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A method for high purity intestinal epithelial cell culture from adult human and murine tissues for the investigation of innate immune function

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Abstract

Intestinal epithelial cells (IECs) serve as an important physiologic barrier between environmental antigens and the host intestinal immune system. Thus, IECs serve as a first line of defense and may act as sentinel cells during inflammatory insults. Despite recent renewed interest in IEC contributions to host immune function, the study of primary IEC has been hindered by lack of a robust culture technique, particularly for small intestinal and adult tissues. Here, a novel adaptation for culture of primary IEC is described for human duodenal organ donor tissue as well as duodenum and colon of adult mice. These epithelial cell cultures display characteristic phenotypes and are of high purity. In addition, the innate immune function of human primary IEC, specifically with regard to Toll-like receptor (TLR) expression and microbial ligand responsiveness, is contrasted with a commonly used intestinal epithelial cell line (HT-29). Specifically, TLR expression at the mRNA level and production of cytokine (IFN γ and TNF α) in response to TLR agonist stimulation is assessed. Differential expression of TLRs as well as innate immune responses to ligand stimulation is observed in human-derived cultures compared to that of HT-29. Thus, use of this adapted method to culture primary epithelial cells from adult human donors and from adult mice will allow for more appropriate studies of IECs as innate immune effectors.

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Keywords

Intestinal epithelial cells; Primary cell culture; Human; Murine; Innate immunity

1. Introduction

The intestinal epithelium is the largest single site of environmental exposure, immune activation, and tolerance induction in the body (MacDonald et al., 2011). With a surface area of approximately 400 m² in humans, a highly dynamic and highly regulated single-cell layer of intestinal epithelial cells (IECs) serves as a first line of defense and mediates an intricate balance between tolerance and host effector responses (Peterson and Artis, 2014). At the individual cell level, IECs exist in a bimodal state — participating in either nutrient absorption or immune activation (Shulzhenko et al., 2011). IECs express a wide variety of molecules crucial for microbial sensing, and as immune educators have the capacity to participate in antigen presentation, co-stimulation, and lymphocyte adhesion and trafficking (Fukata and Arditi, 2013; Nakazawa et al., 1999, 2004; Perera et al., 2007; Mayer, 2000). In addition, IECs are a source of a wide array of cytokines and chemokines (Stadnyk, 1994). Together these properties allow IEC to regulate intestinal immune populations, and thereby initiate, propagate, and regulate intestinal inflammation (Mayer, 2005; Shen and Turner, 2006).

There has been recent renewed interest in understanding the environment–IEC–host immune system triologue (Shulzhenko et al., 2011). Although IECs have the capacity to shape the functional outcomes of these dialogues, investigations of these interactions are hindered by a lack of robust primary cell culture techniques. To overcome this hurdle, epithelial biologists have relied on human or murine-derived cell lines, which are often of tumorigenic origin (Langerholc et al., 2011). While these cell lines have numerous benefits, they may exhibit defects in transcription factors downstream of multiple microbial sensing pathways (Sato et al., 2009). In addition, due to the limited numbers and sources of the cell lines, there is a restriction of those HLA/MHC interactions which can be investigated (Sato et al., 2009; Melmed et al., 2003). Similarly, these cell lines limit the evaluation of disease-specific IEC phenomena. Thus, the establishment of robust primary IEC cultures to evaluate innate immune function under conditions of and disease will facilitate a more intricate evaluation of IEC-specific contributions to disease processes.

Although primary IEC cultures have been periodically described, these systems have largely focused on IEC derived from fetal sources (Perreault and Beaulieu, 1998) or organoid-type studies (Gracz et al., 2012). Similarly, while recent advances have been made in IEC monolayer culture systems from murine colonic sources (Moon et al., 2013), small intestinal and human primary IEC monolayer culture remains a challenge. The development of adult primary IEC culture techniques is important for the analysis of IEC function in disease progression, as well as under conditions of exposure to environmental and microbial diversity, rather than under conditions which lack antigenic experience as is the case with fetal-derived IEC cultures (Perreault and Beaulieu, 1998). The development of methods to study small intestinal epithelial cell innate immune function is particularly important due to

the fact that the small and large intestines have been shown to express different levels of innate immune receptors, likely resulting in functional differences between these two populations (Abreu et al., 2003). Thus, in order to most accurately study innate immune function of the intestinal epithelium, it is crucial to work toward developing more robust primary IEC culture methods from a variety of sources, including human adult donor tissues and mouse adult small and large intestines.

Here we present an adapted method which yields high purity IEC cultures from duodenum of adult human organ donors and adult murine duodenum and colon (Booth and O'Shea, 2002). In addition, we compare and contrast the Toll-like receptor (TLR) expression and responsiveness of IEC derived from the duodenum of adult human organ donors to a commonly utilized intestinal epithelial cell line, HT-29 (Fogh, 1975), whereby significant differences were observed. While we have focused on TLR responsiveness of primary IEC cultures, these culture methods will serve as the foundation for future studies to elucidate environment-IEC-host immune system communication and interactions, and can be utilized to evaluate a myriad of innate immune functions.

2. Materials and methods

2.1. Animals

C57BL/6 mice were maintained at the breeding facilities of the University of Florida. All procedures were performed at 10–12 weeks of age, and were conducted in accordance with the guidelines of the University of Florida Institutional Animal Care and Use Committee.

2.2. Human intestinal tissue

Adult human organ donor duodenal tissue was obtained in collaboration with the Network for Pancreatic Organ Donors (nPOD, Gainesville, FL) (Campbell-Thompson et al., 2012). Approximately 8 g (wet weight) of duodenum was received in DMEM/Ham's F-12, Nutrient Mixture with 3.15 g/L glucose, L-Glutamine, Phenol Red, HEPES and Sodium Pyruvate (Thermo Fisher Scientific, Waltham, MA). nPOD cases 6212, 6284 and 6292 were used for imaging and flow cytometric studies while nPOD cases 6230 ("case A") and 6279 ("case B") were used for TLR expression and responsiveness studies. All procedures and protocols were reviewed and approved by the University of Florida Institutional Review Board.

2.3. Primary IEC isolation and culture

Primary IECs were isolated using a protocol adapted from Booth and O'Shea (2002). Human duodenal and murine duodenal and colonic tissues were prepared by removing the longitudinal muscle layer and washing with ice-cold Mg^{2+} - and Ca^{2+} -free Hank's Balanced Salt Solution (HBSS) (Mediatech, Manassas, VA) containing 100 U penicillin, $100 \mu g ml^{-1}$ streptomycin (Mediatech), $25 \mu g ml^{-1}$ gentamycin (MP Biomedicals, Solon, OH) and 0.5 mM dithiothreitol (DTT) (Thermo Fisher Scientific), hereafter referred to as HBSS wash solution. Tissue was cut into two cm^2 pieces, suspended in 50 ml HBSS wash solution, and inverted vigorously ten times, and the contents were allowed to settle for 1 min. The supernatant was removed and the settled contents were washed an additional four times. After the fifth wash, the settled contents were removed, minced with a sterile surgical

scalpel, and suspended in 50 ml of the HBSS wash solution. The resulting suspension was passed over a 1000 μm^2 mesh filter. Remaining tissue was digested in 50 ml of a digestion buffer containing 75 U ml^{-1} collagenase type XI (Sigma-Aldrich, St. Louis, MO), 20 μg ml^{-1} dispase neutral protease II (Roche, Indianapolis, IN), 0.5 mM DTT, and 1% v/v fetal bovine serum (FBS) (Thermo Fisher Scientific) in Dulbecco's Modification of Eagles Medium with 4.5 g/l glucose and L-glutamine, without sodium pyruvate (DMEM) (Corning, Corning, NY). The digestion buffer containing the tissue was then evenly divided, placed in a 37 °C incubator and allowed to shake at 180 rpm for 3 h. The resulting digestion mixture was again passed over a 1000 μm^2 filter, and the tissue fragments atop the filter were washed with 25 ml complete growth media (DMEM, 8.5 g/l sodium pyruvate [Mediatech], 2.5% v/v FBS, 0.25 U ml^{-1} insulin [Sigma-Aldrich], 100 U penicillin, 100 μg ml^{-1} streptomycin, 25 μg ml^{-1} gentamycin, 5 μg ml^{-1} transferrin [Sigma-Aldrich], and 10 ng ml^{-1} epidermal growth factor [Sigma-Aldrich]) containing 2% w/v D-sorbitol (S-DMEM) (Sigma-Aldrich). Tissue debris remaining in the filter was discarded, and the effluent containing proliferative crypt structures was centrifuged at 200 $\times g$, for 4 min, at 4 °C. The remaining pellet containing isolated intestinal crypts was suspended in S-DMEM; this process was repeated four times. After the final wash, the crypts were suspended in complete growth media, plated at a density of approximately 800 crypts/ml/well in a 24-well type I collagen-coated culture dish (EMD Millipore, Billerica, MA) and incubated at 37 °C, 7.5% CO_2 . A complete media change was performed after 48 h in culture, and the epithelial cells outgrown from the crypts were allowed to grow to confluence for 7 days; 50% media changes were conducted every 5 days thereafter.

2.4. HT-29 cell culture

HT-29 cells (ATCC, Manassas, VA, USA) were cultured in McCoy's 5A medium with L-glutamine (ATCC) containing 100 U penicillin and 100 μg ml^{-1} streptomycin, supplemented with 10% FBS, and maintained at 5% CO_2 in a 37 °C incubator. A complete media change was performed every three days.

2.5. Immunofluorescence microscopy

Crypts isolated from human duodenal, murine duodenal, and murine colonic tissues were either plated on type-I collagen coated glass coverslips or stained directly in collagen-coated 24-well plates, while HT-29 cells were plated directly on glass coverslips. Once confluent, cells were fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature. Cells were washed 3 times with phosphate buffered saline (PBS), blocked and permeabilized with a blocking buffer containing 1% bovine serum albumin and 0.1% Triton X-100 for 1 h at room temperature. Either 10% normal goat serum or 10% normal donkey serum was added to the blocking buffer. When antibodies raised in mouse were applied to mDEC or mCEC cultures anti-mouse CD16/CD32 Fc Block (BD Biosciences, San Diego, CA) was included in the blocking buffer. Cells were then incubated overnight at 4 °C with either FITC-conjugated anti-pan cytokeratin antibody (1:100, clone C-11, Abcam, Cambridge, EN), rabbit anti-ZO1 (1:100, polyclonal [Z-R1], Life Technologies, Carlsbad, CA), or mouse anti-cytokeratin 18 (1:100, clone C-04, Abcam) diluted in blocking buffer. Cells were then washed with PBS and either AlexaFluor® 647 goat anti-rabbit IgG antibody (1:400, Life Technologies) or AlexaFluor® 488 donkey anti-mouse IgG antibody (1:400, Life

Technologies) diluted in PBS was applied for 1 h at room temperature (RT). Following washing, cells were then incubated for 5 min at RT with 4',6-diamidino-2-phenylindole (DAPI) diluted in PBS (300 nM) and imaged directly in PBS or coverslips were mounted with VECTASHIELD® HardSet™ Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Incubation with secondary antibodies only or the proper isotype antibodies were used as negative controls. Fluorescent images were captured with either a Zeiss Axiovert 200 M Microscope fitted with a Zeiss AxioCam MRm camera or an EVOS® FL imaging system. Image analysis was performed using ImageJ (Bethesda, MD, USA) and OMERO (Goldberg et al., 2005). Acquisition settings were identical across groups. Adjustment of contrast and/or brightness was applied evenly over the entire image field.

2.6. Periodic acid–Schiff stain

Cells were cultured in a collagen-coated plastic dish as previously described. After 5 days in culture, media was removed and cells were fixed with 4% PFA for 30 min at RT. Following fixation, cells were washed with PBS and then stained using a Periodic acid–Schiff Kit (Sigma-Aldrich) according to manufacturer's instructions. Following staining, cells were imaged using a Micromaster inverted light microscope (Fisher Scientific) and images were captured using a 5.0 megapixel Digital Microscope Eyepiece Camera (Premier HiROCAM MA88, C&A Scientific Co, Manassas, VA) and accompanying TSView software (C&A Scientific).

2.7. Flow cytometry

Primary IECs were cultured as described on bovine type I collagen-coated Nunc™ Upcell™ dishes (Sigma, Thermo Fisher Scientific) while HT-29 were cultured as described on Nunc™ Upcell™ dishes (Thermo Fisher Scientific). Cells were lifted from the Nunc™ Upcell™ dishes by incubating at 4 °C for 20 min, after which cell suspensions were passed over a 70 µm cell strainer (Thermo Fisher Scientific) and blocked with either 10% human serum (Sigma Aldrich) or anti-mouse CD16/CD32 Fc Block for 30 min at 4 °C. When streptavidin conjugated secondary antibodies were used, cells were additionally blocked using avidin–biotin blocking kit (Vector Laboratories). Cells were then fixed, permeabilized (BD Cytotfix/Cytoperm, BD Biosciences) and stained with FITC-conjugated anti-pan cytokeratin antibody (clone C-11, Abcam), biotinylated anti-mouse CD104 (clone 346-11A, BioLegend, San Diego, CA, USA) or biotinylated anti-human CD104 (clone 439-9B, Novus Biologicals, Littleton, CO, USA) followed by streptavidin-PE (BD Biosciences), FITC-conjugated anti-CD3 (mouse: clone 145-2C11, human: clone HIT3a), APC-conjugated anti-CD11b (mouse: clone M1/70, human: clone ICRF44), APC-conjugated anti-CD11c (mouse: clone N418, human: clone BU15 [Thermo Fisher Scientific]), or PE-conjugated anti-CD19 (mouse: clone eBio1D3, human: clone J3-119 [Beckman Coulter, Indianapolis, IN]). All antibodies were purchased from eBioscience (San Diego, CA, USA) unless otherwise noted, and used at manufacturer's recommended dilutions. Manufacturer's recommended isotype controls were used as negative controls for all antibodies used. A total of 200,000 ungated events per sample were acquired using a BD Accuri C6 flow cytometer (BD Biosciences) and data were analyzed using BD Accuri C6 software (BD Biosciences).

2.8. Real-time PCR

Total RNA from unstimulated primary IEC or HT-29 cultures was harvested using an RNeasy extraction kit (Qiagen, Valencia, CA). RNA was reverse transcribed to generate cDNA. Primers specific for 18rRNA, hTLR-1, h-TLR2, hTLR-3, hTLR-4, hTLR-5, hTLR-6, hTLR-7, hTLR-8 and hTLR-9 RT² qPCR primers (SABiosciences, Frederick, MD) along with SsoAdvanced™ SYBR Green Supermix (BioRad, Hercules, CA) were used for qPCR. Standard curves were used to determine mRNA transcript copy number in individual reactions. Data were collected using CFX Connect (BioRad) and analyzed using CFX Manager™ Software (BioRad). Data were normalized to 18rRNA.

2.9. IEC TLR stimulation

7 days following plating of crypts, IEC cultures were primed or not with 20 ng/ml of species-specific human interferon gamma (IFN γ) (Invivogen, San Diego, CA, USA) or mouse IFN γ (Thermo Fisher Scientific) for 12 h after which IEC cultures were stimulated or not with 1 μ g/ml of ultrapure TLR agonists (FSL1 [TLR2/6], Pam(3)CSK(4) [TLR 2/1], LPS [TLR4], ODN-2395 [TLR9], LPS [TLR2], and FLA-ST [TLR5]) (Invivogen). Culture supernatants were collected 6 h following TLR agonist stimulation to evaluate soluble mediator expression.

2.10. ELISA

Human cytokine ELISA kits (BD Biosciences) were used to quantify IFN γ and tumor necrosis factor alpha (TNF α) in culture supernatants according to manufacturer's instructions. Concentrations were determined using a spectrophotometer and standard curves. Data were normalized to total RNA.

3. Results

3.1. Primary IEC cultures display characteristic intestinal epithelial morphology

Here a novel use and adaptation of a protocol for culture of high purity intestinal epithelial cells from the duodenum of human organ donors (hDEC) and from the duodenum (mDEC) and colon (mCEC) of adult mice is presented (Langerholc et al., 2011). Specifically, duodenal tissues from adult human cadaveric donors or from the duodenum and colon of adult C57BL/6 mice were processed to obtain crypt organoid structures (Fig. 1A). Notably, organoids derived from colonic tissues often form donut-like clusters. After 48 h in culture, cells with epithelial-like cobblestone morphology were observed growing outward from the central crypt structure (Fig. 1B). After seven days in culture, confluent fields of epithelial cells were apparent (Fig. 1C). Crypt structures frequently remained visible in confluent cultures.

3.2. Characterization of primary IEC cultures

Initial characterization of the primary IEC culture monolayers was performed by immunofluorescence (IF) and compared to the HT-29 human intestinal epithelial cell line. IF was performed 7 days following the initial plating, whereby IECs derived from human (hDEC) and murine (mDEC and mCEC) adult tissues exhibit expected cobblestone

morphology and cytokeratin immunoreactivity, similar to that observed in the HT-29 cell line (Fig. 2A–E). In addition, further characterization of the cultures by flow cytometric analysis also revealed expression of cytokeratin and the epithelial cell marker CD104 in greater than 90% of the primary cultures, similar to that observed in the HT-29 cell line (Fig. 2F–I).

Interestingly, a variety of morphologies were apparent in the IEC cultures (Fig. 3). In addition to the expected cobble stone morphology (Fig. 3A), a subpopulation of cells exhibited large, rounded phenotypes or granular morphologies accompanied by a distally situated nuclei indicative of goblet cells. In order to better characterize the apparent goblet cell phenotype, production of mucopolysaccharides and expression of the goblet cell-specific mucin, mucin 2 (Muc2), were evaluated in hDEC cultures by Periodic acid–Schiff (PAS) staining and IF, respectively. PAS staining as well as Muc2 expression was detected specifically in cells with the observed goblet cell morphology (Fig. 3B, C).

Further characterization of these cultures again utilized IF to probe for cytokeratin 18 (K18), whose mRNA expression is more associated within the intestinal crypt than the villus, and ZO-1, a well characterized marker of tight junctions (Flint et al., 1994). Similar to the pan-cytokeratin reactivity, K18 expression was observed in hDEC, mDEC and mCEC cultures, although it was more strongly associated with cells nearer the crypts and leading edges of the cultures (Fig. 4A, D, G and data not shown). In addition, ZO-1 expression was also detected at the junction between individual cells similar to that observed in HT-29.

Together, these data demonstrate phenotypic similarities between primary IEC cultures and HT-29, although primary cultures result in a variety of cellular morphologies. This variety of morphologies suggest that the primary culture system described here has the capacity to give rise to mixed cultures of fully differentiated intestinal epithelial cells, thereby allowing for studies to better recapitulate *in vivo* mechanisms.

3.3. Primary IEC cultures exhibit high purity and absence of contaminating phenotypes

To evaluate the purity of primary IEC cultures, monolayers were characterized by flow cytometric analysis which revealed an absence of cell markers indicative of contaminating immune cells including B cells (CD19), T cells (CD3), macrophages and dendritic cells (CD11b, CD11c) (Fig. 5A–C). Notably, while we did not detect CD11b in any of our cultures by flow cytometry, we did observe expression of this marker on ~30% of the HT-29 cells as previously reported (Fig. 5D) (Bouhlal et al., 2002). These data demonstrate a small phenotypic difference between primary IEC and HT-29, although whether this results in a functional difference has yet to be determined.

3.4. Human primary IECs differentially express TLRs 1–9 compared to HT-29 cell line

As previously mentioned, IECs participate in microbial sensing through TLR (Fukata and Arditi, 2013) and while immortalized cell lines are frequently used for functional and microbial responsiveness studies, alterations in transcription factor expression and function may limit their use for the evaluation of IEC-TLR responsiveness (Melmed et al., 2003; Furrie et al., 2005). Thus, the TLR expression and function in our primary hDEC was compared and contrasted to that of the cell line HT-29. First, the expression of TLRs 1–9 at

the mRNA level in unstimulated hDEC was evaluated (Fig. 6). hDEC cultures expressed all queried TLRs, although at varying levels. For instance, TLR1 was most the abundantly expressed TLR for case A (black bars) while TLR5 was the most abundantly expressed TLR for case B (white bars) (Fig. 6). Most importantly, there were significant differences in the expression levels of multiple TLRs between primary hDEC and HT-29. Specifically, there were significantly higher levels of all TLRs queried in primary hDEC compared to HT-29 with the exception of TLR7, where the expression of TLR7 was significantly higher in HT-29 compared to primary hDEC (Fig. 6). Again these data demonstrate a phenotypic difference between primary cultures and the HT-29 cell line, this time with a potential effect on innate immune function.

3.5. Human primary IECs differentially respond to a variety of microbial ligands compared to the HT-29 cell line

To characterize the innate immune function of primary cultures, the TLR responsiveness of primary hDEC cultures was compared to that of HT-29. Here, it was observed that unstimulated expression of both TNF α and IFN γ was significantly higher in hDEC cultures than that of the HT-29 cell line (Fig. 7A, B). In addition, stimulation induced a significant amount of TNF α and IFN γ in response to some TLR ligands while suppressing their expression in response to others. Importantly, this pattern of reactivity differed than that observed in HT-29 cultures (Fig. 7A, B). For instance, regardless of TLR ligand used, stimulation of HT-29 cells did not induce nor suppress TNF α or IFN γ , with the exception of *E. coli* LPS, which induced a small amount of TNF α (Fig. 7B). On the other hand, stimulation of primary cultures from case B (white bars), with FLS1 or FLA-ST resulted in the upregulation of TNF α , while stimulation with FLA-ST or ODN-2395 resulted in the suppression of IFN γ (Fig. 7A, B). Interestingly, the opposite pattern of reactivity was observed in cultures from case A (black bars) whereby stimulation with FLA-ST or ODN-2395 resulted in the upregulation of IFN γ (Fig. 7B). This difference may be due to the statistically significant difference in the expression of soluble mediators (both TNF α and IFN γ) expressed in the absence of stimulation in these two cultures (Fig. 7A, B).

Because IFN γ priming of HT-29 has been demonstrated to induce LPS responsiveness and other immune-modulating molecules (Angrisano et al., 2010), we assessed IFN γ and TNF α production of hDEC and HT-29 cultures following IFN γ priming and microbial ligand stimulation. Interestingly, while IFN γ priming had no effect on hDEC responsiveness, IFN γ did confer HT-29 responsiveness to FLS1, *E. coli* LPS and FLA-ST in the context of IFN γ expression (Fig. 7C, D). Together, these data demonstrate that the phenotypic differences observed between primary hDEC and HT-29 do translate into functional differences in their innate immune responsiveness, highlighting the importance of using primary culture systems for this type of evaluation. Importantly, additional studies are needed to evaluate the variability in primary cultures from different donors.

4. Discussion

An emerging view of inflammation-associated diseases focuses on impaired immune regulation at the intestinal level, highlighting the intestinal immune system as a potentially

critical component of disease etiology. Indeed, there has been recent renewed interest in the immune functions of the intestinal tract, and more specifically the role IECs play in initiating and shaping subsequent immune responses locally and systemically (Purchiaroni et al., 2013; Tomasello and Bedoui, 2013; Brown et al., 2013). Despite its importance, cellular techniques for assessing IEC phenotype and function as immune educators remain limited. Here, we present an adapted method for robust, pure primary IEC culture utilizing adult tissues of both humans and mice, which will serve as the foundation for future studies to elucidate environment–IEC–host immune system interactions.

Poised at the host–environment interface, IECs serve as active participants in shaping mucosal immune responses and employ a variety of host defense mechanisms, including microbial sensing through TLR (Hecht, 1999). TLRs 1–9 are expressed at the mRNA or protein level in IEC and utilize NF- κ B in their signaling pathway (Abreu et al., 2003). In health, a variety of mechanisms are employed by IEC to avoid exuberant inflammatory responses to nonpathogenic microbial signals, yet the mechanisms and totality of the functional outcomes of IEC-TLR signaling in health remain unclear (Shibolet and Podolsky, 2007). In addition to physiologic regulation in health, TLR, TLR-related signaling adaptors, and the downstream transcription factor NF- κ B have been recognized as key components in the initiation and progression of multiple inflammatory conditions of the gastrointestinal tract including colorectal cancer (Sato et al., 2009; Vaipopoulos et al., 2013; Wang et al., 2009; O’Leary et al., 2012; Aviello et al., 2014; Luddy et al., 2014). Therefore, a system that would allow for the delineation of IEC-TLR signaling under conditions of both health and disease is needed.

As previously mentioned, to date IEC-TLR responsiveness has been primarily evaluated in cell lines (Bocker et al., 2003; Cario et al., 2000). Unfortunately, functional differences in NF- κ B regulation have been demonstrated among common IEC lines with the most robust differences seen between cell lines and primary colonic epithelial cells (Jobin et al., 1997). Here, we present evidence of significant differences in TLR expression between hDEC cultures of two donors and the HT-29 cell line, whereby TLR 1, 2, 3, 4, 5, 6, 8, and 9 were expressed significantly higher in the primary cell cultures, while HT-29 cell cultures expressed significantly higher TLR7. In addition, we found that hDEC not only had a differential response to microbial ligand stimulation than the HT-29 cell line, but also expressed a higher level of both TNF α and IFN γ prior to stimulation. Recently it has been suggested that the traditionally pro-inflammatory cytokine IFN γ can contribute to intestinal homeostasis in an IEC specific manner, although the totality of the mechanisms remain undefined (Thelemann et al., 2014). To our knowledge, the constitutive cytokine expression of primary IECs, or IECs *in vivo* is not fully understood. This is in part due to heavy reliance on tumorigenic cell lines, which we and others have demonstrated to express very low levels of cytokines at the protein level in general. In addition, IEC-specific expression of cytokines *in vivo* is difficult to assess even under conditions of health considering the number of resident immune cells within the gastrointestinal tract. Taken together, this again highlights the need for a system that would allow for this delineation.

Here we also demonstrate that TLR ligation had negligible effects on HT-29 cultures but was able to induce and suppress cytokine expression by hDEC cultures in a ligand-

dependent manner. Most notably, this response pattern was also different between the hDEC cultures assessed. Interestingly, our data suggests that TLR stimulation of primary epithelial cell cultures with a high IFN γ to TNF α ratio (case B) results in a suppression of TLR-induced IFN γ responses, while those with a lower IFN γ to TNF α ratio promote expression of TLR-induced IFN γ . These data along with the current literature (Melmed et al., 2003) demonstrate the potential issues associated with assessing TLR-responsiveness in cell lines and highlight the need to use primary cell culture systems.

TLR expression differences have been noted between small and large intestines, suggesting that the expression and engagement of these receptors at these anatomical sites may result in differing functional outcomes (Abreu, 2010). In addition, the small intestine contains the highest density of immune cells within the intestine, and is an important site for maintaining immune balance, whereby epithelial cells themselves may contribute to the process of tolerance (Karlsson et al., 2001). For instance, M cells of Peyer's patches and goblet cells in the small intestine appear uniquely equipped for antigen sampling and maintenance of local and systemic oral tolerance (McDole et al., 2012; Fujihashi et al., 2001). Moreover, IL-17 expressing CD4 $^{+}$ T cells, which provide anti-microbial immunity at epithelial surfaces and are often implicated in autoimmune disease, are expressed at a higher proportion in the small intestine than in the colon; additionally, their development appears to require intestinal microbiota (Goto et al., 2014; Esplugues et al., 2011). Together, these findings suggest that IECs from different compartments of the gastrointestinal tract may have different innate immune functions, and highlight the need for primary cultures of small intestinal epithelial cells for the study of IEC-immune regulation. While recent advances have been made in IEC monolayer culture systems from murine colonic sources (Moon et al., 2013), small intestinal primary IEC in monolayer culture remains a challenge. Here we present a protocol that allows for the cultivation of primary IEC from the small intestine of both mouse and man. While we demonstrate that this technique is also suitable for murine colonic tissues, we have not utilized this approach with human colonic tissue due to limited sources of these tissues and thus cannot speak to its efficacy in this cell population at this time.

To date, adult-derived IEC culture systems have also remained problematic (Chopra et al., 2010; Leushacke and Barker, 2014; Kaeffer, 2002). The development of adult primary IEC culture presented here will facilitate the analysis of IEC function under multiple experimental conditions. For instance, an adult primary IEC culture will allow for evaluation of IEC function at different stages of disease progression. Similarly, use of these cultures will improve study of how the aging process affects IEC education and functional outcomes thereof — indeed age-associated differences in TLR expression and function have been reported (Panda et al., 2010; van Duin et al., 2007; Boehmer et al., 2004; Dunston and Griffiths, 2010). Importantly, it will also allow for evaluation of IEC function under conditions of exposure to a rich environmental and microbial diversity, rather than under conditions which lack antigenic experience as is the case with fetal-derived IEC cultures (Perreault and Beaulieu, 1998). This point highlights a caveat of the method presented here whereby *in vivo*, IECs are normally exposed to microbiota and microbial products during differentiation from the crypt, which may affect the way these cells are programmed to respond to TLR-ligation (Ewaschuk et al., 2007) — a consideration our method does not

incorporate. With that said, this method could be used to evaluate the effect of natural flora biofilms under conditions of health and disease on the differentiation and shaping of the innate immune function of IECs.

Intestinal immune activation has been implicated not only in local inflammatory diseases such as celiac disease, irritable bowel syndrome, inflammatory bowel disease, and colon cancer, but also in systemic inflammatory diseases including rheumatoid arthritis, type 1 diabetes, and multiple sclerosis (Nissinen et al., 2004; Vaarala et al., 2008; Alkhawajah et al., 2013). The role of IEC innate immune function has been implicated in this process. For instance, it has been observed that local gastrointestinal inflammation can contribute to upregulation of IEC-TLR expression (de Kivit et al., 2011). What is unclear due to the lack of primary models is whether the converse is also true — if increased IEC-TLR expression can result in local inflammation rather than tolerance. For instance, it has been shown that IEC can induce IFN γ secretion by CD8 $^{+}$ T lymphocytes in IBD although the role of IEC-TLRs in the process is unclear (Bisping et al., 2001). On the other hand, models have demonstrated that IEC-TLR2 expression can modulate tight and gap junctions of the intestinal epithelium (Cario et al., 2004; Fasano, 2012). Understanding the contribution of IEC function (or dysfunction) to the intestinal immune system during or prior to such diseases may be a critical component of understanding the natural progression of both local and systemic diseases. As such, the development of adult primary IEC culture from murine sources as presented here is important in that it will allow the analysis of IEC function at specific stages of disease progress, where appropriate murine models of human disease are available. Additionally, primary IEC culture from adult human donors will allow for translation of relevant findings from murine models. Together this will promote scientific discovery specific to human biology and disease.

In summary, here we present a method that will allow for the isolation and propagation of IEC from adult human and murine sources. The protocol presented can be applied to adult organ donor tissue, as well as both small intestinal and colonic sources of adult murine tissue. Most importantly, we describe key differences in the phenotype and function of primary IEC cultures as compared to a conventional cell line. Moreover, we demonstrate some phenotypic and functional heterogeneity in hDEC cultures derived from human donors, which may reflect in vivo differences among human donors. While functional data was presented on human cultures, similar results were seen with those derived from murine models (data not shown). The presented methods will serve as the foundation for future studies to elucidate environment–IEC–host immune system interactions, and although the presented studies investigated TLR-responsiveness of primary cultures, these methods can be easily adapted to include a variety of stimuli including, but not limited to, manual, chemical, and biological insults.

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Fig. 1. Epithelial outgrowth from isolated crypts. DIC imaging at different time points during culture reveals the following: (A) Intestinal crypts containing proliferative stem cells are isolated by enzymatic digestion. (B) After 48 h in culture, primary IECs begin to grow outward from the crypt. (C) After 7 days in culture, confluent monolayers with cobblestone morphology are formed. Scale bar 20 μm.

Fig. 2.

Characterization of primary IEC cultures. Monolayers of (A) human duodenal epithelial cells (hDEC), (B) murine duodenal epithelial cells (mDEC), (C) murine colonic epithelial cells (mCEC) and (D) the human intestinal cell line HT-29 were probed with DAPI (blue) to visualize nuclei and anti-pan-cytokeratin antibody (green). (E) An appropriate isotype control antibody verified absence of non-specific labeling. (F–I) Seven days following plating, hDEC, mDEC, mCEC and HT-29 cultures were analyzed by flow cytometry for the expression of two independent markers of epithelial cells — cytochrome c and CD104.

Fig. 3.

Multiple cellular morphologies in hDEC cultures. Human primary IEC cultures grown to confluence contain cells with cobblestone morphology, large, rounded cell body, and distally situated nuclei with vesicles, which lack cytokeratin expression. (B) hDEC were stained using a Periodic acid–Schiff (PAS) staining procedure to visualize expression of mucopolysaccharides. (C) hDEC were labeled with DAPI (blue) to visualize nuclei and anti-Muc2 (red) to evaluate expression of mucin 2. Incubation with secondary antibodies only or the proper isotype antibodies verified absence of non-specific labeling. Scale bar 5 μ m.

Fig. 4.

Immunocytochemical characterization K18 and ZO1 expression. Monolayers of (A, B, C) human duodenal epithelial cells (hDEC), (D, E, F) murine duodenal epithelial cells (mDEC), (G, H, I) murine colonic epithelial cells (mCEC) and (J, K, L) the human intestinal cell line HT-29 were probed with DAPI (blue) to visualize nuclei and either (A, D, G, J) anti-keratin 18 (red) or (B, E, H, K) anti-ZO1 antibody (green). (C, F, I, L) Incubation with secondary antibodies only or the proper isotype antibodies verified absence of non-specific labeling. Scale bar 5 μ m.

Fig. 5.

Human and mouse primary IEC cultures do not express common immune cell markers. Seven days following plating, hDEC, mDEC, mCEC and HT-29 cultures were analyzed by flow cytometry for markers of immune cell contamination CD19, CD3, CD11b, and CD11c (red line; black line = isotype control). The data are representative of 3 independent experiments.

Fig. 6.

Human primary IECs express TLR1–9. Two cases of primary hDEC (case A = black; case B = white) and HT-29 cell lines (gray) were probed by qPCR for TLR mRNA expression. Data are an average of triplicates. * $p < 0.05$ HT29 vs. case A and case B; ^ $p < 0.05$ HT29 vs. case B; # $p < 0.05$ case A vs. case B. Student's *T* test.

Fig. 7.

Human primary IECs are responsive to a variety of microbial ligand agonists. Two cases of primary hDEC (case A = black; case B = white) and HT-29 cell lines (gray) were cultured to confluence in a 24 well plate. (A–B) IEC responsiveness to $1 \mu\text{g ml}^{-1}$ of TLR agonists indicated was evaluated after 6 h of stimulation. (C–D) Primary hDEC cultures were primed with $20 \text{ ng } \mu\text{l}^{-1}$ of human $\text{IFN}\gamma$ 12 h prior to TLR agonist stimulation, whereas HT-29 cells were primed for 72 h prior to TLR agonist stimulation. Data are an average of triplicates. TLR agonists were FSL1 (TLR2/6), Pam(3)CSK(4) (TLR 2/1), *E. coli* LPS (TLR4), ODN-2395 (TLR9), *P. gingivalis* LPS (TLR2), and FLA-ST (TLR5). * $p < 0.05$ HT29 vs. case A and case B; ^ $p < 0.05$ HT29 vs. case B; # $p < 0.05$ case A vs. case B. † $p < 0.05$ HT-29 unstimulated vs. *E. coli* LPS (A, D), FLS1 (D), and FLA-ST (D). ‡ $p < 0.05$ case B unstimulated vs. FLS1 (A), FLA-ST (A, B), and ODN-D-2395 (B). ∇ $p < 0.05$ case A unstimulated vs. *E. coli* LPS (7A), FLA-ST (7D) and ODN-2395 (7B, D). Student's *T* test.