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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).

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Author contribution

D.M. conceived and supervised this study. A.R., B.S.R., R.A.K. and D.M. designed experiments. A.R., B.S.R. and R.A.K. performed experiments, prepared figures. A.R. and D.M. wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

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 (α E integrin), which interacts with E-cadherin on intestinal epithelial cells (Kilshaw and Murant, 1990) and most IELs express CD8 $\alpha\alpha$ homodimers (Leishman et al., 2002). The murine ligand for CD8 $\alpha\alpha$ is the thymus leukemia antigen (TL), a non-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 CD4CD8 $\alpha\alpha^+$) 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; Sato et al., 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 ("R23">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 *C. 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 1cm pieces and incubated in a Falcon tube containing 25ml of cold PBS (Corning) with 5mM 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 25ml of cold PBS with 5mM EDTA and incubated for 45 min at 4°C in a HulaMixer (Invitrogen) set to 30rpm 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. 25ml of cold 1× RPMI 1640 (Gibco) was added to the supernatant, which was then centrifuged at 1400rpm (approx. 400g) for 5min at 4°C. The resulting pellet containing detached crypts was washed with 50ml of cold RPMI 1640 and centrifuged again at 1400rpm for 5min 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 10ml of cold RPMI 1640. The crypts were further purified by filtration through 70µm mesh followed by centrifugation at 600rpm (approx. 200g) for 5min at 4°C. The pellet containing purified crypts was resuspended in 2ml 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% 1M HEPES (Gibco 15630), 50µM 2-Mercaptoethanol (Sigma M6250)) containing 50ng/ml recombinant murine EGF (Invitrogen PMG8041), 100ng/ml recombinant murine Noggin (Peprotech 250-38), 500ng/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 100µl of crypts with 40µl of additional complete culture medium and 60µl of Matrigel (for a total of 200µl per well) in pre-warmed at 37°C 24-well plate. After polymerization of Matrigel (about 20 minutes), 300µl 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 minutes, and 1ml 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 50ml Falcon tube. The Matrigel was then diluted out with additional 25ml of cold PBS. The enteroids were then centrifuged at 600rpm for 5min at 4°C and re-plated.

2.3 In vitro T cell culture

Naïve 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 2µg/ml of anti-CD3 (17A2) with 1µg/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 10ng/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 10ng/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 1400rpm at 4°C for 5min and counted. Where indicated, T cells were stained before co-culture with CellTrace Violet (Invitrogen) for proliferation assays, as per manufacturer protocol. For co-culture, a total of 100,000 T cells and roughly 20 enteroids/well were co-cultured in 200µl per well of complete culture medium with 30% Matrigel in 24-well plates. Where indicated, soluble 1nM OTI (SIINFEKL) or 1µM OTII (ISQAVHAAHAEINEAGR) peptide was included in the 300µl of complete culture medium that was added to each well after Matrigel polymerization (Fig. 1A). Every 2 days, the complete culture medium was gently pipetted off and replenished with fresh complete culture medium (with or without peptide). The co-culture was re-plated in fresh Matrigel and complete culture medium every 6 days. To re-plate the co-cultures, the plate was incubated on ice for 30 minutes and 1ml of cold PBS was added to each well. Using a p1000 pipet, the contents of each well were mechanically disrupted and collected in a 15ml Falcon tube. The Matrigel was then diluted with additional 10ml of cold PBS. The enteroids and T cells were centrifuged at 1400rpm for 5min at 4°C and re-plated in fresh complete culture medium with 30% Matrigel in 24-well plates. 300µl of complete culture medium was then added after Matrigel polymerization.

2.5 T cell harvesting

The plates were incubated on ice for 30 minutes and 1ml of cold PBS was added to each well. Manual pipetting was used to disrupt the Matrigel and dissociate the enteroids. Contents of each well were transferred to 15mL Falcon tubes and diluted with additional 10mL of cold PBS. Next, cells were centrifuged at 1400 rpm for 5min at 4°C and washed again with 15ml of cold PBS. After final centrifugation, cells were stained for flow cytometry or microscopy. In co-culture experiments, no hematopoietic cells were recovered from the enteroid counterpart.

2.6 Antibodies and flow cytometry analysis

For FACS staining, cells were re-suspended in staining buffer (PBS, 2 % BSA, 10mM EDTA, 0.1% NaN₃) and stained with the antibodies. Fluorescent-dye-conjugated antibodies

were purchased from BD-Pharmingen (anti-CD4, 550954; anti-CD25, 553866; anti-CD103, 557495) or eBioscience (anti-CD8a56-0081; anti-CD45.1, 25-0453; anti-CD45.2, 47-0454; anti-CD69, 11-0691; anti-V α 2, 1705812; anti-CCR9, 46-1991). Flow cytometry data was acquired on an LSR-II flow cytometer (Becton Dickinson) and analyzed using FlowJo software (Tree Star).

2.7 Microscopy (Immunofluorescence)

To evaluate T cell-enteroid interaction, two approaches were employed. For live imaging, T cell-enteroid co-culture was prepared as previously described using wild type enteroids and *in vitro* CellTrace-labeled CD8⁺ T cells isolated from OTI mice. CD8⁺ T cells were stimulated or not with RA prior to addition to the co-culture. Where indicated, 1nM OTI peptide was added to the top layer of complete culture medium. Live cell imaging was performed on the CellVoyager (Yokagawa/Olympus) spinning disk confocal microscope at 37°C and with CO₂. A z-stack was acquired every 5min for 18hs. Reconstruction of images was done using Yokagawa CellVoyager cv1000 beta software.

For confocal analysis, enteroids were derived from UBC-GFP mice and co-cultured with *in vitro* activated CD4⁺ T cells isolated from DsRed mice, as described above. Three-dimensional reconstitution image was generated using Imaris software.

3. Results

3.1 Activated T cells survive in enteroid co-culture

We first investigated the ability of enteroids to grow and expand in T cell complete culture medium containing enteroid factors, as described above. We were not able to detect noticeable differences in the enteroid formation and expansion in comparison to enteroids grown in the previously suggested medium (Advanced DMEM/F12) (Sato et al., 2009) (Fig. 1B). Next, we proceeded with co-culturing naïve and activated T cells with mature enteroids. We chose to use peripheral CD4⁺ and CD8⁺ T cells, rather than *ex vivo* isolated IELs, in order to establish a system that would allow the study of T cell activation, maturation and IEL programming when exposed to particular gut environmental cues thought to influence peripheral IEL (pIEL) differentiation (Reis et al., 2014). For the visualization of cultured T cells, we used peripheral RFP⁺ CD4⁺ T cells sorted from mice that express RFP variant DsRed under the control of the chicken beta actin promoter (DsRed), co-cultured with enteroids derived from UBC-GFP mice, which express enhanced GFP under the human ubiquitin C promoter. We found that peripheral CD4⁺ T cells only survived in enteroid co-cultures when they were previously activated *in vitro* (in the presence of TCR stimulation with or without differentiating cytokines) (Fig. 1C). We were able to detect live CD4⁺ T cells associated with enteroids for at least four weeks, after three passages of the co-culture, and the number of incorporated T cells varied from 1 to 20 cells/enteroid (*data not shown*). Incorporated T cells acquired membrane projections (dendrites) (Gallucci et al., 1982) resembling actual IELs (Fig. 1D). Since our initial conditions did not include secondary TCR stimulation, we concluded that this system allows the prolonged study of IEC-T cell interactions without the requirement of continuous TCR engagement.

3.2 T cell proliferation and differentiation in enteroid co-cultures

We next investigated the effects of enteroid-mediated antigen presentation on T cell proliferation and differentiation using complementary approaches. Proliferation dye (CellTrace Violet)-labeled sorted naïve or *in vitro* activated CD45.1⁺ CD8⁺ T cells isolated from mice carrying a transgenic TCR specific for ovalbumin (OVA) peptide presented in the context of MHC-I (OTI mice) were co-cultured with wild type or iFABP-tOVA-derived enteroids. iFABP-tOVA mice express a truncated, cytosolic form of OVA under the control of the intestinal fatty acid binding protein promoter, restricting membrane-OVA to mature small intestine enterocytes (Vezys et al., 2000). As expected, no proliferation was observed when naïve OTI cells were co-cultured with wild type enteroids in the absence of exogenous OVA peptide (Fig. 2A, B). In contrast, iFABP-tOVA-derived enteroids induced about 20% and 60% naïve and activated T cell proliferation, respectively, even in the absence of exogenous peptide (Fig. 2A, B). Addition of exogenous OTI peptide induced similar T cell proliferation in wild type and iFABP-tOVA-derived enteroids, although naïve T cells showed enhanced proliferative capacity when compared to previously activated T cells, suggesting that IEC-mediated presentation suppresses or prevents proliferation of previously activated T cells in these conditions (Fig. 2A, B). Upon addition of exogenous peptide, MHC-I presentation between CD8⁺ T cells also induced proliferation, as expected. We also analyzed upregulation of gut homing and IEL-related molecules by CD8⁺ T cells in these co-culture conditions. iFABP-tOVA-derived enteroids induced high levels of integrin CD103 (α E β 7), as well as gut homing (CCL25 receptor) CCR9 expression by OTI cells (Fig. 2C, D). Supplementation with exogenous OTI peptide induced similar levels of CD103 expression by T cells co-cultured with wild type or iFABP-tOVA-derived enteroids, although previously-activated T cells displayed less CD103 upregulation (Fig. 2B, C). Of note, we were unable to detect proliferation of naïve or previously activated OVA-specific CD4⁺ (OTII) T cells when OTII (MHC-II-restricted) peptide was added to wild type enteroids. We also did not observe CD4⁺ T cell activation as measured by CD25 and CD69 expression, indicating that naïve, wild type mice-derived enteroids are inefficient in MHC-II-mediated antigen presentation (*data not shown*). The above data indicate that enteroid co-cultures can be applied to study a variety of effects of IECs and IEC-mediated antigen presentation on T cells.

3.3 TCR engagement changes T cell behavior in enteroid co-cultures

Finally, we sought to visualize T cell-enteroid interactions upon T cell activation. Wild type enteroids were co-cultured with *in vitro*-activated CellTrace-labeled CD8⁺ T cells isolated from OTI mice, allowing visualization of OTI cells in enteroid co-cultures. We compared OTI cells previously activated in the presence or not of retinoic acid, a vitamin A metabolite involved in gut homing (Iwata et al., 2004) that was recently shown to induce an IEL phenotype in both CD4⁺ and CD8⁺ T cells (Huang et al., 2011; Reis et al., 2014). Co-cultures were imaged on a Yokagawa/Olympus CellVoyager for 18 hours allowing visualization of T cell-enteroid interactions (Fig. 3, Videos 1-4). We observed T cell incorporation into enteroids and constant movement between IECs. This movement pattern resembles a recently described IEL behavior observed in wild type mice (Edelblum et al., 2012). Addition of OTI peptide seemingly led to stable arrest in T cell movement between

IECs, likely consequent of TCR engagement (Video 1, no peptide; Video 2, OTI peptide). As expected by its gut-homing induction properties, retinoic acid exposure apparently enhanced T cell incorporation into enteroids (Video 3, RA; Video 4 RA plus OTI peptide). Please note that the time-lapse experiments were not designed with the intent of quantification of T cell behavior. The above results illustrate that a simple 3-D enteroid-based co-culture system can be utilized to study IEC-T cell interactions, allowing the study of activation, proliferation and cell behavior.

4. Discussion

The description of Lgr5⁺ IEC stem cell progenitors allowed the development of a variety of tools to study IEC biology (Sato and Clevers, 2013). Of particular interest to mucosal immunology, murine and human-derived intestinal enteroids have provided the framework for basic research studies and clinical applications of intestinal stem cells, both of which have shown remarkable advances in the past few years (Sato and Clevers, 2013). In this study, we described a simple method to study T cell-IEC interactions that allows gain- and loss-of-function studies using mouse strains targeting either IECs (Koo et al., 2012) or T cells using primary cells in long-term 3-D cultures. We focused our studies on peripheral, primary T cells; however, the methods described here could be adapted for the study of innate immune cells, including dendritic cells (Farache et al., 2013), macrophages (Mazzini et al., 2014) and innate lymphoid cells (Fuchs et al., 2013), all recently described to functionally interact with IECs. Additionally, we envision that this culture system could also be applied to the study of mature IELs, although optimizations are likely required since these cells are very sensitive to isolation and culture.

Several observations made in this study indicate that enteroid-based co-cultures may recapitulate features of *in vivo* T-cell-IEC interactions. First, T cells acquired dendrites that resemble IEL membrane extensions (Gallucci et al., 1982). Although the functional relevance of IEL dendrites is obscure, it is possible that these extensions are used as sensing mechanisms for detecting environmental cues (Cheroutre et al., 2011). In addition, it is possible that IEL dendrites play a role in their movement pattern, recently described to be dynamic (Edelblum et al., 2012) rather than static, as previously thought. In that regard, we believe that enteroid cultures could also serve for studies evaluating factors that may influence IEL behavior in the epithelial layer. Furthermore, the study of IEC-mediated antigen presentation could be facilitated by this system. Although we were unable to visualize IEC-dependent MHC-II antigen presentation, it is possible that the addition of factors that activate IECs, such as IFN- γ (Dotan et al., 2007), would allow functional CD4⁺ T cell activation and differentiation to be induced by IECs. Finally, this system could provide a simplified system to study the influence of commensal bacteria species on IEC-T cell interactions. In this case, the use of heat-killed bacteria, selective antibiotics or bacteria classes with reduced proliferative capacity would likely be required, as enteroids were observed to be highly susceptible to cell death upon exposure to bacteria.

5. Conclusion

The use of crypt-derived 3-D enteroid co-cultures may facilitate the investigation of the 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Enteroids grow in T-cell complete media
- Peripheral T cells survive in long term enteroid co-cultures
- T cells upregulate IEL markers in enteroid co-cultures
- T cells show IEL movement patterns in enteroid co-cultures

Figure 1. T cell-enteroid co-culture

(**A**) Schematic view of enteroid and T cell isolation for co-cultures (see Methods for detailed explanation). (**B**) 20× objective light microscopy image of enteroid cultures taken on days 0, 1, 3 and 6 (scale bar=50μm). (**C**) Confocal tri-dimensional reconstruction of a CD4⁺ T cell (in red, white arrow) inserted in an enteroid (in green) after 14 days of co-culture (two passages). Enteroids were derived from UBC-GFP mice and *in vitro* activated CD4⁺ T cells from DsRed mice (scale bar=25μm). (**D**) CellVoyager microscopy image of T cells (blue) and enteroid taken on day 14 of the co-culture. Enteroids were derived from WT mice and co-cultured with pre-activated (OVA peptide plus DCs) OTI CD8⁺ T cells. Black arrows indicate CellTrace-labeled OTI CD8⁺ T cells (scale bar=5μm). Data are representative of at least three experiments.

Figure 3. T cell movement in enteroids co-culture

OTI CD8⁺ T cells were pre- activated with OVA peptide (SIINFEKL) plus DCs in the presence or not of RA for 3 days and co-cultured with WT enteroids. T cell-enteroid co-cultures were recorded by CellVoyager microscopy every 5 minutes for 18 hours. Time-lapse images of co- cultures at 0, 6, 12 and 18 hours are depicted (scale bar=40μm). For movies refer to supplemental material.