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A 3-D enteroid-based model to study T-cell and epithelial cell interaction

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ABSTRACT

The constant interaction between intestinal epithelial cells (IECs) and intraepithelial lymphocytes (IELs) is thought to regulate mucosal barrier function and immune responses against invading pathogens. IELs represent a heterogeneous population of mostly activated and antigen-experienced T cells, but the biological function of IELs and their relationship with IECs is still poorly understood. Here, we describe a method to study T-cell–epithelial cell interactions using a recently established long-term intestinal "enteroid" culture system. This system allowed the study of peripheral T cell survival, proliferation, differentiation and behavior during long-term co-cultures with crypt-derived 3-D enteroids. Peripheral T cells activated in the presence of enteroids acquire several features of IELs, including morphology, membrane markers and movement in the epithelial layer. This co-culture system may facilitate the investigation of complex interactions between intestinal epithelial cells and immune cells, particularly allowing long term-cultures and studies targeting specific pathways in IEC or immune cell compartments.

1. Introduction

The intestinal epithelium is a vital network of single-layer epithelial cells (IECs) and interspersed intraepithelial lymphocytes (IELs) (Guy-Grand et al., 2013). This interaction is a tightly regulated, complex interplay that is crucial for maintenance of intestinal homeostasis, barrier function and immune responses at the mucosal site (Cheroutre et al., 2011). Dysregulation of IEC–IEL interaction generally leads to intestinal pathological disorders such as ulcerative colitis, Crohn's disease and celiac disease (Jabri and Sollid, 2009; van Wijk and Cheroutre, 2009).

In addition to performing key functions in digestion and absorption, the IECs represent the foremost physical barrier against pathogenic and commensal microorganisms that reside within the lumen of the gut (Peterson and Artis, 2014). Structurally organized into *villi*, the epithelium is composed of three major cell types: enterocytes, goblet cells, and enteroendocrine cells. At the base of each *villus* are crypts of Lieberkuhn containing Paneth cells and actively proliferating stem cells that are the source of the ever-renewing epithelium (Sato et al., 2011b).

Interposed between the epithelial cells and in close proximity to the lumen of the gut are the IELs, which represent a heterogeneous population of mostly activated and antigen-experienced T cells.

IECs and IELs are in close contact with each other, and each cell population is able to influence the other in a variety of ways (reviewed by Cheroutre et al., 2011). It is thought that one of the main physiological functions of IELs is to preserve the integrity of the intestinal epithelial barrier; however, prevention of pathogen invasion must be tightly regulated to avoid unnecessary or excessive responses that result in inflammatory conditions. IELs constitutively express CD103 (αE integrin), which interacts with E-cadherin on intestinal epithelial cells (Kilshaw and Murant, 1990) and most IELs express CD8αα homodimers (Leishman et al., 2002). The murine ligand for CD8αα is the thymus leukemia antigen (TL), a non-

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classical MHC class I molecule expressed on mouse small intestinal epithelial cells (Hershberg et al., 1990). IELs are classified into natural or thymus-derived IELs ($CD8\alpha\alpha^+$ $TCR\alpha\beta^+$or $TCR\gamma\delta^+$), and peripherally-induced ($CD8\alpha\beta^+\alpha\alpha^+$ and $CD4^+CD8\alpha\alpha^+$) IELs (Cheroutre et al., 2011). Given the extent of T cell-epithelial cell proximity and interactions, it stands to reason that these cells may have important influences on each other; however, the biological function of IELs and their relationship with IECs is still poorly understood.

Since *ex vivo* isolated IECs show poor survival in culture, most of the *in vitro* models developed to study IEC–IEL interaction rely on immortalized IEC lines. In the past several years, long-term intestinal "enteroid" murine and human culture systems have been established, resembling the three-dimensional crypt-villus architecture/structure of the small intestine (Sato et al., 2009, 2011a). These enteroids contain self-renewing stem cells and, when grown on laminin-rich Matrigel with necessary growth factors, undergo expansion and generation of *villi* composed of single-layer epithelial cells with all four cell lineages present *in vivo*, including enterocytes, Paneth cells, enteroendocrine cells and Goblet cells (Sato et al., 2009). Here, we describe a method to study T-cell-epithelial cell interactions using an intestinal enteroid-based culture system.

2. Methods

2.1. Mice

C57BL/6 (000664), UBC-GFP (004353), Actb-DsRed (006051), OTI (003831), OTII (004194) and CD45.1 (002014) mice were purchased from the Jackson Laboratories and maintained in our facilities. iFABP-tOVA transgenic mouse line was generously provided by Dr. V. Vezys (Vezys et al., 2000). Mice were maintained at the Rockefeller University animal facilities under specific pathogen-free conditions and sentinel mice were tested to be negative for *Helicobacter* spp. and *Citrobacter rodentium*. Mice were used at 7–15 weeks of age for most experiments. Animal care and experimentation were consistent with NIH guidelines and were approved by the Institutional Animal Care and Use Committee at the Rockefeller University.

2.2. Enteroid culture

Crypts were isolated from mouse small intestine as described previously (Sato et al., 2009) with some modifications. Isolated small intestines were kept on ice throughout entire manipulation. The intestines were cut longitudinally and feces were washed off with cold 1× PBS. Using a scalpel, *villi* were gently scraped off and discarded, tissue was cut into 1 cm pieces and incubated in a Falcon tube containing 25 ml of cold PBS (Corning) with 5 mM EDTA (Ambion) for 5 min on ice. After this incubation, the tube was briefly shaken by hand and the tissue was transferred into new Falcon tube with fresh 25 ml of cold PBS with 5 mM EDTA and incubated for 45 min at 4 °C in a HulaMixer (Invitrogen) set to 30 rpm for orbital rotation with 60° turning angle for reciprocal rotation. After incubation, the tube was vigorously shaken by hand and the tissue was collected on a sieve and discarded. 25 ml of cold 1× RPMI 1640 (Gibco) was added to the supernatant, which was then centrifuged at 1400 rpm (approx. 400*g*) for 5 min at 4 °C. The resulting pellet containing detached crypts was washed with 50 ml of cold RPMI 1640 and centrifuged again at 1400 rpm for 5 min at 4 °C. All manipulations of the culture after this centrifugation were performed in cell culture hood. The supernatant was aspirated and the pellet was resuspended in 10 ml of cold RPMI 1640. The crypts were further purified by filtration through 70 μm mesh followed by centrifugation at 600 rpm (approx. 200*g*) for 5 min at 4 °C. The pellet containing purified crypts was resuspended in 2 ml of cold T-cell culture medium (RPMI 1640, 10% FBS (Sigma F0926), 1% Pen/Strep (Gibco 15140), 1% L-glutamine (Gibco 25030), 1% sodium pyruvate (Gibco 11360), 2% non-essential amino acids (Gibco 11130), 2.5% 1 M HEPES (Gibco 15630), 50 μM 2-mercaptoethanol (Sigma M6250)) containing 50 ng/ml recombinant murine EGF (Invitrogen PMG8041), 100 ng/ml recombinant murine Noggin (Peprotech 250–38), 500 ng/ml recombinant human R-spondin (R&D Systems 4645-RS), which we refer as complete culture medium. Initial 70–80% crypt seeding confluency in 30% of Matrigel (BD Bioscience) was obtained after plating on average of 100 μl of crypts with 40 μl of additional complete culture medium and 60 μl of Matrigel (for a total of 200 μl per well) in pre-warmed at 37 °C 24-well plate. After polymerization of Matrigel (about 20 min), 300 μl of pre-warmed (37 °C) complete culture medium was gently added to each well. Every 2 days, the complete culture medium was gently pipetted off and replenished with fresh complete culture medium. The expanding enteroids were passaged every 6 days and re-plated with fresh Matrigel and complete culture medium. To extract the enteroids, top medium was gently pipetted off, the plate was placed on ice for 30 min, and 1 ml of cold PBS was added to each well to dissolve the Matrigel. Using a p1000 pipet, the contents of each well were mechanically disrupted and collected in a 50 ml Falcon tube. The Matrigel was then diluted out with additional 25 ml of cold PBS. The enteroids were then centrifuged at 600 rpm for 5 min at 4 °C and re-plated.

2.3. In vitro T cell culture

Naive splenic $CD4^+$ or $CD8^+$ ($CD25^-CD62^{hi}CD44^{lo}$) T cells were isolated via negative selection using magnetic beads (MACS, Miltenyi Biotec) or sorted using a FACS Aria cell sorter (Becton Dickinson). $CD4^+$ T cells were cultured in T-cell culture medium as described above in 96-well plate for 3 days in 96-well plates pre-coated with 2 μg/ml of anti-CD3ε (17A2) with 1 μg/ml of soluble anti-CD28 (37.51) or co-cultured with magnetic bead-isolated (MACS, Miltenyi Biotec) $CD11c^+$ splenic DCs. The activation/stimulation was followed by a resting period of 2 days with 10 ng/ml of IL-2 (R&D 402-ML). $CD8^+$ T cells were treated similarly but with 2 days of activation/stimulation followed by 2 days of rest with 10 ng/ml of IL-2. Where indicated, the $CD8^+$ T cells were also stimulated with 1nM of retinoic acid (RA) (Sigma R2625).

2.4. T cell–enteroid co-culture

Enteroids were extracted from Matrigel as described earlier and washed with cold RPMI 1640. $CD4^+$ and $CD8^+$ T cells were collected from 96-well plates, washed with RPMI 1640, centrifuged at 1400 rpm at 4 °C for 5 min and counted. Where