed to establish a novel and reproducible method to prolong the survival of mouse and human cells. Cells were isolated following a standard procedure, and cultured on ordered-cow's collagen membranes. A prolonged cell life span was achieved; cells covered the complete surface of bio-membranes and showed a classical enterocyte morphology with high expression of enzymes supporting the possibility of cryopreservation. Apoptosis was dramatically reduced and cultured enterocytes expressed cytokeratin and LGR5 (low frequency). Cells exposed to LPS or flagellin showed the induction of TLR4 and TLR5 expression and a functional phenotype upon exposure to the probiotic *Bifidobacterium bifidum* or the pathogenic *Clostridium difficile*. The secretion of the homeostatic (IL-25 and TSLP), inhibitory (IL-10 and TGF- β) or pro-inflammatory mediators (IL-1 β and TNF) were induced. In conclusion, this novel protocol using cow's collagen-ordered membrane provides a simple and reproducible method to maintain intestinal epithelial cells functional for cell-microorganism interaction studies and stem cell expansion. This article is protected by copyright.

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INTRODUCTION

The intestinal epithelium is a highly organized system with key functions acting as a barrier to an antigen-rich lumen and as an orchestrator of the underlying mucosal immune system (Nusrat et al. 2000). Enterocyte culture systems are ideal for physiologic and functional studies, including nutrient absorption, permeability, prediction of intestinal metabolism, drug development for therapy, cell cycle control, gene expression, interactions with the indigenous components of the microbiota, etc. Unfortunately, culturing small bowel epithelial cells (IECs) from animal tissues has been a difficult task (Bader et al. 2000; Perreault & Beaulieu 1998), mainly due to the short life span (3-5 days) of differentiated cells once they are detached from the extracellular matrix (Kim JM, Eckmann L, Savidge TC, Lowe DC, Withhöft T 1998; Evans et al. 1992). Therefore, most studies rely on the use of tumoral cell lines and animal models. Available cell lines are usually from mice or human intestinal cancer. Given that the small intestine cell line mouse ICc1-2 was generated through viral transformation and cells reflect a crypt-like phenotype (Bens et al. 1996), it fails to reflect the physiology of absorptive cells. Most research on small intestine disorders, such as food allergies, rely mostly on the use of animal models (Smaldini et al. 2012), which are costly and time consuming. The same is true for human colon cancer cell lines, such as Caco-2 and HT-29 (Engle et al. 1998; Sambuy et al. 2005; Awortwe et al. 2014). Although these cell lines are widely used and have contributed enormously to the knowledge of human IEC behavior, they have a tumoral origin and a limiting metabolic competence with enterocytes.

There are few reports in the literature regarding the generation of primary IEC cultures. Despite the wide use of cells from fetuses or very young individuals (Perreault & Beaulieu 1998; Macartney et al. 2000), novel methods to culture adult IEC have been accomplished for short periods (Sharbati et al. 2015; Graves et al. 2014; Hahn et al. 1987) and most of them include the use of a feeder layer or a collagen-based supplement. Therefore and based on these concerns, the approach of this study was to characterize a novel, simple and reproducible method for obtaining IECs in culture from young or mature mice using an ordered membrane of bovine collagen. IECs survived at least 8 days

in culture with a classical enterocyte morphology, they could undergo freeze/thaw cycles, and they proved to be useful for cell-microorganism interactions' studies.

MATERIALS AND METHODS

Isolation and culture of intestinal epithelial cells

Intestinal epithelial cells were isolated from adult Sv129 mice (5-12 weeks old). Mice were housed and cared for in the animal facilities of the School of Sciences (University of La Plata), according to institutional guidelines. Experimental protocols were approved by the local Institutional Animal Care and Use Committee CICUAL (Protocol Number: 017-00-15). Animals were sacrificed by cervical dislocation. A midline abdominal incision was made, the first 5 cm of the intestine were removed under sterile conditions and washed 5 times with phosphate buffer saline (PBS) supplemented with antibiotics (penicillin 100 U.ml⁻¹ and streptomycin 100 µg.ml⁻¹) and 20 % fetal bovine serum (inactivated by heating 30 min at 65°C) (FBS, Gibco Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at room temperature. The gut was opened longitudinally and mucus was removed by incubation with medium and 1 mM dithiothreitol for 10 min at 4 °C. Afterwards, the tissue was incubated with medium and 0.5 mM EDTA with orbital agitation for 30 min at 37°C to remove the epithelial compartment (the remaining tissue was discarded).

Intestinal epithelial cells were also isolated from colonic biopsies of adult patients (n=5, 3 females and 2 males, mean age 47 years old) undergoing colonoscopy for abdominal pain, constipation, irritable bowel syndrome, screening of colorectal cancer or polyp resection. The protocol described was carried out under compliance with the Ethics Committee at the Gastroenterology Hospital Dr Bonorino Udaondo (# 0398) Buenos Aires, Argentina. All the patients provided informed consent. Human biological specimens were processed as described in Muglia et al. 2015 (Muglia et al. 2015). Briefly, biopsies were placed in ice-cold HBSS supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin. Samples were incubated with HBSS/penicillin/streptomycin plus 0.5 mM

EDTA, after washing with HBSS, in order to obtain epithelial cells. Cells were pelleted, resuspended in RPMI 1640 supplemented with 5% FBS and penicillin/streptomycin, and used immediately.

Human and mouse cell suspensions were spun (5 minutes at 300xg at room temperature) and pelleted cells were washed with RPMI 10% FBS. Vital staining with trypan blue (ICN Biomedical Inc. Aurora OH, USA) was performed for cell count and viability assessment. Wells were coated with rat collagen or covered with collagen membrane. DMEM-Glutamax medium (Gibco, Gibco Thermo Fisher Scientific, Waltham, MA, USA) supplemented with non-essential amino acids (Gibco), 0.17 mM insulin, 0.0068 mM transferrin and 0.038 mM selenium (ITS-X, Gibco Thermo Fisher Scientific, Waltham, MA, USA), 20 % FBS and antibiotics (penicillin 100 U.ml⁻¹ and streptomycin 100 µg.ml⁻¹) were used for cell culture. Cells (5x10⁵) were plated in 24-well or 96-well culture plates (Cell Star Greiner Bio-One, Monroe, NC, USA) and incubated in a culture chamber at 37°C with 5% CO₂.

Cells were harvested with trypsin (TrypLE Express, Thermo Fisher Scientific, Waltham, MA USA) at different days of culture (DOC), and stained with trypan blue.

Isolation of rat collagen

Wistar rats were anesthetized with an intramuscular injection of 50-60 mg/kg ketamine (Fort Dodge Laboratories, Fort Dodge, IA, USA) and killed by cervical dislocation. Tails of 3 animals were removed and washed with 70% ethanol for 15 min. The skin was removed and tendons were dissected and incubated with 1% acetic acid solution (100 ml.g⁻¹ tendon). The resulting mixture was stirred during 48 h at 4°C in horizontal rotator at 50 rpm and the solution was centrifuged at 35000xg for 1 h. The supernatant was collected and kept at 4°C. Sterile conditions were maintained throughout the whole procedure.

The collagen solution was placed on ice and mixed with DMEM medium. Neutralization of pH with 1N NaOH promoted collagen polymerization (Habermehl et al. 2005). The solution was quickly

distributed onto culture plates.

Elaboration of ordered collagen membranes

Collagen from bovine Aquila's tendons was used to synthesize ordered bio-membranes as described in Ruderman et al. (Ruderman G, Mogilner IG, Tolosa EJ, Massa N, Garavaglia M 2012). Briefly, tendons were dried and collagen was extracted with 0.5M acetic acid. The resulting solution was centrifuged at 5000xg (30 min at 0°C) and the middle phase was recovered and spun (5000xg during 30 min at 0°C). Protein concentration of the supernatant was measured with the modified Lowry method (Komsa-Penkova et al. 1996). The collagen suspension was dialyzed to pH 5-6, centrifuged and poured on acrylic striated plates. Plates were dried with silica gel at room temperature until solid membrane was formed. Membranes were cut using a 6mm diameter puncher for 96-well culture plates. Membranes were sterilized by UV exposure during 30 min prior to use.

Alkaline phosphatase activity assay

Cultured cells on collagen membranes were harvested by pipetting and lysed with 2% triton-X100 (Fisher Biotech, New Jersey, USA) for 30 min at 37°C. Debris was removed by centrifugation at 14000xg for 2 min and supernatants were stored at -20°C until use. A 50 µl aliquot of the lysate was incubated with 50 µl of 1 mM *para*-nitrophenylphosphate, pNPP (Fluka Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C, and absorbance was evaluated at 409 nm.

Microscopic characterization of cultured cells

Cultured cells on collagen membranes were evaluated using an inverted light microscope (Nikon TI-Eclipse). For electron microscopy, cells were harvested by trypsinization at different time-points of culture, pelleted by spinning (6000xg for 10 min at 4°C), fixed and dehydrated according to standard procedures (Jurado et al. 1998). Observations were performed using a JEM-1200 EX II Electron Microscope (Jeol, Tokyo, Japan).

Confocal microscopy was carried out in a SP5 Leica Microscope (Leica, Wetzlar, Germany). Cells were harvested from membranes by trypsinization at different DOC and citospinned on polylysine positive charged slides (Pearl, Carlsbad, CA, USA). Slides were fixed with acetone for 10 min, washed with PBS, and blocked with 2% BSA. Slides were incubated for 16 h with monoclonal antibodies anti-pan-cytokeratin (undiluted, Sigma-Aldrich, St. Louis, MO, USA), anti-LGR5 (1/100, Abcam, Cambridge, UK) or anti-vimentin (1/1000, Sigma-Aldrich, St. Louis, MO, USA) as primary antibody, followed with Alexa 488-conjugated anti-mouse γ globulin (1/2000) (Abcam, Cambridge, UK) for 1 h at 37 °C. Nuclei were counterstained with $1\mu\text{g}\cdot\text{ml}^{-1}$ propidium iodide (PI). Slides were mounted and visualized in the microscope.

Apoptosis assays

Cultured cells were detached from the collagen membrane by trypsinization and apoptosis was evaluated by fluorescence microscopy and flow cytometry. The former was done using acridine orange:ethidium bromide solution (0.01%:0.003% v/v). Cells were incubated with the dyes for 5 min at room temperature and fluorescence was visualized with a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan). The latter was carried out by flow cytometry with Annexin V-FITC (BD Pharmingen, San Diego, CA, USA) and PI ($1\mu\text{g}\cdot\text{ml}^{-1}$). Early apoptotic cells (Annexin V⁺PI⁻ cells) and non-apoptotic cells (Annexin V⁻PI⁻ cells) were quantified. Fluorescence acquisition was performed with a FACScalibur cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA) using QuestProCell software. Data were analyzed with the FlowJo software.

Quantitative RT-PCR for epithelial enzymes and innate receptors

Total RNA was extracted from detached cells using the NucleoSpin RNA II kit (Macherey Nagel, Easton, Pennsylvania, USA) according the manufacturer's specifications. The amount of the extracted RNA was determined by UV absorption and the optical density ratio of $\text{OD}_{280\text{nm}}/\text{OD}_{260\text{nm}}$ was used as a purity measure. Reverse transcription was performed using random primers and

MMLV-Reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qPCR was performed on an *ABI prism* sequence detection system using *SYBRGreen* fluorescence (BioRad, Hercules, CA, USA) in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Primer sequences for alkaline phosphatase (Apk3) and maltase glucoamylase (Mgam) were as follows: Fwd primer Apk3: 5'-gtcattccagtgaggaggagaac-3', Rv primer Apk3: 5'-agaatcccagataagctttcc-3' and Fwd primer Mgam: 5'-ccactttaagtgactgacc-3' and Rv primer Mgam: 5'-gattcggatcagccagct-3'. Results were normalized to β -actin using the $2^{-\Delta\Delta CT}$ method according to Smaldini et al. (Smaldini et al. 2015).

TLR4 and -5 mRNA were evaluated in 8 DOC cultures that were stimulated with 1 ug/ml *Escherichia coli* lipopolysaccharide (LPS) or 1 ug/ml flagellin (FliC) from *Salmonella typhimurium* for 16 h according to Smaldini et al. (Smaldini et al. 2014). Cells were harvested, mRNA was extracted and qPCR was performed as indicated. Primer sequences were for TLR4: Fwd 5'-cca gtg agg atg atg cca gaa t-3' and Rv 5'- gcc atg gct ggg atc aga gt-3'; for TLR5: Fwd 5'- tgc ett gaa gcc ttc agt tat g-3' and Rv 5'- cca acc acc acc atg atg ag-3' as in Iraporda et al. (Iraporda et al. 2015).

Bacterial cultures

Bifidobacterium bifidum CIDCA5310, was isolated from healthy infant feces as indicated in Gomez Zavaglia et al. (Gómez Zavaglia et al. 1998; Trejo et al. 2006; Trejo et al. 2010). Bifidobacteria were grown in MRS (Man, Rogosa and Sharpe) medium (BIOKAR, Biokar Diagnostics, Beauvais, France) plus cysteine (0.05% w/v) at 37°C for 20 h under anaerobic conditions (AnaeroPak; Mitsubishi Gas Chemical Co, Inc.). The pathogenic *Clostridium difficile* strain 117 is a clinical isolate obtained from the Servicio de Bacteriología, Hospital Muñiz (Buenos Aires, Argentina) and characterized by their virulence factors *in vivo* e *in vitro* (Carasi et al. 2012; Trejo et al. 2013). *Clostridium* were grown under anaerobic conditions in BHI (Brain Heart Infusion) (BIOKAR, Biokar Diagnostics, Beauvais, France) plus cysteine (0.05% w/v) at 37°C for 20 h. Plate counts were performed by plating serial dilutions of the cultures on BHI-agar or MRS-agar for *C difficile* and MRS-agar respectively. Plates were incubated for 72 h at 37°C in anaerobic conditions.

Quantification of secreted chemokine and cytokines

Intestinal epithelial cells were plated in complete medium on collagen membranes for 8 DOC and then exposed to *Clostridium difficile* (ratio 1:100) or *Bifidobacterium bifidum* (ratio 1:100) at 37°C for 24 h. Supernatants were collected and concentration of homeostatic (IL-25 and TSLP), immunosuppressive (IL-10 and TGF- β) and pro-inflammatory (IL-1 β) cytokines were assessed by ELISA. Commercial kits were employed (IL-10, IL-25 and TGF- β - eBioscience, San Diego, CA, USA; TSLP-Biolegend, San Diego, CA, USA) according to manufacturer's specifications.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5 software. The significance of the difference was determined using the *t*-student test. A p-value <0.05 was regarded as statistically significant.

RESULTS

IECs remain in culture at least 8 days

Intestinal epithelial cells were isolated from mouse small intestine and cell suspension was cultured on rat collagen-covered wells or striated cow's collagen membranes placed on wells. The number of viable cells was counted and we found a significant higher viable cell count in collagen membranes compared with rat collagen-covered wells (Fig. 1A). As depicted in Figure 1B, living cells remained attached to the collagen membranes for 8 days, while non viable cells were detached and remained in the supernatant. The number of viable cells on membranes stabilized at day 2 of culture and thereafter, remained alive and attached to the membrane (Fig. 1 A,B). To further characterize the morphology of viable cells, optic and electron microscopy was performed. After 8 DOC, attached cells to the collagen membrane showed the classical epithelial cell morphology (Fig. 1C), covering the complete surface of the membrane. Electron microscopy showed the classical columnar cell

shape of IEC with brush border (Fig. 1D). The appearance of apoptotic cells on collagen membranes through staining of attached cells with acridine orange and ethidium bromide was subsequently investigated (Fig. 1E). Apoptotic cells (bright orange nuclei) were visualized at 21 DOC, while at 8 DOC no apoptotic cells were found. To further quantify the number of apoptotic cells attached to the membrane we carried out Annexin V and propidium iodide staining by flow cytometry (Fig. 1 F). Results showed that cells remained viable (Annexin V⁻PI⁻ cells) for 8 DOC ($45 \pm 4\%$) and a significant frequency of early apoptotic cells was observed longer than 14 DOC.

We next assessed viability of cells cultured during 8 days on collagen membranes and then frozen in liquid nitrogen for a month. Thawed cells cultured on collagen membranes during 8 DOC showed a similar morphology to cells that had not gone through the frozen-thawed cycle (Fig. 1G). Cells grew and covered the complete surface of the membrane in 3 DOC.

Cultured cells express markers of epithelial cells

In view of the viability of attached cells to the ordered collagen membrane, the transcript expression of markers associated with IECs by qRT-PCR was examined (Blais et al. 2014). At 8 DOC we found that cultured primary and frozen/thawed cells maintained a significant expression of maltase and alkaline phosphatase (Fig. 2A). The transcript level of enzymes in the whole tissue was reasonable lower compared to that of epithelial cells. To further evaluate protein expression, we analyzed alkaline phosphatase enzymatic activity in cell extracts. Figure 2B depicts that cells maintained on collagen membrane during different DOC have similar enzymatic activity, comparable with frozen/thawed cells, and lower than Caco-2 cells grown during 15 DOC on plastic culture flasks (Fig. 2B). Finally, we characterized the cell phenotype by confocal microscopy using anti-cytokeratin, anti-vimentin and anti-LGR5 antibodies (Fig. 2C). We observed that cells cultured during 8 DOC expressed membrane cytochrome and LGR5 (a minority of cells), and were negative for vimentin.

Cultured IEC respond to microbial stimuli

We finally assessed a functional characterization of IECs by exposing membrane-attached cells to different bacteria. We compared by qPCR TLR expression in resting cells and cells exposed to TLR agonists. We found that transcript level of TLR5 was 21.7 ± 3.2 fold higher in cells exposed to FliC and that of TLR4 was 4.1 ± 1.1 fold higher in cells exposed to LPS (Fig. 3A). Fold change in mRNA expression was calculated in activated cells with respect to resting cells.

Thereafter, enterocytes cultured during 8 DOC were exposed to *Clostridium difficile* or *Bifidobacterium bifidum* during 24 h. Supernatants were harvested and different cytokines and chemokines were assessed by ELISA. We found that the secretion of the intestinal homeostatic factors IL-25 and TSLP were significantly induced with the probiotic *B. bifidum*, while supernatants of cells incubated with the pathogenic bacteria *C. difficile* had same levels as cells incubated with medium (Fig. 3B). Moreover, cells incubated with the *B. bifidum* secreted increased IL-10 ($p < 0.01$) and TGF- β ($p < 0.05$) as tolerogenic factors, compared with cells incubated with *C. difficile* or medium (Fig. 3C). Finally, cells incubated with *C. difficile* produced significant higher amounts of the pro-inflammatory IL-1 β compared with IEC incubated with *B. bifidum* or medium (Fig. 3D).

A similar procedure was done with human IEC isolated from colon biopsies of control patients. Cells were culture during 8 DOC and then exposed to bacteria. We found that soluble TNF was 0.05 ± 0.02 pg.ml⁻¹, 0.04 ± 0.03 pg.ml⁻¹ and 49.23 ± 3.51 pg.ml⁻¹ for cells incubated with medium, *B. bifidum* and *C. difficile*, respectively.

DISCUSSION

In this study we describe a method for the establishment of intestinal epithelial cell cultures from adult mice based on cell growth on collagen ordered bio-membranes. There is consensus that primary IECs are difficult to maintain in culture and remain viable for hours or few days. Epithelial cells receive important survival signals from the extracellular matrix and undergo detachment-induced apoptosis or anoikis (Hofmann C, Obermeier F, Artinger M, Hausmann M, Falk W, Schoelmerich J, Rogler G 2007) Although several methods of organoid and cell culturing have recently been published, most of them are rather intricate and time consuming (Mahe et al. 2015; Pereira et al. 2016). The procedure described above is very simple, reproducible and promotes the proliferation of stem and matured intestinal epithelial cells. A rich collagen membrane with collagen fibers aligned in one direction, parallel to each other, contributed to arrange IECs in ordered manner during growth. Consequently, the formation of tight junctions between cells may be promoted and hence survival is enhanced. Additional studies will be required to more in depth characterization of the molecules involved. Furthermore, this study aims at attempting to define the efficacy of bio-membranes to allow frozen/thawed cells to grow covering the whole membrane surface.

Intestinal epithelial cells play important functions, amongst them nutrient absorption and barrier between the outside world and inside environment. We have characterized the alkaline phosphatase expression and activity in cultured cells on striated membranes and in frozen/thawed cells. This protein is an integral component of the outer membrane of the microvilli and plays a central role in absorptive functions (Narisawa et al. 2003) and homeostasis maintenance (Malo et al. 2014; Moss et al. 2013; Goldberg et al. 2008). In addition, the absorptive phenotype of cultured cell has also been here characterized by the expression of maltase, an enzyme involved in carbohydrate metabolism which is expressed in the brush border region of IEC. Therefore, cells grown on collagen-ordered bio membranes maintained their absorptive functions and established cell-cell contacts. The intestinal epithelial cells play a crucial role as a regulator of intestinal immune homeostasis. In response to pathogens, IECs may produce a variety of factors that play a crucial

role in both the innate and adaptive immune responses. However, under steady-state conditions, IECs contribute to the tolerogenic environment that avoids inflammatory-mediated tissue damage. These two functions of IECs – distinguishing among the diverse elements of the intestinal microbiota and responding to pathogenic microorganisms – are determined by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) (Wells et al. 2011).

In this context, there is a growing interest regarding IEC interactions with the microbiota. Here we have used 8-week old mouse and human IECs to study their response to commensal and pathogenic bacteria and assayed the release of cytokines and tolerogenic factors. We showed that cultured cells were sensitive to bacterial components through the expression of specific innate receptors (TLR4 and TLR5), thus suggesting that cultured primary enterocytes would be appropriate for functional studies. The pathogenic role of the *Clostridium difficile* flagellum has been attributed to adherence and colonization of human and mouse intestinal epithelial cells (Baban et al. 2013). In addition, *C. difficile* flagellum is active in the context of innate immune by recognition through TLR5 (Yoshino et al. 2013). Other TLR agonists, such as peptidoglycans and surface layer proteins, have been described in *C. difficile* and may mediate IEC activation (Hasegawa et al. 2011; Bianco et al. 2011). In this sense, TLR4 null mice were more susceptible to *C. difficile* infection compared to wild type (Ryan et al. 2011), while Caco-2 cells infected with *C. difficile* up-regulated the transcript level of components of the NF- κ B pathway (Janvilisri et al. 2010) and human HEK-293 cells transfected with TLR4 and exposed to *C. difficile* surface components induced NF- κ B downstream intermediates (Ryan et al. 2011). With respect to *Bifidobacterium*, several strains have shown the ability to stimulate the immune system inducing anti-inflammatory response. Bacterial cell wall components, peptidoglycan and lipoteichoic acid, recognize TLR2 (Vizoso Pinto et al. 2009); the *B. breve* MCC-117 interacts with TLR2 on IEC and modulates the subsequent TLR4 activation by inhibition of MAPK and NF- κ B pathways and the production of pro-inflammatory cytokines (Tomosada et al. 2013); *B. breves* C50 secretes IL-10 through binding to TLR2 (Tomosada et al. 2013; Hoarau et al. 2006). Our results showed that cultured IEC on ordered bio-membranes are

immunologically active since they respond upon exposure to pathogen or probiotic intestinal bacteria.

Although cultured primary intestinal epithelial cells responded to *B. bifidum* and *C. difficile* we cannot assure that TLR5 or TLR4 were implicated in our assays. This is not the focus of this work and further research is needed to address this relevant issue. As most of these studies are performed using tumoral human and animal cell lines the protocol here studied provides a novel and useful tool to investigate different aspects of cell-microorganism interactions.

Although small bowel columnar epithelial cells or enterocytes are the most numerous cell subset of the intestine, we were able to detect a low frequency of LGR5⁺ cells upon 8 DOC, which is likely to likely correspond to intestinal stem cells located in the small bowel crypts. We propose this simple culture technique using the ordered collagen membrane to allow expansion of these cells from the whole epithelial compartment, which could further be used in stem cell-based therapy and studies of stem cell biology. Additional studies are required to specifically identify the self-renewed capability of these cells and the possibility of long-term cultures of sorted single multipotent LGR5⁺ cells.

Other authors have developed different methodologies based on the use of collagen suspension to isolate and culture mouse crypt LGR5⁺ stem cells (Sato et al. 2009), or entire crypts in a laminin-rich gel system that support the 3D growth of the crypts (Yui et al. 2012).

In conclusion, we have developed a valuable method based on the use of collagen-rich and ordered synthetic membranes to avoid anoikis of isolated intestinal cells from small and large bowel and support cell growth. Cultured cells maintained classical morphology, absorptive characteristic features, and normal growth after freeze/thaw cycles. Therefore, the use of collagen-rich and ordered bio-membranes provides a useful tool with potential applications in biomedicine.

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CONFLICTS OF INTEREST

Authors have no conflict of interest.

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LEGENDS TO FIGURES

Figure 1: Characterization of mouse intestinal epithelial cells cultured on collagen. A, Evaluation of live cells on rat tail collagen (RTC) compared to synthetic collagen membranes (CM). B, Live and dead cells on CM. C, Optic microscopy images of intestinal epithelial cells (IECs) at 1 and 8 days of culture (DOC). Scale bars: 25 μm . D, Electron transmission microscopy images of IECs at 1 and 8 DOC. Scale bars: 1 μm . E, Acridine orange/ethidium bromide staining of IEC cultures (8 and 21 DOC). White light (left) and fluorescence images (right). Arrows indicate orange apoptotic nuclei. Representative images are shown. Scale bars: 25 μm . F, Annexin V/PI staining of IECs in culture (1 and 8 DOC). Dot-plot and statistical analysis are shown. Results are representative of four experiments. G, Microscopy images of IECs frozen in liquid N_2 , thawed and cultured on collagen membranes using routine procedures (representative pictures are shown). From left to right: scale bars: 50, 25 and 5 μm , respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 2. Characterization of cultured mouse intestinal epithelial cells. A, Alkaline phosphatase and maltase gene expression of cultured IECs at different DOC. Cells from the whole gut and frozen/thawed cells were included in the analysis. B, Alkaline phosphatase activity of cultured IECs, frozen/thawed cells and Caco-2 cells as control. C, Confocal microscopy and staining for pan-cytokeratin, vimentin and LGR5 in cultured cells at different DOC. Caco-2 cells were included as positive control for LGR5 staining (representative pictures are shown). Scale Bars: 10 μm for cytokeratin and vimentin; 5 μm for LGR5. Results are representative of three experiments. * $P < 0.05$.

Figure 3: Analysis of intestinal epithelial cell-bacteria interaction. IECs were cultured during 8 DOC and exposed to *Bifidobacterium bifidum* CIDCA5310 and *Clostridium difficile* 117 for 24 h. A, TLR4 and TLR5 transcript levels were evaluated by qPCR in IECs cultured during 8 DOC and exposed during 16 h with Flic (1 $\mu\text{g}\cdot\text{ml}^{-1}$), LPS(1 $\mu\text{g}\cdot\text{ml}^{-1}$) or medium. B, Quantification of homeostatic factors, IL-25 and TSLP by ELISA in supernatants of cultured IECs. C, Quantification

of tolerogenic factors, IL-10 and TGF- β by ELISA in supernatants of cultured IECs. D, Quantification of IL-1 β by ELISA in supernatants of cultured IECs. Results are representative of three experiments. * P<0.05, ** P<0.01.

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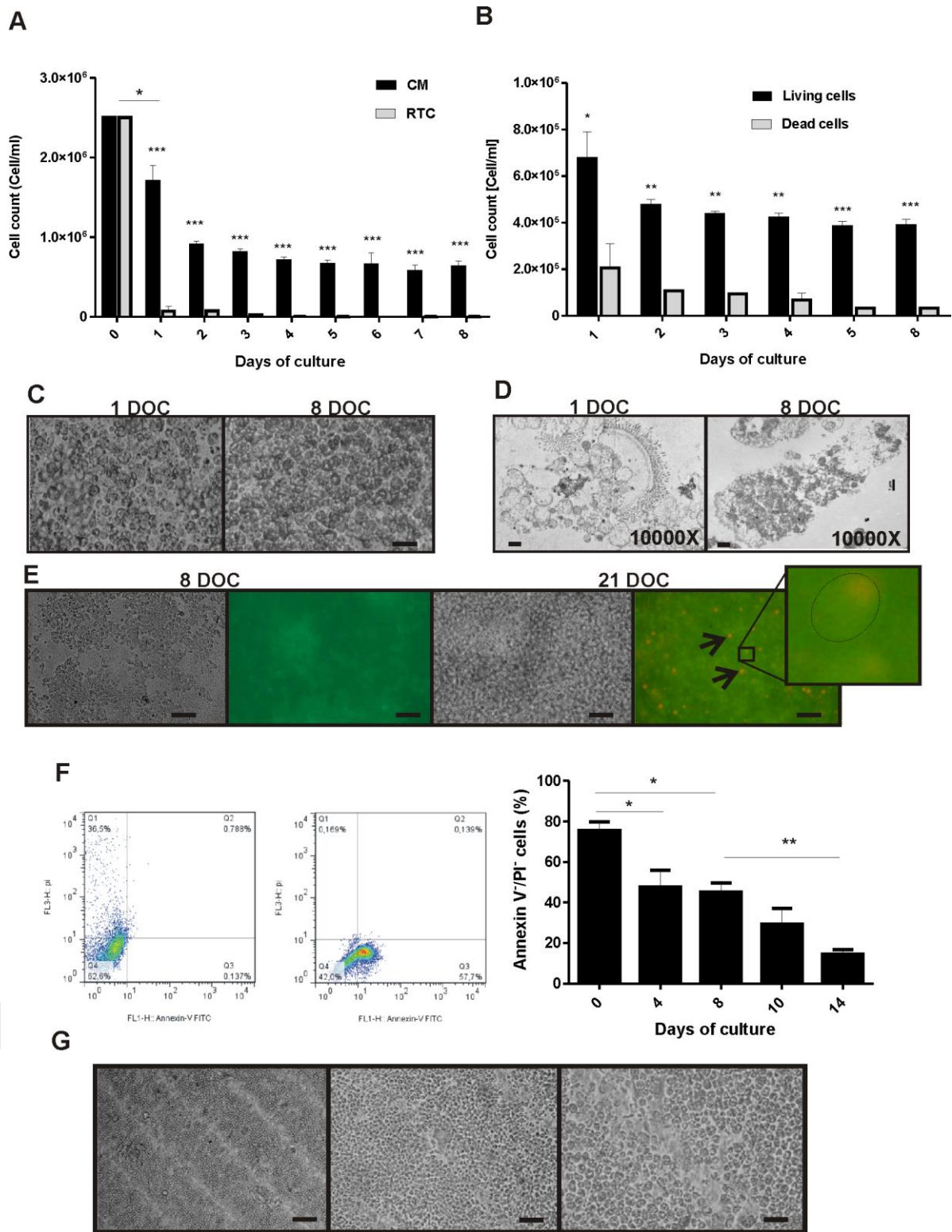


Figure 1

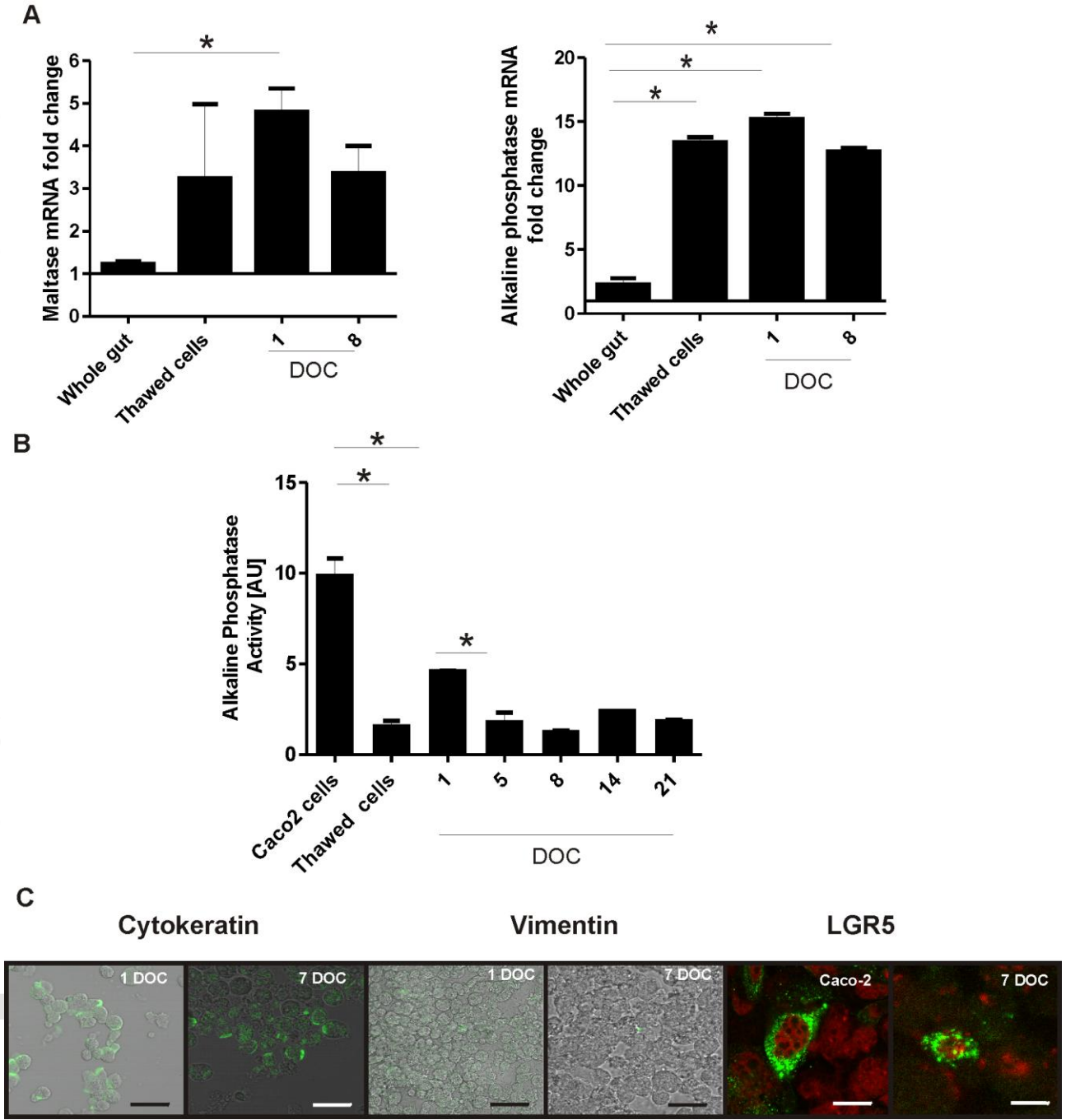


Figure 2

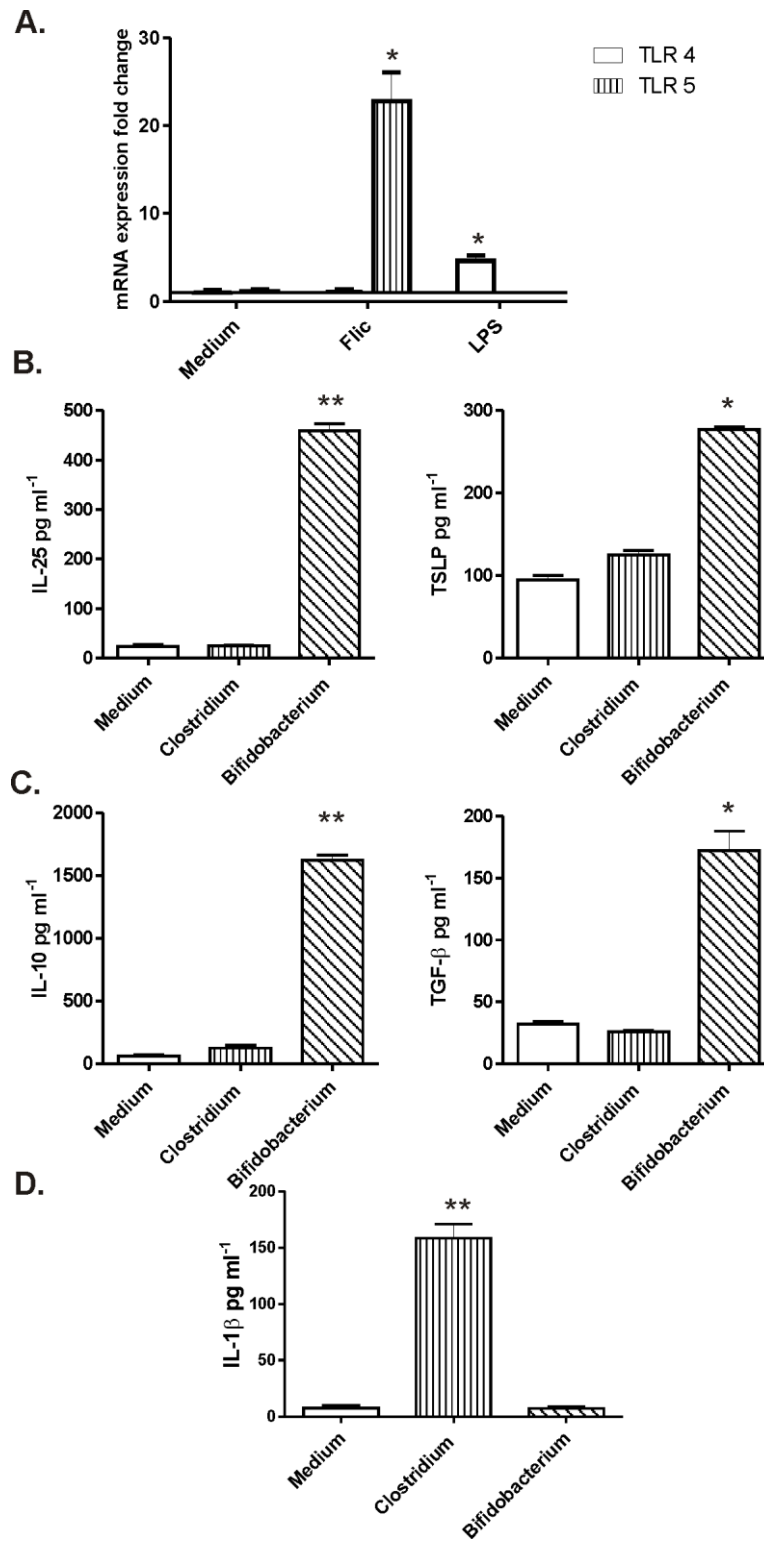


Figure 3