Wallet,Shannon Margaret

From: ees.jim.b.2b7c32.27c43456@eesmail.elsevier.com on behalf of M.C. Nussenzweig <nussen@rockefeller.edu>
Sent: Monday, August 25, 2014 10:08 AM
To: Wallet,Shannon Margaret
Subject: Your Submission

Ms. Ref. No.: JIM-D-14-00164R1
Title: A METHOD FOR HIGH PURITY INTESTINAL EPITHELIAL CELL CULTURE FROM ADULT HUMAN AND MURINE TISSUES FOR THE INVESTIGATION OF INNATE IMMUNE FUNCTION Journal of Immunological Methods

Dear Dr. Wallet,

I am pleased to confirm that your paper "A METHOD FOR HIGH PURITY INTESTINAL EPITHELIAL CELL CULTURE FROM ADULT HUMAN AND MURINE TISSUES FOR THE INVESTIGATION OF INNATE IMMUNE FUNCTION" has been accepted for publication in Journal of Immunological Methods.

Comments from the Editor and Reviewers can be found below.

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Thank you for submitting your work to this journal.

With kind regards,

M.C. Nussenzweig, M.D., Ph.D.
Editor in Chief
Journal of Immunological Methods

The Journal of Immunological Methods is the official journal of the Association of Medical Laboratory Immunologists (AMLI). A major focus of the AMLI is to convene an annual meeting exploring various aspects of immunology and medical laboratory immunology. If you are not already a member of the AMLI and wish to know more about it's activities, please visit http://www.amli.org

Comments from the Editors and Reviewers:

Reviewer #1: The authors have adequately addressed the reviewer's major comments in the revision. It is a little disappointing that the IEC monolayers are not polarized, since that would have provided a much more robust model to study TLR signaling and epithelial barrier function. However, the method is still a significant technical contribution and meets the ongoing need for a facile in vitro culture model to study mouse IECs responses.

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Article Type: Research Report

Keywords: intestinal epithelial cells, primary cell culture, human, murine, innate immunity

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Abstract: Intestinal epithelial cells (IEC) serve as an important physiologic barrier between environmental antigens and the host intestinal immune system. Thus, IEC 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 and TNF) 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 IEC as innate immune effectors.

*Cover Letter

UF UNIVERSITY of FLORIDA

M.C. Nussenzweig, M.D., Ph. D.
Editor-in-Chief
Journal of Immunological Methods

July 8, 2014

Dear Dr. Nussenzweig,

Re: Ms. Ref. No.: JIM-D-14-00164

We greatly appreciate your willingness to review our revised manuscript entitled, “A METHOD FOR HIGH PURITY INTESTINAL EPITHELIAL CELL CULTURE FROM ADULT HUMAN AND MURINE TISSUES FOR THE INVESTIGATION OF INNATE IMMUNE FUNCTION,” for publication in the Journal of Immunological Methods.

As a reminder, here we present a novel protocol and experimental data which has not been submitted for publication anywhere else. Specifically this manuscript reports on an adapted method to culture primary epithelial cells from adult human donors and from adult mice. Importantly the innate immune responsiveness of these cultures were compared and contrasted with traditional intestinal epithelial cell lines. Data presented here provide rationale and methods for the use of primary epithelial cell cultures for the studies of intestinal epithelial cells as innate immune effectors.

We appreciate the insightful comments from the reviewer which has significantly improved the manuscript. We have responded in a point-by-point fashion to the reviewer. Based on these revisions we hope our manuscript is now acceptable for publication in Journal of Immunological Methods.

If you have any questions, or need any further information, please feel free contact me.

Sincerely,

Shannon Wallet

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***Response to Reviewers**

JIM-D-14-00164 Response to the Reviewer: We appreciate the insightful comments from the reviewer which has significantly improved the manuscript. We have responded in a point-by-point fashion to the reviewer.

This manuscript describes a promising technique for isolating and culturing primary human and murine intestinal epithelial cells (IECs). A facile and reproducible source of primary IEC with physiological expression of innate sensing molecules (i.e., TLRs and) would be an asset to the mucosal immunology field and facilitate molecular and cellular studies of intestinal epithelial responses. The strength of the manuscript is that is clearly describes a simple procedure for isolating IEC. The manuscript could be strengthened by adding more human donor tissue specimens to address donor-dependent variability/robustness and the cultured IECs were better characterized in terms of phenotype and function.

1. TLR expression was only examined by RT-PCR. It would enhance the manuscript if, in a limited number of experiments, cultured IECs were stained with antibodies to some of the TLRs (TLR1, TLR4 or TLR5). Also, confocal microscopy would give better resolved images in Figure 5 and would make it possible to show that the TLRs are expressed and localize normally (i.e., basolateral or apical). *Because the cultures described in this manuscript have not been cultured for polarity we cannot evaluate the localization of the TLRs at this time, thus we feel that qPCR is the most appropriate for quantification of these receptors. Future experiments will evaluate proper localization following polarization of the cell cultures.*

2. In figure 5, it looks like unstimulated IEC express cytokines constitutively. Is this physiological? Also, assuming that IEC are normally exposed to microbiota *in vivo*, would they be expected to respond to LPS (for example) by making inflammatory cytokines such as TNF-a? Please address these concerns in the discussion. *To our knowledge, the constitutive cytokine expression of primary IEC, or IEC in vivo is not fully understood. In part, this is due to heavy reliance on tumorigenic cell lines, which are known to and which we have demonstrated to express very low levels of "proinflammatory" cytokines at the protein level. 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 GI tract. Future experiments utilizing this system will evaluate the effect of natural flora biofilms on the differentiation and innate immune function of these cultures. This manuscript is a description of the culture system and a demonstration that primary IECs do express innate immune receptors and have innate immune responsiveness (constitutive and induced) which differs from traditionally utilized cell lines. For these reasons we feel description of this culture system is important to this field. We have added these concepts to the discussion.*

3. Figure 5 would be easier to read if the TLR ligands were identified in the figure caption (currently only in the methods section). *This has been amended as requested.*

4. Moreover, comparisons are made between IECs and HT-29 cells and between both IEC cultures, but not between unstimulated and stimulated cultures. It seems important to compare cytokine secretion between unstimulated and TLR ligand stimulated cultures within each group to make the point that TLR ligand stimulation is robust in the culture system. *This comparison and interpretation has been added to the results and discussion section.*

5. "Trialogues"… very cool! But, "interaction" might be clearer in the context, and the culture system (as described) doesn't warrant a new term, since it doesn't include normal flora or host immune cells for the trialogue. *We would prefer to keep the "trialogue" term as referenced as part*

of the introduction, thus have limited its use to the two sentences on Page 4. We agree that our results do not investigate the relationship between IECs, flora and immune cells, so we have refrained from utilizing the term "trialogue" when discussing our results.

6. It would have been nice to show data for a few more human tissue donors (1-2 more). This would provide insight into just how variable the system is in terms of TLR expression and cytokine secretion. *We agree with the reviewer and we are currently in the process describing the inherent variability along with the ability to evaluate changes when cultured from conditions of disease (such as diabetes). Due to the limited number of 'healthy' donors, we will be compiling this data for a follow-up manuscript.*

7. Also, can this approach be used with human colon tissue? *Although we have utilized this technique for MURINE colonic tissues, at this time we have not utilized this approach with HUMAN colonic tissue due to our limited sources of human tissues. Thus we have focused the scope of this manuscript on HUMAN small intestinal tissues. We have added this caveat to the discussion.*

8. IECs are normally exposed to TLR ligands in the gut, is there concern that culturing the IEC in the absence of these factors might alter their development and function? For example reduced TLR expression or responsiveness? Please discuss this caveat. *We completely agree with this insightful point. We have included this concept in the revised discussion.*

9. On page 8, it states that "the preparation was processed for immune cells" at two different places in the protocol. This begs the questions: how, why and what immune cell types were recovered in the fractions? Since the focus is on IEC, it might be better to omit this detail or add a sentence in the discussion to the effect that variations of the protocol might be used to isolate viable immune cells for further study. *We wholeheartedly agree. Since our focus is IEC innate function, we have chosen to omit the details concerning resident immune cell analysis, and will save this information for a future publication.*

10. The IEC monolayers could be better characterized. *We appreciate these suggestions by the reviewer which significantly improve our characterization.*

a. Do the cells stain with cytokeratin-18 or just pan cytokeratin? *We have utilized IF to interrogate CK-18 expression in the primary cells as well as HT-29 cells. We have added these to the results and discussion of the manuscript.*

b. Also, tight junction and adherence junction proteins were not examined. For example staining the cell monolayers for ZO-1 or E-Cad and imaging with a confocal microscope would go a long way towards showing the cells are behaving like intestinal epithelial cells. *We have also utilized IF to interrogate ZO-1 in the primary cells as well as HT-29. We have added these data to the results and discussion of the manuscript.*

c. It might also be worthwhile to perform a PAS stain or stain for Muc-2 to see if goblet cells are present in the culture. *We have performed both the PAS and MUC2 staining and have added these data to the results and discussion of the manuscript.*

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 (IEC) serve as an important physiologic barrier between environmental antigens and the host intestinal immune system. Thus, IEC 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 (IFNγ and TNFα) 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 IEC as innate immune effectors.

Keywords: intestinal epithelial cells, primary cell culture, human, murine, innate immunity

Highlights

- Method results in high purity human and murine primary intestinal epithelial cell cultures.
- Method can be utilized with adult human duodenum and murine duodenum and colon.
- Human primary cultures express higher levels of TLRs than the HT-29 cell line.
- Human primary cultures are more TLR-responsive than the HT-29 cell line.
- Primary cultures allow for more appropriate study of IEC innate immune function.

1. Introduction

The intestinal epithelium is the largest single site of environmental exposure, immune activation, and tolerance induction in the body (1). With a surface area of approximately 400 m^2 in humans, a highly dynamic and highly regulated single-cell layer of intestinal epithelial cells (IEC) serve as a first line of defense and mediate an intricate balance between tolerance and host effector responses (2). At the individual cell level, IEC exist in a bimodal state – participating in either nutrient absorption or immune activation (3). IEC 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 (4-8). In addition, IEC are a source of a wide array of cytokines and chemokines (9). Together these properties allow IEC to regulate intestinal immune populations, and thereby initiate, propagate, and regulate intestinal inflammation (10, 11).

There has been recent renewed interest in understanding the environment-IEC-host immune system trialogue (3). Although IEC have the capacity to shape the functional outcomes of these trialogues, 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 (12). While these cell lines have numerous benefits, they may exhibit defects in transcription factors downstream of multiple microbial sensing pathways (13). 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 (13, 14). 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 heath 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 (15) or organoid-type studies (16). Similarly, while recent advances have been made in IEC monolayer culture systems from murine colonic sources (17), small intestinal and human primary IEC monolayer culture remain 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 (15). 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 intestine have been shown to express different levels of innate immune receptors, likely resulting in functional differences between these two populations (18). 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 intestine.

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 (19). 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 (20), 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) (21). Approximately 8 grams (wet weight) of duodenum was received in DMEM/Ham's F-12, 1:1 Nutrient Mixture with 3.15g Glucose per Liter, 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 IEC were isolated using a protocol adapted from Booth and O’Shea (19). 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 ug ml^{-1} streptomycin (Mediatech), 25 ug 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, inverted vigorously ten times, and the contents allowed to settle for one minute. 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 ug 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 Dubelcco'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 hours. 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 ug ml^{-1} streptomycin, 25 ug ml^{-1} gentamycin, 5 ug 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 *x g*, 4 minutes, 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 hours 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 ug 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 minutes 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 hour 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 AlexaFlour® 647 goat anti-rabbit IgG antibody (1:400, Life Technologies) or AlexaFlour®488 donkey anti-mouse IgG antibody (1:400, Life Technologies) diluted in PBS was applied for 1 hour at room temperature (RT). Following washing, cells were then incubated for five minutes 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 200M Microscope fitted with a Zeiss AxioCam MRm camera or an EVOS® FL imaging system. Image analysis was performed using Image J (Bethesda, MD, USA) and OMERO (22). 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 minutes at RT. Following fixation, cells were washed with PBS and then stained using a Periodic acid-Schiff Kit (Sigma-Aldrich) according to manufacturer instructions. Following staining, cells were imaged using a Micromaster inverted light microscope (Fisher Scientific) and images 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 IEC were cultured as described on bovine type I collagen-coated NuncTM Upcell^{TM} dishes (Sigma, Thermo Fisher Scientific) while HT-29 were cultured as described on Nunc^{TM} Upcell^{TM} dishes (Thermo Fisher Scientific)**.** Cells were lifted from the Nunc^{TM} Upcell^{TM} dishes by incubating at 4°C for 20 minutes, 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 a avidin-biotin blocking kit (Vector Laboratories). Cells were then fixed, permeabilized (BD Cytofix/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 recommended dilutions. Manufacturer 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 or was harvested using an RNeasy extraction kit (Qiagen, Valencia, CA). RNA was reverse transcribed to generate cDNA. Primers specific for 18s rRNA, hTLR-1, h-TLR2, hTLR-3, hTLR-4, hTLR-5, hTLR-6, hTLR-7, hTLR-8 and hTLR-9 RT^2 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 18s rRNA.

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 (IFNγ) (Invivogen, San Diego, CA, USA) or mouse IFNγ (ThermoFisher Scientific) for twelve hours after which IEC cultures were stimulated or not with 1 ug/ml of ultra-pure TLR agonists (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]) (Invivogen). Culture supernatants were collected six hours following TLR agonist stimulation to evaluate soluble mediator expression.

2.10. ELISA. Human Cytokine ELISA kits (BD Biosciences) were used to quantify IFNγ and tumor necrosis factor alpha (TNFα) 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 (12). 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 (**Figure 1A**). Notably, organoids derived from colonic tissues often form donut-like clusters. After 48 hours in culture, cells with epithelial-like cobblestone morphology were observed growing outward from the central crypt structure (**Figure 1B**). After seven days in culture, confluent fields of epithelial cells were apparent (**Figure 1C**). Crypt structures frequently remained visible in confluent cultures.

3.2. Characterization of primary IEC cultures. Initial characterization of the primary IEC cultures 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 IEC 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 (**Figure 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 (**Figure 2F-I**). Interestingly, a variety of morphologies were apparent in the IEC cultures (**Figure 3**). In addition to the expected cobble stone morphology (**Figure 3A**), a subpopulation of cells exhibited large, rounded phenotypes (**Figure 3A^{I}**) or granular morphologies accompanied by a distally situated nuclei (**Figure 3A^{II}**) indicative of goblet cells. In order to better characterize the apparent goblet cell phenotype, production of mucopolysaccharides and expression of the goblet cell-specfic mucin, mucin 2 (Muc2), was 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 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 (23). 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 (**Figure 4 A, 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) (**Figure 5 A-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 (**Figure 5D**) (24). 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 IEC differentially express TLR1-9 compared to HT-29 cell line. As previously mentioned, IEC participate in microbial sensing through TLR (4) 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 (14, 25). 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 (**Figure 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)

(**Figure 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 primary hDEC (**Figure 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.

3.4. Human primary IEC 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 TNFα and IFNγ was significantly higher in hDEC cultures than that of the HT-29 cell line (**Figure 7 A, B**). In addition, stimulation induced a significant amount of TNFα and IFNγ 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 (**Figure 7 A, B**). For instance, regardless of TLR ligand used, stimulation of HT-29 cells did not induce nor suppress TNFα or IFNγ, with the exception of *E. coli* LPS, which induced a small amount of TNFα (**Figure 7B**). On the other hand, stimulation of primary cultures from case B (white bars), with FLS1 or FLA-ST resulted in the upregulation of TNFα, while stimulation with FLA-ST or ODN-2395 resulted in the suppression of IFNγ (**Figure 7 A, B**). Interestingly, the opposite pattern of reactivity was observed in cultures from case A (black bars) where by stimulation with FLA-ST or

ODN-2395 resulted in the upregulation of IFNγ (**Figure 7B**). This difference may be due to the statistically significant difference in the expression of soluble mediators (both TNFα and IFNγ) expressed in the absence of stimulation in these two cultures (**Figure 7 A, B**).

Because IFNγ priming of HT-29 has been demonstrated to induce LPS responsiveness and other immune-modulating molecules (26), we assessed IFNγ and TNFα production of hDEC and HT-29 cultures following IFNγ priming and microbial ligand stimulation. Interestingly, while IFNγ priming had no effect on hDEC responsiveness, IFNγ did confer HT-29 responsiveness to FLS1, *E. coli* LPS and FLA-ST in the context of IFNγ expression (**Figure 7 C, D**). Together, these data demonstrate that the phenotypic differences observed between primary hDEC and HT-29 does 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 IEC play in initiating and shaping subsequent immune responses locally and systemically (27-29). 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, IEC serve as active participants in shaping mucosal immune responses and employ a variety of host defense mechanisms, including microbial sensing through TLR (30). TLRs 1-9 are expressed at the mRNA or protein level in IEC and utilize NF-κB in their signaling pathway (18). 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 (31). In addition to physiologic regulation in health, TLR, TLR-related signaling adaptors, and the downstream transcription factor NF-κB have been recognized as key components in the initiation and progression of multiple inflammatory conditions of the gastrointestinal tract including colorectal cancer (13, 32-36). 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 (37, 38). Unfortunately, functional differences in NF-κB regulation have been demonstrated among common IEC lines with the most robust differences seen between cell lines and primary colonic epithelial cells (39). 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 TNFα and IFNγ prior to stimulation. Recently it has been suggested that the traditionally pro-inflammatory cytokine IFNγ can contribute to intestinal homeostasis in an IEC specific manner, although the totality of the mechanisms remain undefined (40) 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 IFNγ to TNFα ratio (case B) results in a suppression of TLR-induced IFNγ responses, while those with a lower IFNγ to TNFα ratio promote expression of TLR-induced IFNγ. These data along with the current

literature (14) 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 intestine, suggesting that the expression and engagement of these receptors at these anatomical sites may result in differing functional outcomes (41). 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 (42). 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 (43, 44). 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 (45, 46). Together, these findings suggest that IEC 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 (17), small intestinal primary IEC in monolayer culture remain 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 (47-49). 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 (50-53). 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 (15). This point highlights a caveat of the method presented here whereby *in vivo*, IEC are normally exposed to microbiota and microbial products during differentiation from the crypt, which may affect the way these cells are programed to respond to TLR-ligation (54) – 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 has also been implicated in systemic inflammatory diseases including

rheumatoid arthritis, type 1 diabetes, and multiple sclerosis (55-57). 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 (58). 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 IFNγ secretion by CD8+ T lymphocytes in IBD although the role of IEC-TLRs in the process is unclear (59). On the other hand, models have demonstrated that IEC-TLR2 expression can modulate tight and gap junctions of the intestinal epithelium (60, 61). 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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Figure Legends:

Fig. 1: **Epithelial outgrowth from isolated crypts.** DIC imaging at different time points during culture reveals **(A)** Intestinal crypts containing proliferative stem cells are isolated by enzymatic digestion. **(B)** After 48 hours in culture, primary IEC 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 – cytokeratin and CD104.

Fig. 3. Multiple cellular morphologies in hDEC cultures. Human primary IEC cultures grown to confluence contain cells with **(A^{I})** cobblestone morphology, **(A^{II})** large, rounded cell body, and **(A^{III})** 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 µm.

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 µm.

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, CD11c (red line; black line = isotype control). The data are representative of 3 independent experiments.

Fig. 6. Human primary IEC express TLR1-9. Two cases of primary hDEC (case A=black; case B=white) and HT-29 cell lines (grey) 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 IEC 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 (grey) were cultured to confluence in a 24 well plate**. (A-B)** IEC responsiveness to 1 ug ml^{-1} of TLR agonists indicated was evaluated after six hours of stimulation. **(C-D)** Primary hDEC cultures were primed with 20 ng ul^{-1} of human IFNγ 12 hours prior to TLR agonist stimulation, whereas HT-29 cells were primed for 72 hours 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.

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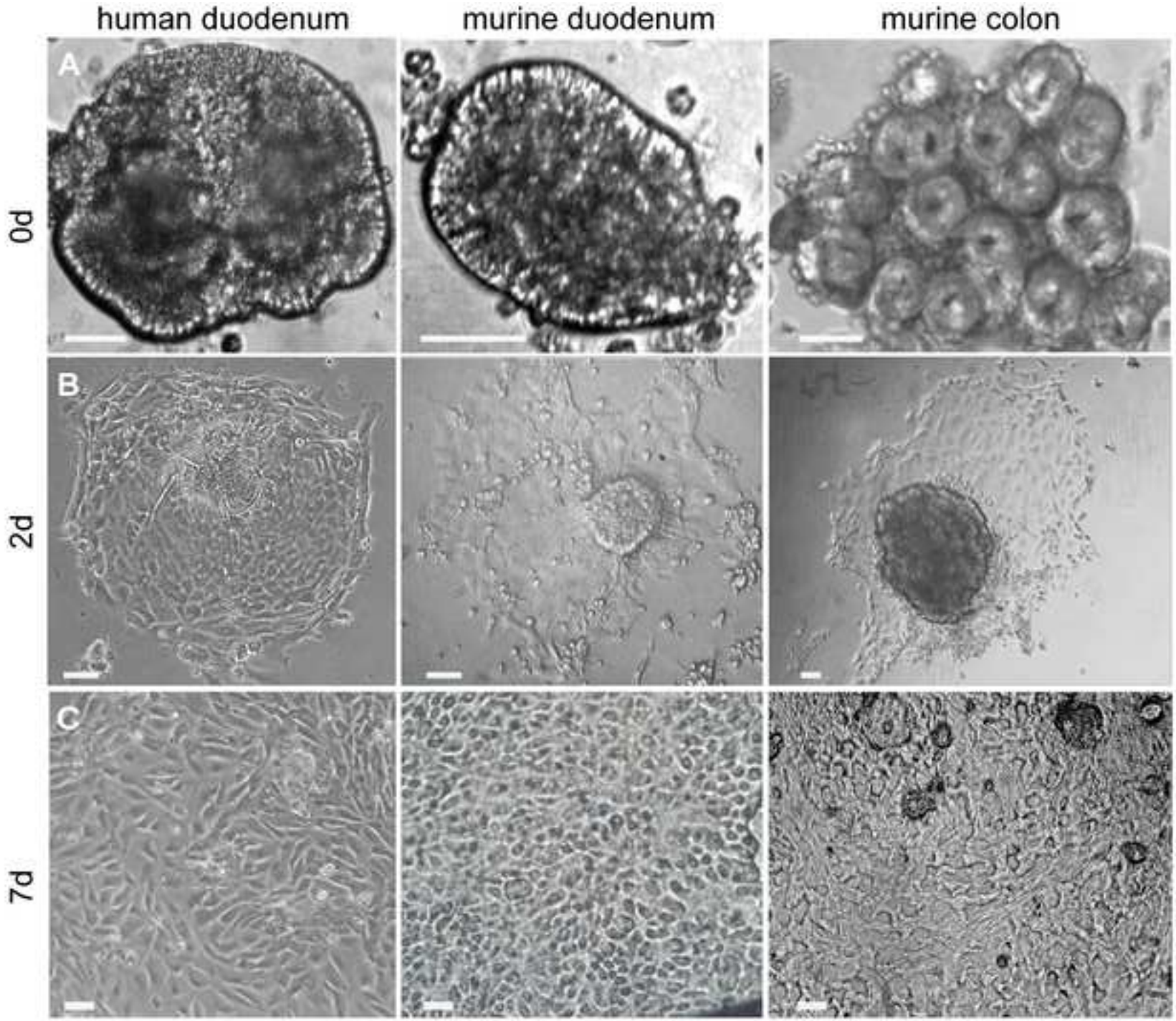

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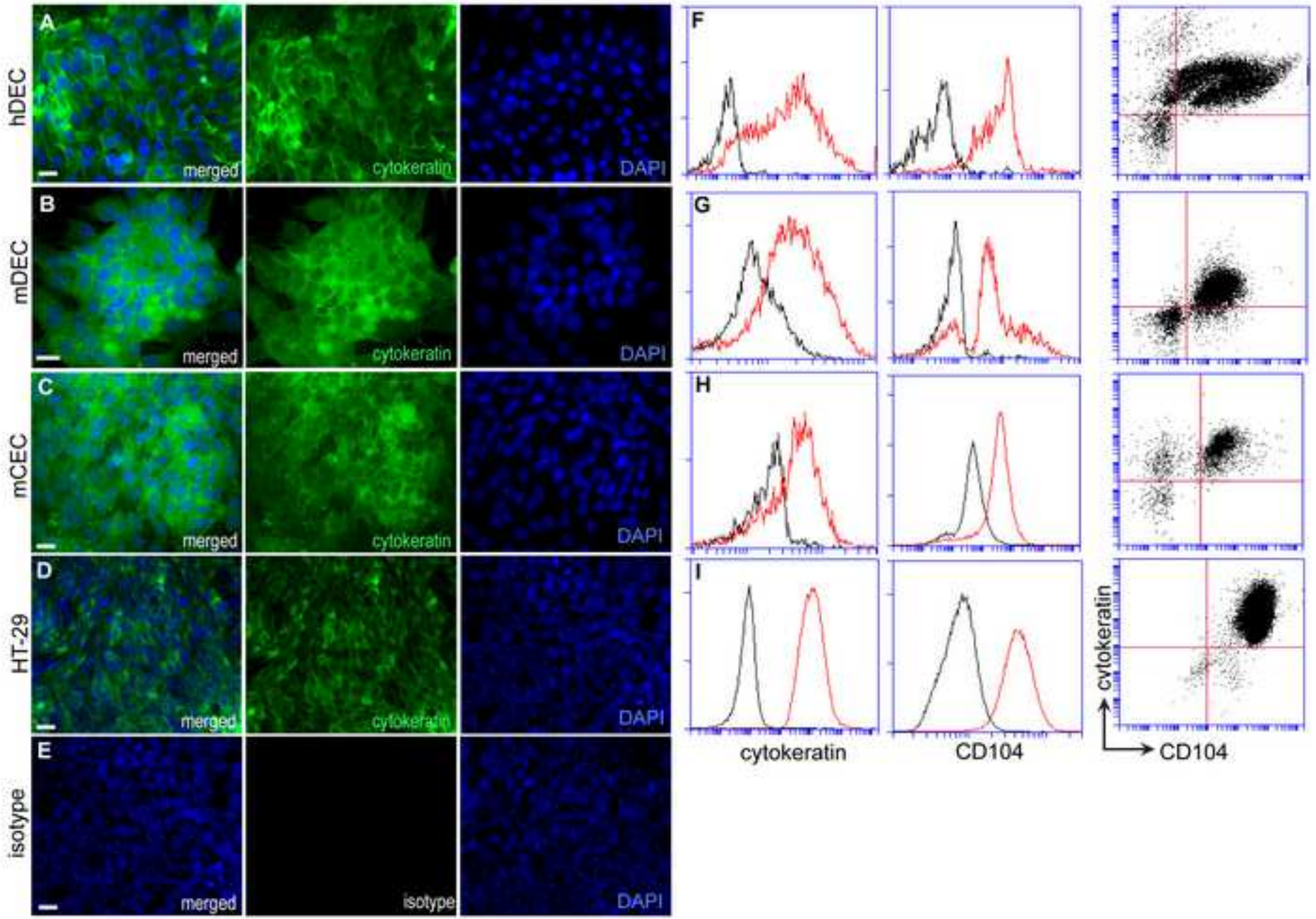

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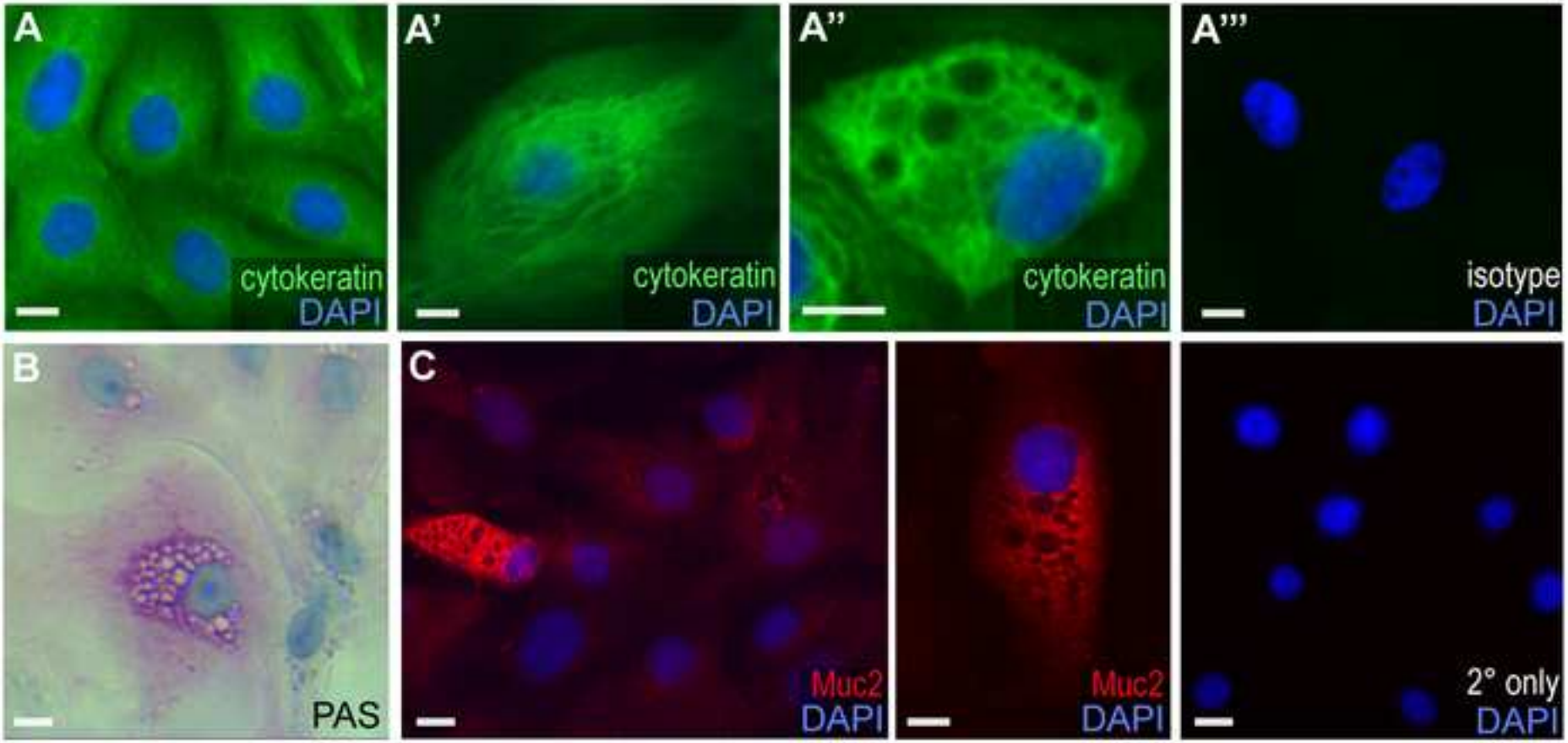

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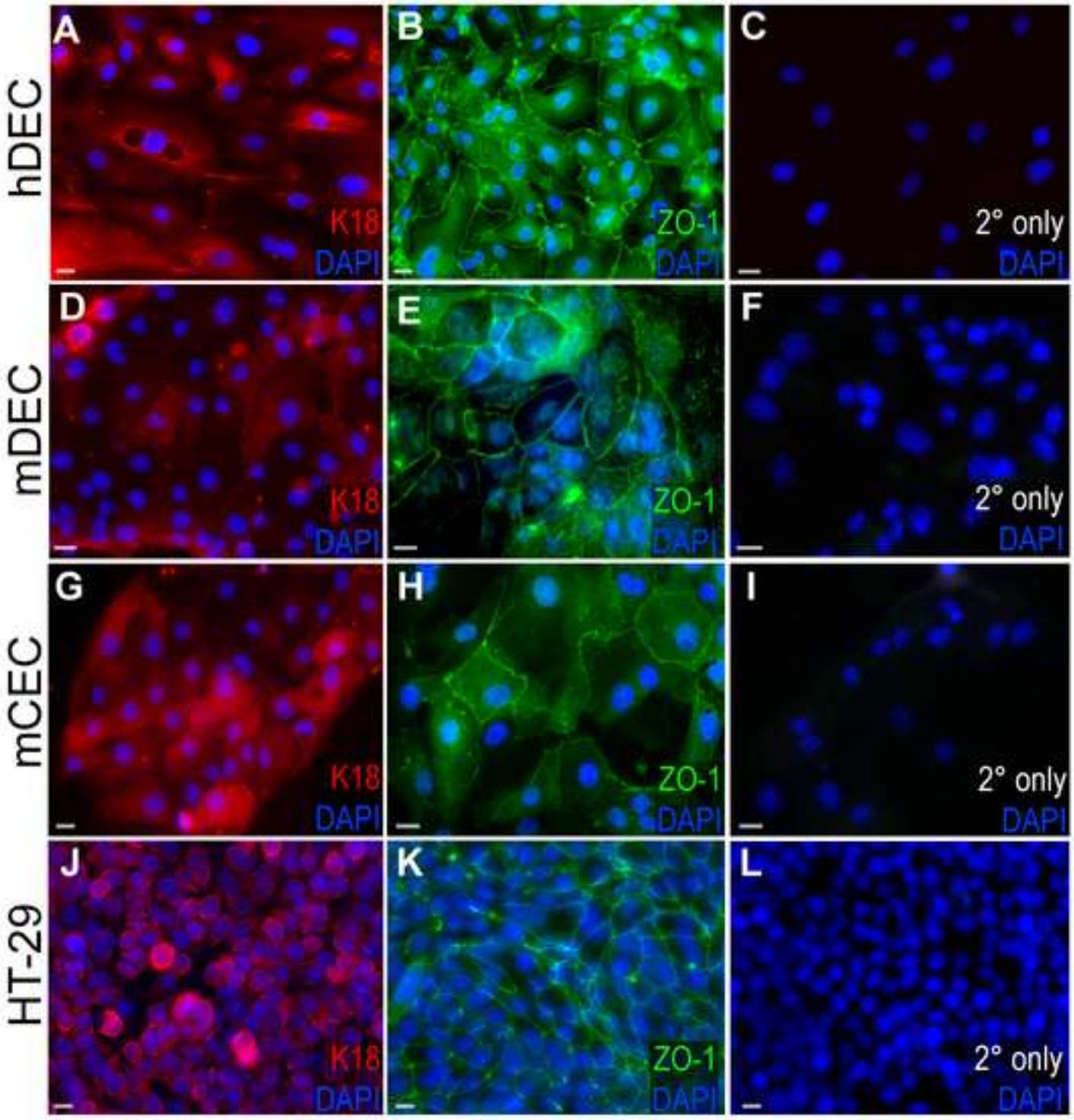

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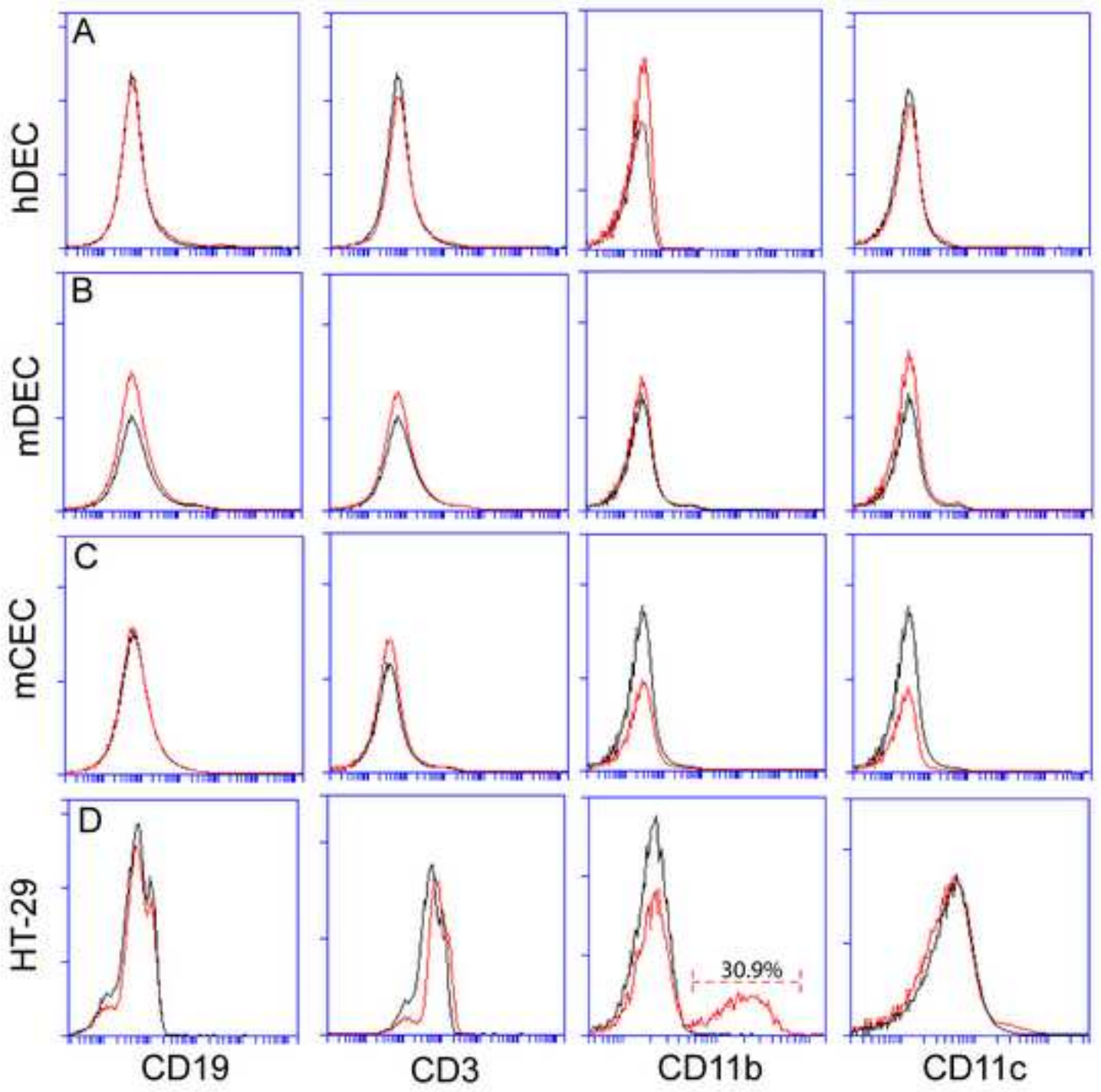

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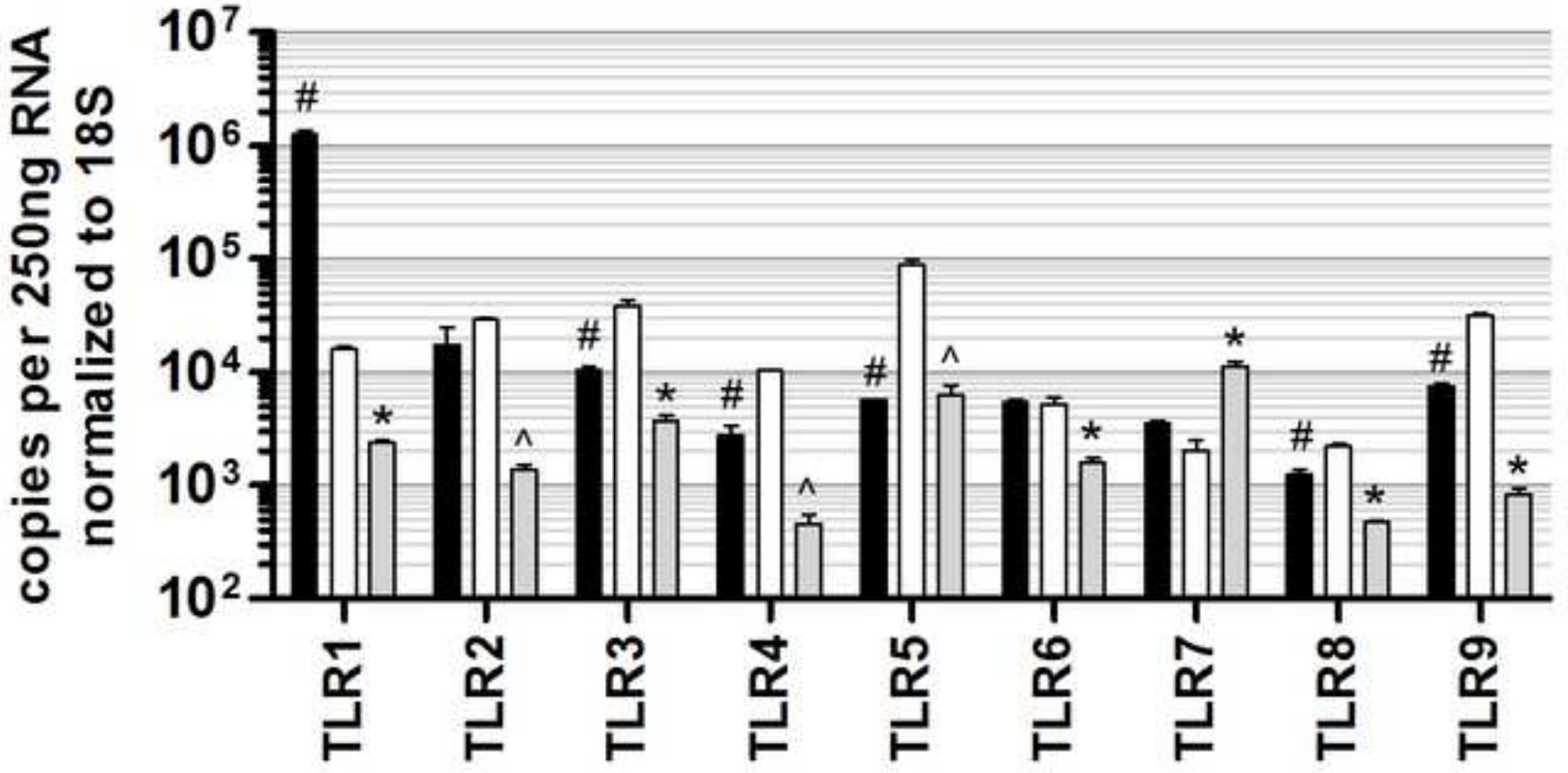

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