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Investigating the movement of intestinal intraepithelial lymphocytes using an organoid co-culture system

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Investigating the movement of intestinal intraepithelial lymphocytes using an organoid co-culture system

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List of abbreviations

IEC	Intestinal epithelial cells
IEL	Intestinal intraepithelial lymphocytes
μL	Microliter(s)
μm	Micrometer(s)
M	Molar
L	Liter(s)
mg	Milligram(s)
mL	Milliliter(s)
mM	Millimolar
ng	Nanogram(s)
PBS	Phosphate-buffered saline
WT	Wild type
PTX	Pertussis toxin
CT	Control
LIS	Lateral intercellular space

Acknowledgement

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Finally, I would like to thank my family for their belief and support in my academic journey.

Declaration

Candidate

I, the candidate, Chi Nguyen, am the author of this thesis: Investigate the movement of intestinal intraepithelial lymphocytes using co-culture system. All references cited have been consulted by the candidate. This thesis contains no material that has been previously submitted for a higher degree.

Student: Chi Nguyen

Supervisor

The conditions of the relevant ordinances and regulations have been fulfilled.

Supervisor: Dr Mahima Swamy

Abstract

The small intestine, which is lined by a single layer of intestinal epithelial cells, is the main site of nutrient absorption. It is continuously exposed to food antigens, and also several bacteria and microorganisms. Intestinal intraepithelial lymphocytes (IELs) are T cells that are interspersed between epithelial cells, above the basement membrane. This makes them one of the first immune cells to encounter and provide defence against invasive microbes. Studies have shown the important contribution of IELs patrolling along the epithelium to the immunosurveillance of the guts. However, little is known about how these immune cells move.

Intestinal organoids are three-dimensional structures of epithelial cells that mimic the gut epithelium *in vitro*. In this study a murine IEL-organoid co-culture system was established to study IEL movement. It has been shown that it is possible to keep them together in culture for long-term and by performing brightfield and confocal microscopy imaging of the co-culture, I was able to visualize IEL movements. Indeed, IELs were observed to be highly motile inside organoids similar to previous studies. IL-15, a chemokine which is known to promote IEL proliferation and survival, can also affect IEL chemokinesis and chemotaxis. When IL-15 bindings was blocked, IEL movement was reduced. I also performed an analysis of IEL proteomic data for molecules regulated by IL-15 that could be involved in IEL migration. The expression of several adhesion molecules and chemokine receptors was either upregulated or downregulated which showed a potential involvement in IEL movement and retention within the epithelium. I showed that one way in which IL-15 drives IEL migration is potentially through a chemokine receptor, CXCR6. Results from the migration assay showed that IEL were chemoattracted and migrated towards CXCL16, the ligand of CXCR6. CXCL16 is expressed by stressed epithelial cells, and could be a mechanism for IELs to be attracted to sites of intestinal damage.

Introduction

1. Intestinal epithelial cells and their roles in small intestine immunity

The small intestine is a part of the gastrointestinal tract, which is an organ that takes in food, digests it and absorbs nutrients. The lining of the gut consists of a single layer of intestinal epithelial cells (IEC), and their main function is to absorb nutrients from food. The epithelium also serves as a barrier that separates the lumen, which contains foreign substances accompanying the intake of food such as bacteria, and the sterile lamina propria which hosts several types of immune cells. Thus, its contribution to the gut immune system is highly significant.

To aid in intestinal immunity, IECs serve two major functions: segregation and mediation (Okumura and Takeda 2017). Segregation is the separation of the gut microbes and host immune cells, while mediation is the delivery of signals between them. Both roles aim to maintain the balance between the intestinal microbiota and the host immunity, thus avoiding intestinal inflammation. IECs also generate two main types of barrier, physical barrier and chemical barrier.

The physical barrier includes the mucus layer and epithelial layer. Mucus is a sticky fluid which is mucin-glycoprotein-rich. It forms a thick layer which covers intestinal epithelial cells. The epithelial layer is a single cell layer, composed mainly of enterocytes joined by tight junctions. It is a contiguous and relatively impermeable membrane, securely separating the sterile environment underneath the epithelium from foreign substances. The epithelium is organized into villi and crypts to increase the surface area (Fig 1). At the bottom of the crypts are pluripotent intestinal stem cells, which constantly generate new epithelial cells every 4 to 5 days (van der Flier and Clevers 2009). Differentiated cells, except Paneth cells, migrate upward and out of the crypts. Afterwards, epithelial cells undergo apoptosis and are shed off the villi into intestinal lumen, while new cells are constantly generated (Hall, Coates et al. 1994). As a result, the epithelium is always renewed (van der Flier and Clevers 2009).

Secretory intestinal epithelial cells, which consist of enteroendocrine, goblet and Paneth cells, are specialized for maintaining the digestive or barrier functions of the epithelium (Peterson and Artis 2014). These cells secrete various gastrointestinal hormones, the mucus layer and antimicrobial peptides respectively. Microfold cells or M cells which

only found in the Peyer's patches in the small intestine, are responsible for the uptake and delivery of antigens from the lumen to antigen-presenting cells (Mabbott, Donaldson et al. 2013). Tuft cells also contribute to intestinal immunity and are enriched in a G protein-coupled receptor, SUCNR1 (succinate receptor) to sense protists and helminths which then can sufficiently activate type 2 inflammation (von Moltke, Ji et al. 2016, Ting and von Moltke 2019). Cup cells are another cell type which are randomly distributed in the villus epithelium and their function in gut immunity remains unclear (Madara 1982).

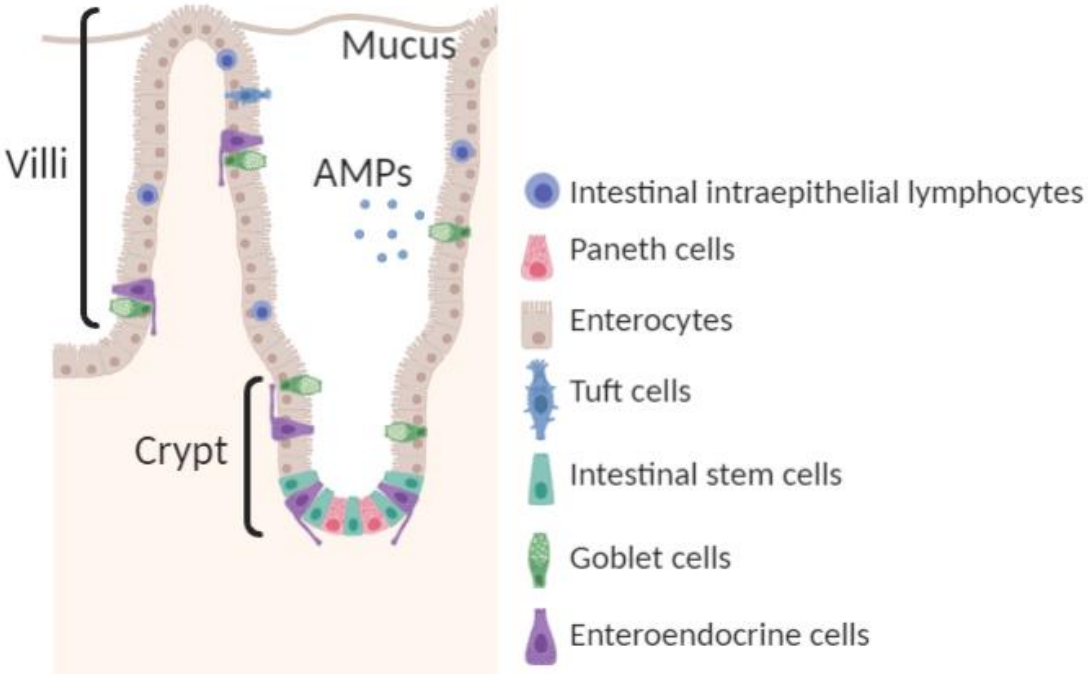

Fig 1: The small intestine is organized into crypts and villi to expand the surface area of the gut. The epithelium is composed of several populations of specialized cells which contribute to the small intestine functions: absorbing nutrients and protecting the body from harmful components. Intestinal stem cells (IESCs) are located at the bottom of the crypts.

To aid in the chemical barrier functions, Paneth cells produce antimicrobial peptides (AMPs) including the regenerating islet-derived 3 (Reg3) family of peptides that have a critical role in segregating bacteria from the epithelial surface of the small intestine (Okumura and Takeda 2017). AMPs are small, basic amino-acid-rich cationic proteins that can protect against bacterial infection by interacting with negatively charged microbial membrane, causing membrane disruption (Brogden 2005). The Reg3 family of proteins was defined as antimicrobial proteins of which Reg3γ is active against Gram-positive bacteria (Cash, Whitham et al. 2006, Vaishnava, Yamamoto et al. 2011). The production of antimicrobial molecules by Paneth cells is regulated partially by

TLR4/Myd88 signalling and NOD2 signalling which is driven by gut microbiome (Vaishnava, Yamamoto et al. 2011, Muniz, Knosp et al. 2012).

The intestinal immune system developed several tools in order to prevent the gut from bacterial invasion. The first one is the immunity provided by gut microbes. The intestinal microbiota has been indicated to be involved in the host immunity by constantly and directly contacting with host cells (Okumura and Takeda 2017). The second one is the protection from the epithelium composed by IECs. IECs can regulate host immune response by pro-inflammatory cytokine and chemokine secretion (Okumura and Takeda 2017). For instance, in humans epithelium, TLR5/Myd88 signalling promotes IECs-derived IL-8, which recruits neutrophils to the intestinal mucosa (Gewirtz, Navas et al. 2001, Yu, Zeng et al. 2003). IECs also respond to bacterial substances by generating factors that enhance cell survival and repair, and immunoregulatory responses such as TGF-β (Bauche and Marie 2017). The third protective level is represented by the innate and adaptive immune cells: dendritic cells, macrophages, phagocytes and the lymphocytes that are observed scattering in the gut. The most abundant lymphocyte population in the intestinal epithelium is the intestinal intraepithelial lymphocytes (IELs).

2. Intestinal intraepithelial lymphocytes

IELs are one component of the intestinal immune system and are present an estimated density of one IEL for every 5 to 10 epithelial cells in the small intestine (Beagley, Fujihashi et al. 1995). IELs are interspersed between epithelial cells, above the basement membrane, which make them one of the first immune cells to provide defence against invasive micro-organisms (Hu, Jia et al. 2018). However, the understanding about how the IELs are fully activated and which factors drive the cytolytic activity of IELs toward infected cells remain unclear.

Most of the IELs, around 90%, are TCR^+ and can be further classified into induced and natural IELs, also known as conventional and unconventional T cells respectively. Induced IELs include $CD4^+$ and $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ subsets, derived from antigen-specific T cells that were activated in the periphery in response to antigens then enter the epithelium. Thus, they typically express a memory-like phenotype (Cheroutre, Lambolez et al. 2011). Natural IELs include $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ subsets, which enter the epithelium as soon as they are generated in the thymus, and typically express the CD8αα receptor (Van Kaer and Olivares-Villagomez 2018). Some natural IELs express natural killer (NK) cell receptors and the CD3 complex (Shires, Theodoridis et al. 2001,

Cheroutre, Lambolez et al. 2011). TCR-negative cells only account for 10% of IELs and consist of subsets that resemble to innate lymphoid-like cells (ILCs) found outside the intestinal epithelium.

As the epithelium is constantly exposed to microbial pathogens and commensal organisms, IELs exert various functions in order to fight against bacterial and moderate overt inflammation (Sumida 2019). IELs can contribute to the innate immune response and fight against bacteria through cytolysis by secreting cytotoxic substances such as granzymes, perforin and Fas ligand or through natural killer receptors (Inagaki-Ohara, Nishimura et al. 1997, Bauer, Groh et al. 1999). Another mechanism is the regulation of mucus and antimicrobial peptides produced by Goblet cells and Paneth cells relatively (Hu, Jia et al. 2018). IELs are also involved in intestinal homeostasis and epithelial cell healing and repair (Cheroutre, Lambolez et al. 2011). For instance, TCR$\gamma\delta^+$ IELs have the capacity to produce keratinocyte growth factor to regulate IEC integrity and healing (Boismenu and Havran 1994, Sheridan and Lefrancois 2010). IELs are known for their immunosurveillance at the epithelial layer to quickly respond to infection in the intestine (Van Kaer and Olivares-Villagomez 2018). So far, there have been several studies of IEL migration because such migration to site of infection provides efficient immune response. However, further investigation is still required to give an insight into the cellular mechanisms that regulate IEL migration at steady-state or in response to infection.

3. Surface receptors on IELs involved in migration

Unlike other lymphocytes, IELs do not recirculate, however, they express several chemokine receptors, including CCR2, CXCR3, CCR5 and CCR9. The process of recruiting natural IELs to epithelium, so-called IEL-homing was shown to rely on the interaction between CCR9 and its ligand CCL25 which presents on epithelial cells under homeostatic condition (Wurbel, Malissen et al. 2001, Uehara, Grinberg et al. 2002). In CCR9-deficient mice, approximately 2-fold of intestinal IELs diminished and this reduction mainly due to the loss of γδ IELs. In addition, the decrease in IEL number in the small intestine was found to depend on *β7* integrin, another gut-specific homing molecule (Gorfu, Rivera-Nieves et al. 2009). Furthermore, other molecules were found to be involved in IEL recruitment to epithelium. CXCR3 is expressed on the surface of activated $CD8^+$ IELs and is thought to activate these cells in response to pathogens (Strauch, Mueller et al. 2001). Lack of this molecule leads to change in the number of cells in each IEL subsets, thus, CXCR3 and its ligand CXCL10 are suspected to be involved in IEL recruitment into infected tissues (Groom and Luster 2011). Another

noticeable chemokine receptor is CCR5 which is highly expressed by IELs. Its ligand is CCL5 which previously known as RANTES (Papadakis and Targan 2000). In $CCR5^{-/-}$ mice, there was increased inflammation and tissue damage upon *Toxoplasma gondii* infection (Luangsay, Kasper et al. 2003). Further, $CD8\beta^+$ IELs isolated from CCR5 deficient mice were impaired in their ability to migrate to infected tissues both *in vivo* and *In vitro*. CCL5 was shown to not have chemoattraction on primed IEL, instead it increases MIP-1α (CCL3) secretion (Luo, Berman et al. 2002). However, blocking CCL5 activity could totally inhibit migration of primed IELs to epithelial cells (Luangsay, Kasper et al. 2003). Thus, the results suggested that CCL5 can mediate IEL migration through MIP-1α and/or MIP-1β (CCL4) (both secreted by enterocytes) via chemokine receptor CCR5. In the same study, CCR2 expression level on primed IELs was found to be significantly lower than CCR5 and addition of an antibody to MCP-1, the CCR2 ligand, was ineffective to inhibit IEL migration. However, it is still a potential chemokine receptor to mediate IEL attraction.

G protein-coupled receptors also play a role in IEL homing. GPR18 is expressed by $CD8\alpha\alpha^+$ IELs and there is a reduction in $CD8\alpha\alpha^+TCR\gamma\delta^+$ IELs in mice lacking this receptor (Wang, Sumida et al. 2014). Another G protein-coupled receptor, GPR55 negatively modulates accumulation of $CD8\alpha\alpha^+TCR\gamma\delta^+$ IELs. In addition, in GPR55-deficient mice, IELs show faster movement and interact more with epithelial cells (Sumida, Lu et al. 2017). These results further confirm the involvement of chemokines in IEL migration.

Fractalkine and its specific receptor CX_3CR1 were reported to direct lymphocyte chemoattraction and adhesion within human intestinal mucosa (Muehlhoefer, Saubermann et al. 2000). This study showed that intestinal epithelial cells are a source of fractalkine and nearly half of the freshly isolated human IELs expressed the fractalkine receptor CX_3CR1 on their surface (which contain approximately 80% $CD8^+$ IELs). Cultured human IELs were previously demonstrated to migrate to polarized intestinal layer *in vitro* (Shaw, Hermanowski-Vosatka et al. 1998). However, IELs need to be activated by IL-2R signalling in advance to migrate in response to fractalkine. This migration can partially be inhibited by pertussis toxin, suggesting IEL migration can potentially be regulated by chemokine receptor-mediated signalling (Shaw, Hermanowski-Vosatka et al. 1998). PTX is an exotoxin with an A-B structure that ADP-ribosylates G_i proteins, interfering with a majority of chemokine receptors (Moss, Stanley

et al. 1983). Previous studies also showed that PTX can inhibit the chemotaxis of neutrophils, lymphocytes and macrophages (Spangrude, Sacchi et al. 1985). IEL recruitment to the intestinal mucosa is also regulated by $\alpha_E\beta_7$ and fractalkine has been shown to mediate an integrin-independent adhesion *in vitro* (Imai, Hieshima et al. 1997, Haskell, Cleary et al. 1999), suggesting that fractalkine contributes to IEL retention within intestinal epithelial layer. In addition, fractalkine is strongly upregulated in the intestinal mucosa of patients with active Crohn's Disease.

Once IEL enter the epithelium, they interact with epithelial cells. Until recently it was not clear whether IEL moved between epithelial cells or stayed sessile. A study from Chennupati showed that IEL moved little while later studies showed that IELs were highly motile within the epithelium (Chennupati, Worbs et al. 2010, Hoytema van Konijnenburg, Reis et al. 2017, Hu, Ethridge et al. 2018)

Crosstalk between IELs and IECs plays a key role in gut immune response (Hoytema van Konijnenburg, Reis et al. 2017). One factor involve in that interaction is occludin, a tight-junction protein was reported to regulate TCRγδ+ IELs migration within epithelial layer (Edelblum, Shen et al. 2012). In occludin-deficient mice, TCRγδ+ IELs accumulation in intraepithelial compartment was impaired and IELs showed less interaction with IECs. This was only observed in TCRγδ+ IELs. Another molecule known to mediate the selective localization and retention of IELs is α_E(CD103)β_7 (Schon, Arya et al. 1999). This integrin is expressed in almost all IELs and its ligand, E-cadherin is found on epithelial cells and their binding mediates the adhesion of IELs to IECs (Cepek, Shaw et al. 1994). CD103 deletion increased migration of TCRγδ+ IELs to the lateral intercellular space (LIS) between epithelial cells, thus reducing pathogens invasion (Edelblum, Singh et al. 2015). When performing parallel transcriptome analyses in both TCRγδ+ IELs and IECs upon infection with *Salmonella*, there were an increase of Wnt/β-Catenin pathway which is responsible for the self-renewal capacity of intestinal stem cells (Hoytema van Konijnenburg, Reis et al. 2017). The Wnt/β-Catenin pathway also links with changes in IECs replacement rate, tissue regeneration and cellular metabolism (Karin and Clevers 2016). Furthermore, enteric infection induced changes in TLR sensing and Myd88 signaling, a pathway which IECs follow to response to pathogens (Hoytema van Konijnenburg, Reis et al. 2017). In Myd88-deficient mice, there was a loss of gene expressions associated with the immune response in isolated IECs and TCRγδ+ IELs. TCRγδ+ IELs behavioural changes were shown to depend on Myd88 expression in IECs.

Altogether, IECs are possibly the first cells sense and response to microbes then communicate with IELs, and are also responsible for TCRγδ+ IELs behaviour regulation during infection in a Myd88-dependant way.

An early study described the migration characteristics of TCRγδ+ IELs, which account for approximately 50 – 60% of IEL population in the murine small intestine (Chennupati, Worbs et al. 2010). They found that, under physiological conditions, intestinal TCRγδ+ IELs showed little movement and relatively confined to the epithelium. However, two later studies proved that under homeostatic conditions, IELs actively move in the space between the basement membrane and the epithelium, and occasionally show transient contact with epithelial cells (Edelblum, Shen et al. 2012, Hoytema van Konijnenburg, Reis et al. 2017). TCRγδ+ IELs showed a serpentine movement and a significant increase number of γδ IELs were found in the lateral intercellular space (LIS) upon infection with *Salmonella enterica Typhimurium* (Edelblum, Singh et al. 2015, Hoytema van Konijnenburg, Reis et al. 2017). In addition, TCRγδ+ IELs were observed to gather near and directly contact with infected cells which would enable these cells to protect the host and provide a quick response against pathogens. Thus, TCRγδ+ IELs patrol the epithelium and migrate between adjacent epithelial cells to do their protective functions and without TCRγδ+ IELs, enteric pathogen invasion increased (Edelblum, Singh et al. 2015). Therefore, IELs behavioral changes possibly depend on gut infection and direct contact with bacteria is required for them to fight against bacteria. However none of these studies have evaluated the movement of TCRαβ+ IELs.

4. Intestinal organoids

As mentioned above, it is shown that IEL migration and close interaction with IECs play an important role in gut immune response, thus IECs might be involved in IEL migration. However, studying interactions of IELs with IECs is challenging as isolated IECs easily undergo a programmed cell death, termed anoikis – a form of apoptosis (Frisch and Francis 1994). Recent advances allow long-term culture IECs as organoids, a culture system derived from intestinal stem cells (ISCs) (Sato, Vries et al. 2009).

An organoid is a three-dimensional structure which can resemble cellular composition and tissue organization of the intestine (Sato, Vries et al. 2009). As mentioned above, intestinal stem cells (ISCs) are located near the crypt bottom, at a density of 4 to 6 cells per crypt. ISCs produce the transit amplifying (TA) cells, which can proliferate rapidly. TA cells then differentiate into enterocytes, goblet cells and enteroendocrine cells that

migrate upwards along the crypt-villous axis. Paneth cells – secretory cells that secret Wnt3a, stay at the bottom of the crypts. According to this breakthrough study, intestinal organoids can be formed by supplementing ISC essential growth signals. First, Wnt signalling was reported to be a pivotal element for crypt proliferation (Pinto, Gregorieff et al. 2003). A Paneth cell then emerged, representing the first symmetry-breaking event in intestinal organoid formation (Serra, Mayr et al. 2019). A Wnt3a gradient is formed around these cells and is believed to determine a crypt site. In addition, Wnt agonist, R-spondin-1 also induced crypt hyperplasia *in vivo*. Second, epidermal growth factor (EGF) was associated with intestinal proliferation by reducing TGFβ-induced inhibition of IEC proliferation (Kurokowa, Lynch et al. 1987, Dignass and Sturm 2001). Third, crypt number expansion was reported to depend on transgenic expression of Noggin (Haramis,

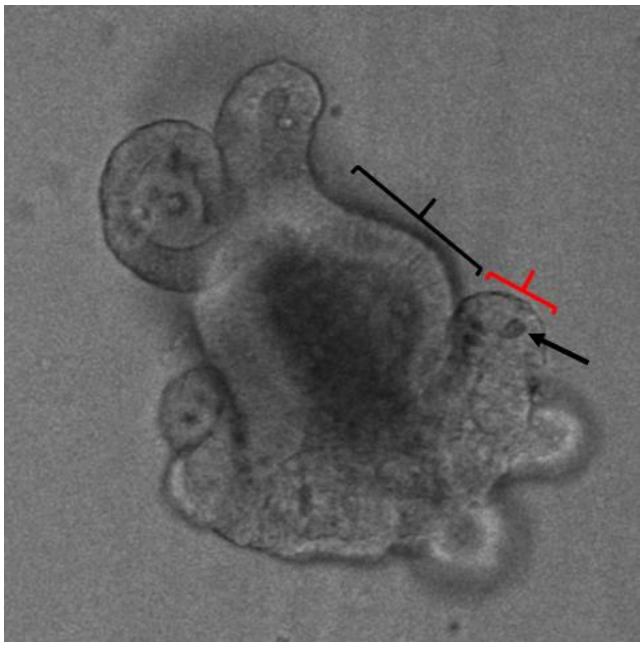

Figure 2: Bright field microscopy of a mouse small intestinal organoid with crypt domains (red bracket) and villus domain (black bracket). Paneth cells are presented at the crypt domain (black arrow). The lumen is filled with dead cells. Organoids were cultured with supplementation of EGF, Noggin and R-spondin.

Begthel et al. 2004). The last element was Matrigel, a mixture of extracellular base matrix to support intestinal organoid growth which have been applied to grow the epithelium of mammal (Stingl, Eaves et al. 2001). Laminin is also an enriched element at the crypt base, thus the use of Matrigel is to create an environment that mimics the stem cell niche *in vivo* (Sasaki, Giltay et al. 2002).

In vitro culture of intestinal organoids, also known as enteroids, with supplementation of all the growth factors above enables a long-term survival of enteroids and a reminiscent of normal intestine. There is a crypt-like structure with Paneth cells reside at the bottom

and TA cells. This crypt region is where the budding events occur. Budding events are similar to crypt fission which is a process in intestinal epithelium expansion (Langlands, Almet et al. 2016). There is also a villus-like domain composed of enterocytes. Apoptotic cells are shed off into the lumens and this process is similar with the dead cell shedding event in normal gut (Fig 2). In addition, IECs expanded by this method can reconstitute normal epithelium when being transplanted back into syngeneic mice, showing that the *in vitro* culture of IEC does not affect the conservation of their *in vivo* features (Nozaki, Mochizuki et al. 2016).

5. Co-culturing IEL with organoids

IELs are highly susceptible to apoptosis after isolation, thus, it has been a challenge to study IEL functions and behaviours *in vitro*. However, survival and proliferation factors derived from IECs can support the maintenance of IEL *in vitro* (Nozaki, Mochizuki et al. 2016). This suggested that IECs have physiological properties that provide a suitable microenvironment for a sustained culture of IELs. Isolated IEC also easily undergo anoikis (a form of apoptosis), but recent advances allow long-term culture of IEC as enteroids, three-dimensional primary culture systems that are derived from intestinal stem cells.

A co-culture system of IELs and intestinal organoids has been developed to study *in vitro* IELs interaction with IECs (Nozaki, Mochizuki et al. 2016). In this study, exogenous addition of cytokines, IL-2, IL-7 and IL-15 were used to test their effects on IEL maintenance and all three were able to expand IEL and maintain IEL survival. IELs were shown to move around enteroids with high motility and constantly changing their contact with enteroids. Some IELs approached enteroids, stayed in it and then egressed from it; others got into enteroids and moved along the epithelial layer in a random direction. Enteroids also support IELs proliferation and IELs have been observed to behave normally inside enteroids. Another group also utilised this method to co-culture TCRγδ+ IELs with enteroids derived from WT mice in order to study the importance of IL-15 (Hu, Ethridge et al. 2018). They performed an intravital microscopy on WT mice and mice with IL-15 overexpression in the epithelial layer found that in those overexpressing murine IL-15, the number of TCRγδ+ IELs associated with enteroids and migrating to the lateral intercellular spaces was increased. IL-15 has been shown to promote IEL survival and proliferation through the trans-presentation of IL-15 by epithelial IL-15Rα to IL-2Rβ presenting on T cells; natural IELs were significantly reduced in IL-15 or its

receptor IL-15Rα and IL-2Rβ-deficient mice (Suzuki, Duncan et al. 1997, Lodolce, Boone et al. 1998, Ma, Acero et al. 2009). Thus, epithelial IL-15 could be a chemoattractant for IEL migration. These studies showed that the IEL-organoid co-culture system can be an efficient tool to study the dynamic nature of IELs. However, very little is known about what controls their migration, their migration is directional or non-directional, there is so much still need to be learned in this area that my project will explore.

6. Project objectives

The main aims of the projects are:

- Establish a stable co-culture between mouse-derived IELs and enteroid.
- Visualize and measure IEL movement by widefield and confocal live imaging of the co-culture.
- Identify potential molecules and determinants involved in the regulation of IEL movement and migration.

Results

1. Studying IEL movement in organoids

The first aim of my project was to develop a stable co-culture between IEL and enteroids. I first tested different available media used for enteroid culture to select which medium will be appropriate and best support both IEL and enteroid growth. With this system, I could further study the IEL migration in enteroids by performing live imaging.

1.1. Testing organoid cultures in different media

Because most of our experiments were dependant on robust intestinal organoid cultures, it was important to find the best conditions to culture them. To that end, the whole intestinal tissue preparation was performed on ice to preserve the yield of crypts. Following the protocol, after a vigorous shake for one minute, a mixture of villus and crypts were obtained as demonstrated below (Fig 3).

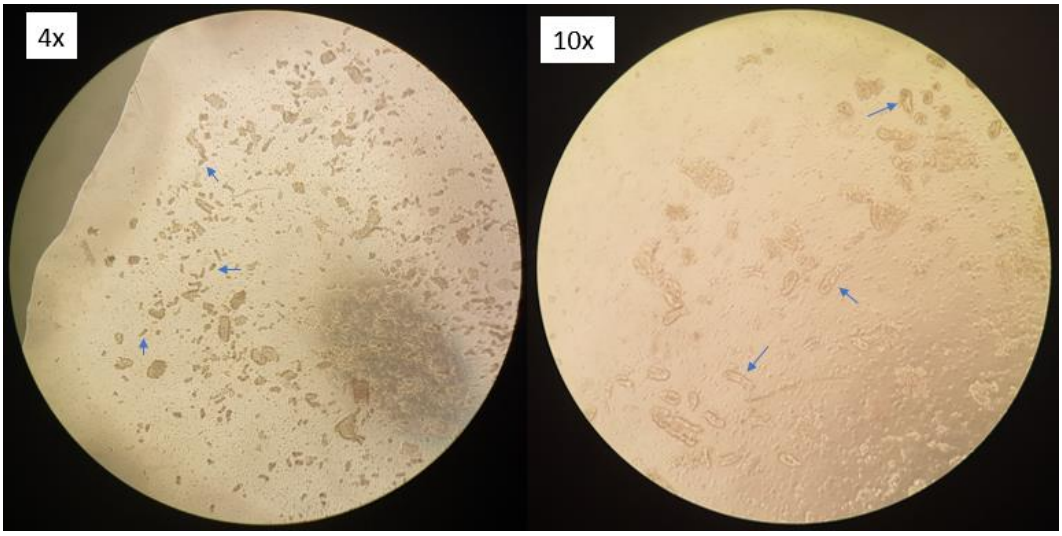

Fig 3: Crypts (blue arrow) after isolation process.

For enteroid culturing, I tested three different medium formulations available: ENR (Sato, Vries et al. 2009), OGM (a commercial organoid growth medium from Stem Cell Technologies) and conditioned L-WRN medium (Miyoshi and Stappenbeck 2013). ENR is a basic crypt medium supplemented with organoid growth factor EGF, R-spondin and Noggin. After one to two days in culture, three dimensional structures were visible.

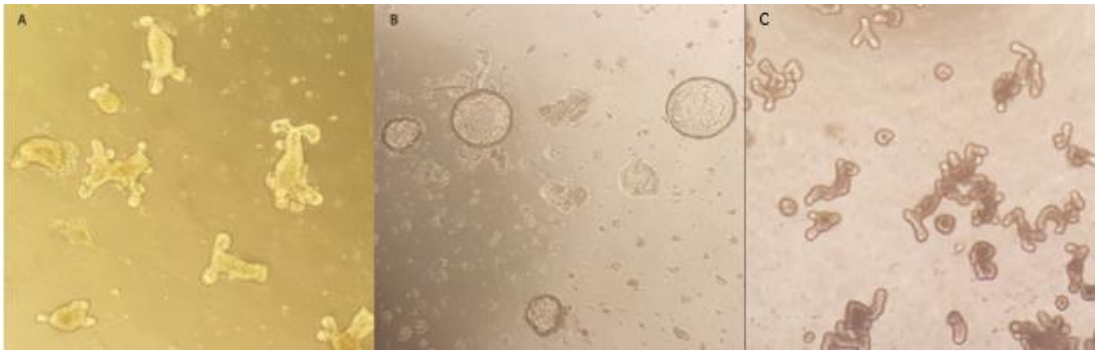

Fig 4: Mouse small intestinal enteroids after 3 – 4 days in culture. A) Enteroids cultured in ENR developed into budding structures. B) Enteroids grew into spheroids when cultured in L-WRN media. C) Enteroids cultured in OGM also have a budding structure, similar to ENR, these enteroids tended to develop in length before bud formation.

Crypts grown in ENR (Fig 4A) developed budding structures approximately 3 to 4 days after culture. It was similar to what I observed with crypts cultured in OGM, the commercial organoid media. A third medium is conditioned L-WRN media. This medium is produced from an L cell line which was engineered to secrete Wnt3a, R-Spondin-3 and Noggin into the medium (Miyoshi and Stappenbeck 2013). Crypts cultured in this conditioned medium formed nearly spherical, non-budding three-dimensional structures, known as spheroids (Fig 4B). Spheroids are highly enriched for proliferating cells, so they expand rapidly. These spheroids started to become visible after just one or two days in culture then quickly developed in size and needed to be passaged after 3 to 4 days (Fig 4B). L-WRN media could be replaced with ENR or OGM after the first passage, we then observed the budding structures in 3 to 4 days after switching to ENR and OGM. Therefore, I was able to identify L-WRN conditioned media as the most suitable medium to promote the formation and proliferation of enteroids which should be used in the first 4 to 5 days. Then ENR or OGM can be used to differentiate and maintain the proper epithelial structure and morphology of the enteroids.

1.2. Establishing the IEL-enteroid co-culture

The next step in the project was to establish a sustainable co-culture in which both enteroids and IELs are healthily growing during the length of experiment, which can up to two weeks. The protocol was followed based on Nozaki et al, 2016. Briefly, enteroids were passaged or cultured two days prior to the co-culture with IELs. When enteroids were passaged, debris which accompanying the crypts in the isolation process would be get rid of, thus passaged enteroids were preferred in my experiment. On the day of the co-culture, IELs were isolated and sorted for CD8-positive cells, the sorting was ensured of 90% purity (by flow cytometry). Enteroids were counted before releasing from

Matrigel by washing the well with cold Advance DMEM/F12 media. Enteroids were disrupted by pipetting vigorously. IELs and enteroids were mixed together with a ratio of 1 enteroid and 500 IELs, warm medium was added, and the mixture was incubated for 30

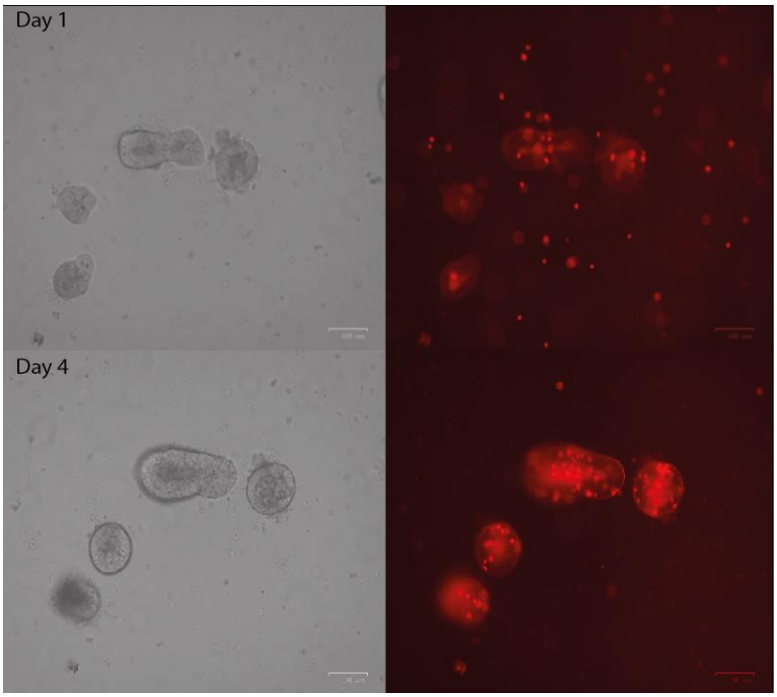

Fig 5: IEL-organoid co-culture establishment. Bright field and merge images showed IEL migration into enteroid after a day and 4 days of plating. IELs were isolated from a td-tomato mouse of which all IELs were red. Crypts were isolated from WT mice and cultured into enteroids.

minutes. Subsequently, the pellet was collected and suspended in Matrigel before adding to a 24-well plate. For this, I used ENR or OGM to develop budding structures. As IL-15 and IL-2 are known to support the proliferation of IEL, they were added to the medium at a concentration of 10ng/mL and 100U/mL respectively (Nozaki, Mochizuki et al. 2016). In these conditions, I was able to maintain a viable co-culture for 12 days. In order to track IELs, I isolated IELs from mice expressing a robust tdTomato fluorescent reporter following GranzymeB-Cre-mediated recombination in IELs. IELs appeared to get into the enteroids right after plating and after 4 days of being co-cultured, almost all IELs moved into enteroids (Fig 5). At day 1, as shown by the figure, there were a total of 55 IELs and 11 of them were inside enteroids; while at day 4, there were 43 IELs and 39 of them were inside enteroids. IEL number reduction would be a result of cell death.

After passaging, IELs were still alive and remained inside the enteroid, indicating that enteroids provided a suitable and essential microenvironment for them to grow. The extrinsic IL-15 added into the medium was believed to support the survival of IELs outside enteroids in the Matrigel.

1.3. IEL movement both inside and outside organoids

Now that I had established the co-culture, the next step was to study how IEL moved into, and within the enteroids. To study the movement of IEL in the co-culture, I performed live imaging with widefield fluorescent microscopy and confocal microscopy. I did the live imaging at day 2 after plating to observe IEL movement both inside and outside enteroids. IELs in Matrigel could be seen moving into the enteroids (Fig 6). Inside the enteroids, by tracking IELs movement, I was able to determine that IEL were moving at the speed of 0.1 microns per sec (Fig 7), which approximately similar to those reported in Nozaki et al, 2016. Interestingly, some IELs appeared to move from one enteroid to another. I also noted the presence of IEL which were immobile in the beginning and then started moving following no specific direction. It should be noted that tracking the movement of IELs was difficult since the live imaging was done with a 3D structure, it can be interrupted when IEL were out of focus and disappeared from the plane of view. As shown from figure 7, the lines of tracking IELs were not seamless due to loss track of IELs.

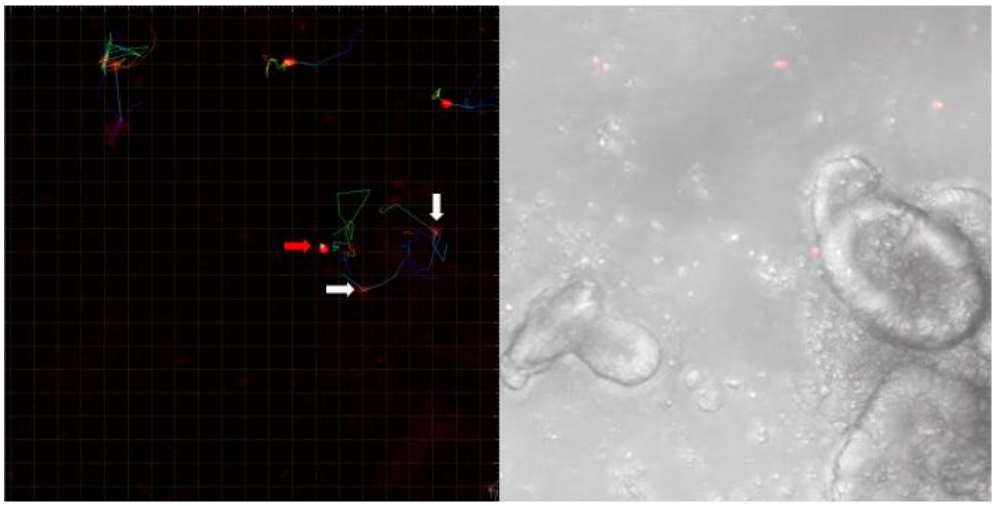

Fig 6: Visualize IEL motility in co-culture using confocal microscopy, supplemented with ENR medium. A figure showed the tracking movement of IELs in the co-culture. Three IELs at the top of the region were moving outside enteroids. Two IELs (white arrow) were moving along the outer layer of enteroid while one IEL (red arrow) stay idle at its place.

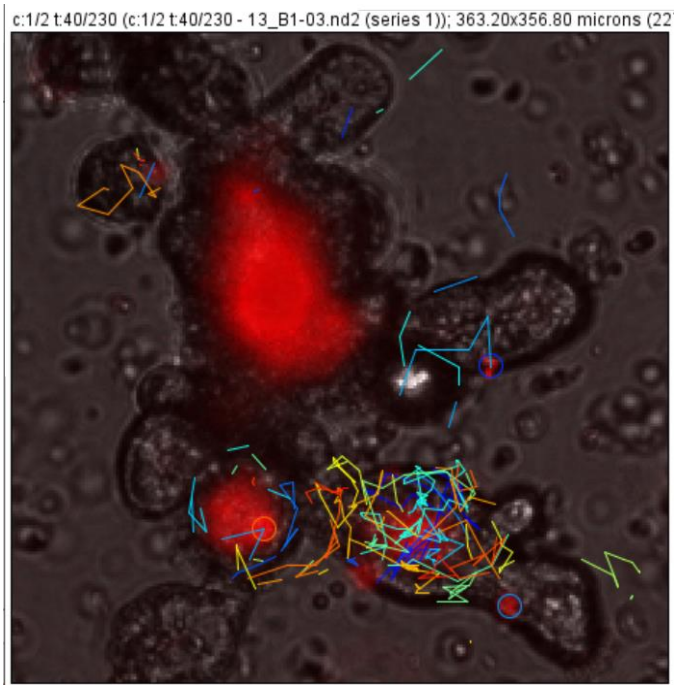

Fig 7: IEL were highly motile in co-culture. The live imaging was done with the widefield fluorescent microscopy. Three IELs were spotted at the ROI. The lines showed the distance of target spots and they were not seamless due to IELs moving out-of-focus and each color showed a different track. IEL tracking was done with Fiji.

1.4. Investigating the importance of IL-15 in IEL migration

Our results so far indicate that IEL are highly motile inside the enteroids, confirming recent *in vivo* data showing that TCRγδ+ IEL were moving within the intestinal mucosa (Hu et al, 2018). This indicates that our IEL-enteroids co-culture is a good model to study IEL movement. IL-15 is known to promote the proliferation and survival of IELs and the trans-presentation of IL-15 by epithelial IL-15Rα to IL-2Rβ expressed on T cells is required (Ma, Acero et al. 2009). IL-15 was stated to be a critical regulator of TCRγδ+ IELs as IL-2Rβ inhibition by a blocking antibody (TM-β1) significantly reduced IEL speed and displacement length after 48-hour treatment (Hu, Ethridge et al. 2018). We wanted to see in our co-culture system, if inhibition of IL-15 signalling would result in any changes in IELs movement. To do so, I set up a co-culture between IELs and enteroids as previously described with IL-2 and IL-15 supplementation, this condition also used as controls. I then treated the IEL-enteroid co-cultures with 40µg/ml of TM-β1, an anti-IL-2Rβ blocking antibody at two timepoints, 1 hour and 48 hours before imaging. IELs treated with the IL-15 alone moved frequently over the course of the live imaging.

However, 48h treatment of TM-β1 lead to a significant decrease in displacement length compared with controls. Within 1h of TM-β1 treatment, the effect of IL-2Rβ inhibition on IEL migration was evident with respect to the number of IEL moving during the

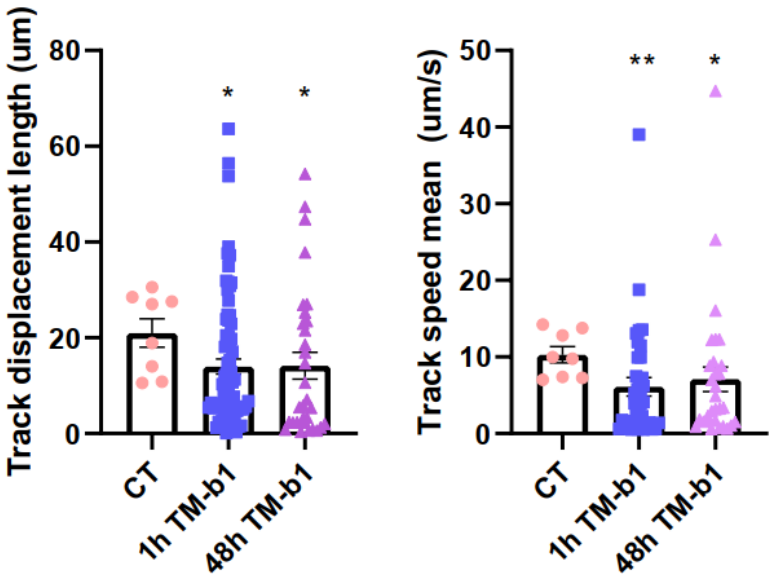

Fig 8: IL-2Rβ inhibition decrease IEL kinetics in co-culture. Mean track speed and displacement length of IEL in co-culture with TM-β1 treatment for 1h or 48h before imaging. Number of tracks: n = 77, 31 and 8, corresponding to the condition presented on the graph). Tracks were generated from time-lase imaging acquired every 2.5 minutes for 105 minutes. Image data was analysed by Imaris and data was shown as individual spot for non-normal distributed data. Statistics done with Graphpad prism, using Mann-Whitney test. *$p < 0.05$, **$p < 0.005$ (n = 1)

imaging. All IELs observed within the enteroid and were highly motile. In the 1h TM- β1 treatment, there were 5 out of 14 IELs did not move, 2 of them were outside enteroids; in other region, 6 out of 20 IELs detected were not motile and all of them were outside enteroid. In 48h TM- β1 treatment, all of the IELs were inside and moving within enteroids. Thus, with TM-β1 treatment, more IELs seemed to idle and this was more likely to happen to IELs in the Matrigel than those within enteroids.

There was a clear reduction in IELs speed between TM-β1 treatment and control in one experiment (Fig 8), however this decrease was not reproduced in the second experiment (data not shown). It appeared that IEL speed in the control conditions was lower than that in TM- β1 treatment condition in my second experiment. This could be explained by the increase in the number of sessile IEL per region of interest observed, longer imaging time and the fact that there were more idle IELs even in the control. IELs which were already inside enteroids seemed to be more active than IELs in the Matrigel. Despite these caveats, blocking IL-2Rβ still reduced the track displacement length in this experiment

as well. Therefore, we were able to conclude that IL-2Rβ inhibition had a negative effect on IEL kinetics, especially on IEL displacement.

2. Molecular determinants involved in IEL intraepithelial migration

We established a co-culture system between IELs and enteroids and used it to visualize and study the impacts of IL-15 inhibition on IEL movement in enteroids. My next aim was to investigate the involvement of any potential molecules such as adhesion molecules or chemokine receptors in IEL migration and retention within the epithelium. To this end, I first analysed the proteomic data of three IEL subsets after 24-hour IL-15 exposure which has recently established by our laboratory. Then, by performing migration assay, I tested IEL response to CXCL16, a ligand to CXCR6 which is a chemokine receptor expressed on IELs and is upregulated by IL-15.

2.1. Analysis of IEL proteomic data for molecules regulated by IL-15 involve in IEL migration

As stated above, we observed a reduction in IEL movement *in vitro* as a response to TM-β1 treatment, suggesting IL-15 might be important for IEL migration. However, previous study (Hu, Ethridge et al. 2018) suggested that IL-15 does not function as a chemotactic agent for IELs albeit that it can increase chemokinesis. The difference between chemokinesis and chemotaxis is the direction of movement; chemokinesis is the movement in any direction while chemotaxis the movement towards an attractant. Therefore, I wanted to address if IL-15 regulates the migration of IEL through other molecules. Our laboratory has recently developed a proteomic map of the global changes induced by IL-15 in IEL. Thus, I analysed this data set to identify proteins regulated by IL-15 that might play a role in IEL migration.

Quantitative label-free mass spectrometry was performed on the three main IEL subpopulations, TCRγδ CD8αα, TCRαβ CD8αα and TCRαβ CD8αβ after 24 hours exposure to 100ng/mL complexed IL-15 (assigned as high levels of IL-15). IELs isolated directly *ex vivo* were used as untreated controls (James, Vanderyken et al. 2020). More than 7100 proteins were identified and quantified in all three subsets. Proteins with a fold change >2 were considered as upregulated, whereas proteins with a fold change <0.5 were downregulated, and the rest were considered unchanged by IL-15 stimulation. Since the error bar of the standard deviation is large due to the inherent issues of label-free proteomic quantitation, we did not consider the statistical significance value, however, we focussed on proteins which had been quantified based on at least two peptides being identified by mass spectrometry, and that were found in at least two replicates (out of four) of each IEL subpopulation. In my analysis for proteins that regulate migration, I

focused only on the upregulated and downregulated proteins that are expressed at the cell surface, as indicated by Uniprot. This would help us to confirm its involvement in IEL migration quickly by adding blocking antibodies. We further narrowed down the number by selecting proteins that are involved in cell adhesion, cell chemotaxis and migration. These analyses lead to a list of 74 upregulated and 26 downregulated potential proteins. In order to corroborate proteins involved in adhesion, migration and chemotaxis, I also used the functional annotation clustering from DAVID database (https://david.ncifcrf.gov/tools) to select the proteins whose topological domains are extracellular; involved in cell-cell adhesion, cell migration and chemotaxis. The commonly identified proteins between the two analyses are presented in Table 1 (Appendix).

Among the upregulated proteins, common chemokine receptors such as CXCR6, CXCR3, CCR9 and CCR5 were detected (Fig 9A). These receptors are all chemokine receptors, involved in cell chemotaxis. CXCR6 functions have not been previously studied on IEL. CCR5 and CXCR3 have been shown to be expressed on all human and murine IEL (Agace, Roberts et al. 2000). CCR5 is implicated in the migration of IEL

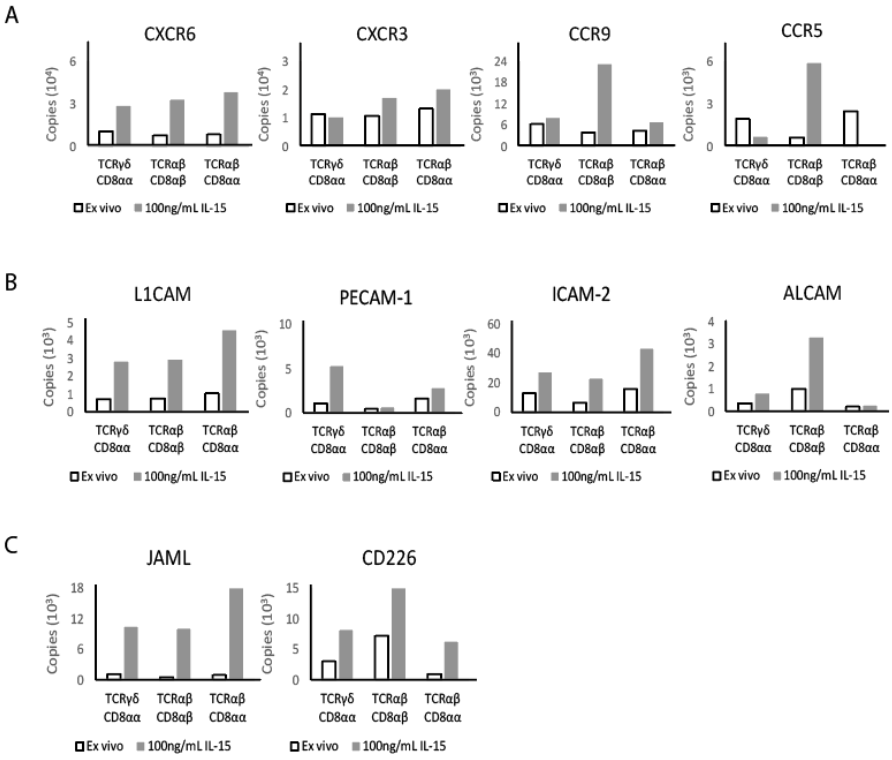

Fig 9: Bar graph shows protein copy numbers upregulated by IL-15 of A) Chemokine receptors. B) Adhesion molecules C) Activating receptors. All of these molecules have been quantified in at least two peptides of at least two replicates (out of four). Fold changes of these molecules were shown in Table 2.

towards *T. gondii* infected cells. CCR5 expression was increased in TCRαβ CD8αβ but decreased in TCRγδ CD8αα and unchanged in the other subset, suggesting potential different impacts of CCR5 in different IEL subsets (Fig 9A). CCR9 as previously mentioned in the introduction, has important contribution to the IEL homing to the gut.

There were a few adhesion molecules like L1CAM, PECAM-1, ICAM-2 and ALCAM which were reported to play a role in adhesion of platelet/endothelial cells, neuronal cells and activated leukocytes (Fig 9B). The upregulation of these molecules in all three IEL populations after culturing with IL-15 suggests its involvement in IEL trafficking.

Activating receptors like JAML and CD226 were upregulated and involved in cell-cell adherence junctions (Fig 9C). CD226 is a member of the poliovirus receptor (PVR)-nectin family, an activating and natural cytotoxic receptor that activates NK cells and regulates its response against tumours (Shibuya, Campbell et al. 1996, Du, de Almeida et al. 2018). CD226 also mediates cell-cell adhesion through binding with its ligands, CD112 and CD155 (Bottino, Castriconi et al. 2003, Tahara-Hanaoka, Shibuya et al. 2004). TGF-β (transforming growth factor beta) is secreted by IELs to maintain intestinal homeostasis (Konkel and Chen 2011) and its receptor type 1 and 2 were found to be strongly upregulated in IELs after exposing to IL-15.

Noticeable among the downregulated proteins were Cadherin-17 (Cdh17), a calcium-dependent transmembrane glycoprotein that concentrates in adherence junctions in IECs and plays a critical role in intestinal homeostasis by limiting epithelium permeability (Wendeler, Drenckhahn et al. 2007, Chang, Yu et al. 2018) (Fig 10). Integrin alpha M was downregulated suggesting IEL adherence was partially integrin mediated. Apart from the cell surface proteins, a cytoplasmic protein, regulator of G-protein signalling RGS1 expression was also decreased. This protein is interesting because RGS proteins are

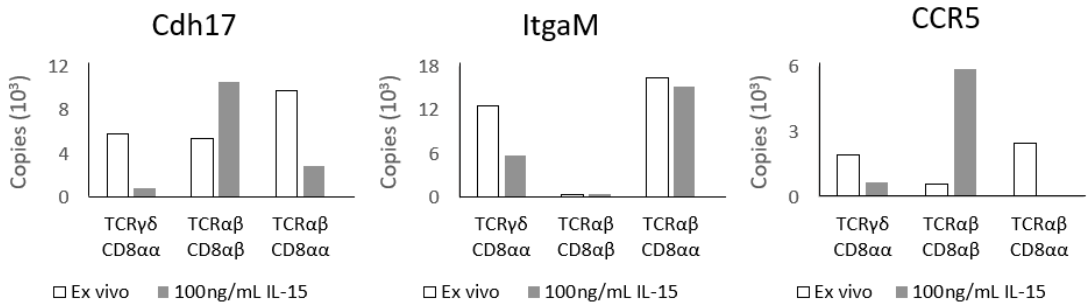

Fig 10: Bar graphs shows protein copy numbers downregulated by IL-15. Cdh17 and ItgaM are involved in tight junction and cell-cell adherence. CCR5 is a chemokine receptor. Fold change of these molecules were shown in Table 2.

GTPase activating proteins and RGS1 is highly expressed in lymphoid organs and acts as a negative regulator of chemokine receptor signalling in lymphocytes (Reif and Cyster 2000, Moratz, Harrison et al. 2004). This observation raised a hypothesis that high-level IL-15 culture might down-regulate adhesion protein expression and increase the ability of IEL moving under chemokine signalling, thus will lead to IEL moving more freely within the epithelium. The expression of some proteins in TCRαβ CD8αβ subpopulation is different from the other two (e.g CCR5, RGS1), indicate other mechanisms may regulate the migration in this induced IEL population.

2.2. Investigating the role of CXCR6 in IEL migration

As CXCR6 is the chemokine receptor that has not been implicated in IEL biology, we further investigate the role of CXCR6. CXCR6 is interesting as it possibly acts as both an adhesion and chemotactic molecule and it is responsible for the retention and circulation of innate lymphoid cell precursors (Chea, Possot et al. 2015, Koenen, Babendreyer et al. 2017). CXCR6 is expressed by subsets of $CD4^+$ T cells, natural killer (NK) cells, NK T cells and plasma cells (Deng, Chen et al. 2010). CXCR6 also involved in recruitment of several immune cells (Butcher, Wu et al. 2016, Ashhurst, Florido et al. 2019). However, its role on IEL has not been studied. First, I checked CXCR6 expression on *ex vivo* IELs and 24-hour cultured IELs with high level of IL-15. I used splenic T cells as a negative

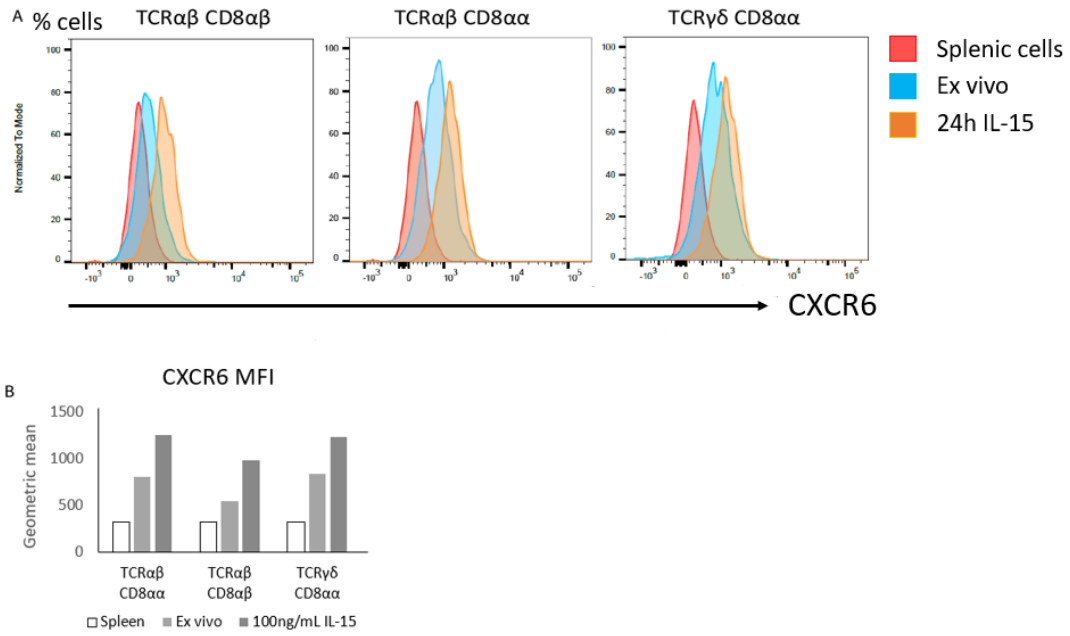

Fig 11: CXCR6 expression in three main IEL subsets. *Ex vivo* and 24-hour cultured with 100ng/mL IL-15 IEL were stained for CXCR6. Splenic T cells were used for negative control. A) Histogram showed CXCR6 expression in spleen T cells (red), *ex vivo* IELs (blue) and 24-hour cultured IELs (orange) of three IELs populations. B) Bar graphs showed the MFI of CXCR6 in spleen T cells, *ex vivo* IELs and 24-hour cultured with 100ng/mL IL-15 (n=1).

control since CXCR6 is expressed on these cells at a low level (Matloubian, David et al. 2000). Clearly, IELs express CXCR6, and this is further upregulated after culture. Figure 11A showed an upregulation of CXCR6 expression comparing to the *ex vivo* and splenic T cells.

Next, we wanted to test if IL-15 regulates IELs migration through CXCR6 expression by a Transwell migration assay. I used CXCL16, the only known ligand for CXCR6 as a chemoattractant. CXCL16 has been found to be expressed by macrophages, dendritic cells, epithelial cells (Matloubian, David et al. 2000, Agostini, Cabrelle et al. 2005). Briefly, IELs were isolated and cultured with 100ng/mL IL-15 to upregulate CXCR6. After 24 hours of incubation, approximately 500,000 IELs were plated into the Transwell insertions, and CXCL16 was added together with medium to the bottom chamber. First thing I observed after incubation time of 1h30 was that IEL had migrated to the bottom in every well (Fig 12). However, there was a significant difference between two conditions: with and without chemokine addition. This suggested that IELs can migrate in response to the gradient of CXCL16. The addition of IL-15 into the cells did not make any significant difference which indicate that IL-15 did not accelerate the migration of

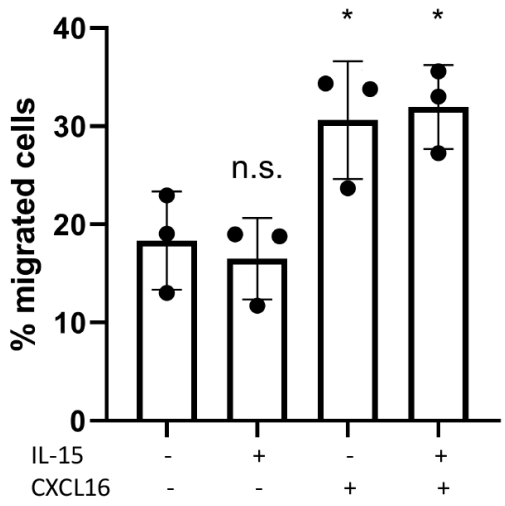

Fig 12: *In vitro* migration assay in the presence or absence of CXCL16. IELs were isolated, sorted for $CD8^+$ T cells and cultured with 100ng/mL IL-15Rα for 24 hours. IELs were collected and washed twice then plated into Transwell inserts, CXCL16 (10ng/mL) and IL-15Rα (100ng/mL) was added to the medium at the bottom. Whole co-culture was incubated for 1h30min. After incubation, IELs at the bottom were quantified. Experiments were done three times independently and each condition was repeated triplicate. Data was shown as percentage of migrated cells relative to number of cells added in each experiment. Data was analysed using one-way ANOVA test with multiple comparisons to control (no cytokines was added). *$p < 0.05$, n.s. not significant (n=3)

IEL, consistent with the previous result from a lab member that CXCR6 was expressed even when cultured with low level of IL-15 (2ng/mL) and at the equivalent level with

100ng/mL of IL-15 (data not shown). These data showed that CXCR6 was expressed in cultured IEL and cultured IELs were chemoattracted by CXCL16. Together, CXCR6 upregulation by IL-15 is involved in IEL migration.

To further address the roles of chemokine signalling pathway on IEL movement, I did an IEL-organoid co-culture as previously described with Pertussis toxin. Pertussis toxin inhibits a majority of chemokine receptors thus inhibit migration of several cells like neutrophils and lymphocytes as mentioned in the introduction. Here I used 10ng/mL Pertussis toxin added in the medium, a concentration when cell migration was shown to be inhibited (Gilder, Wang et al. 2016). As shown in figure 13, at day 2 after plating, there were more IEL migrated into organoids in the control than when Pertussis toxin was added. This was also observed at day 4 of the co-culture. By counting the number of cells in all regions of interest, at day 2, there were 20% of the IELs outside of enteroids in the control while with PTX treatment, the percentage was nearly 60%. At day 4 of the co-culture, due to the autofluorescent of the dead cells, it was difficult to count the cells but I can still observe there were no IELs outside enteroids in the control, and there were still

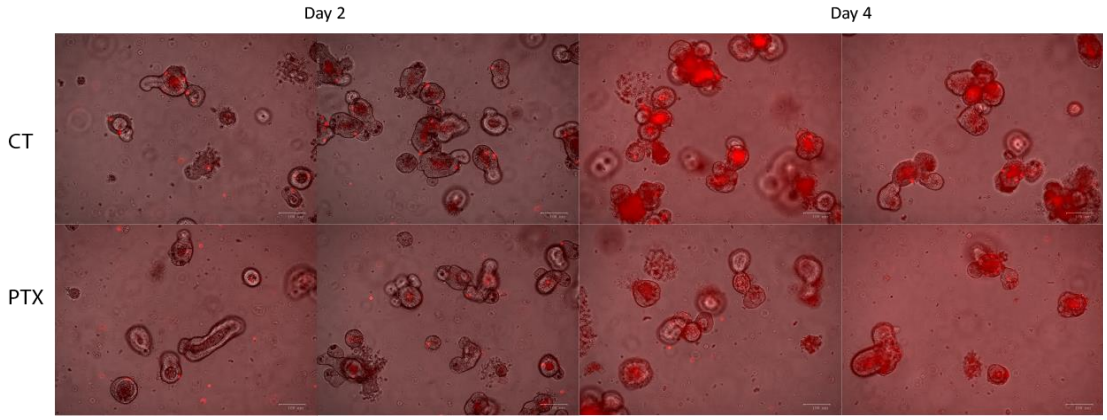

Fig 13. IEL-organoid co-culture with PTX treatment. IEL-organoid co-culture was established as described, supplemented with 100U/mL IL-2 and 10ng/mL soluble IL-15 as controls; to inhibit chemokine receptors, 10ng/mL pertussis toxin was added in the medium. Photos were taken at day 2 and day 4 after plating. The first row was the control (CT) and the second row showed the co-culture with Pertussis toxin (PTX). The first two columns were 2-day and the other two were 4-day old co-culture (n=1).

a few IELs outside enteroids with PTX treatment. The reduction of free IELs outside enteroids might also indicate cell death over time. Despite that, I was able to observe that the number of IELs moved into organoids was less with the pertussis toxin addition. It suggests chemokine signalling pathway might be involved in IEL migration *in vitro*.

Discussion

There have been several studies about IEL migration as IEL patrolling along the epithelium plays a key role in the intestinal immunosurveillance of immune cells. Despite its importance, little is known about the mechanism that regulate IEL motility within epithelial compartment. One study has shown that epithelial IL-15 is a critical regulator for the migration of γδ IELs into the epithelium. However, IL-15 is only sufficient to affect IEL chemokinesis. Co-culture between IEL and intestinal organoid was a suitable system to study the IEL intraepithelial movement as intestinal organoid has been shown to provide a microenvironment that sustains IEL survival *in vitro*. Thus, I wanted to investigate in more detail how IEL motility was regulated within the epithelium using this co-culture system.

In my project, I successfully established long-term IEL-enteroid co-cultures that could be used for the study of IEL intraepithelial movement. I tested three different media for enteroid culturing. L-WRN conditioned medium was used to facilitate the proliferation process to rapidly develop the number of spheroids. Epithelial cells grown in L-WRN conditioned media, developed into spheroids which are spherical in shape and have thin outer walls. Since IEL interacted with more differentiated enterocytes, I switched to medium containing R-Spondin-1 (instead of R-Spondin-3), Noggin and EGF, without Wnt3a (ENR), or OGM to obtain a budding structure of differentiated organoids. I also tested these media for the study of IEL motility in the co-culture. Generally, I observed that there were less IELs migrated into spheroids than differentiated organoids (data not shown). IEL might prefer intestinal organoids with budding structures as they resemble the structure of the gut *in vivo* and the outer wall of enteroids were similar to the epithelial layer where IELs reside. In addition, since differentiated enteroids mimic the microenvironment of the gut, it can produce some cytokines or growth factors that are favourable to IELs. In contrast, spheroids have thinner outer walls which can hardly support IEL retention. They are composed of mainly progenitor cells, thus, growth factors and signalling molecules secreted might be suitable for spheroid proliferation but not IELs. A former lab member showed that there were no significant difference in IEL survival with the addition of IL-7, thus for my co-culture, I only used IL-15 and IL-2 to support the survival of IELs. Further, IELs might respond to the endogenous IL-15 produced by enterocytes, and it is not clear how much IL-15 the undifferentiated

spheroids can produce. Therefore, we focussed on using differentiated organoids in ENR media for co-culture with IEL.

I could also show that the co-culture model was a good model to study IEL intraepithelial movement as IELs were highly motile in my experiments. I observed a slight reduction in the number of IELs after few days in co-culture, which probably cell death, but I was not able to measure the rate of cell death over time. Despite that, the avarage speed of IELs was approximately similar to that seen by Nozaki et al (Nozaki, Mochizuki et al, 2016). In vivo movement of IELs were measured to be at 3 - 8μm/min (Edelblum, 2015). It can also be used to test the functions of surface receptors or proteins that might involve in IEL migration by adding blocking antibodies. However, to visualize IEL behaviours within enteroids under infection, bacteria needs to be microinjected into enteroids which makes it more complicated.

As we have established a co-culture system which we can use to observe IEL movement, we then investigated any factors might involve in IEL movement. IL-15 was previously shown to regulate TCR$\gamma\delta^+$ IELs motility within the intestinal mucosa *in vivo* and in enteroid co-cultures (Hu, Ethridge et al. 2018). I also found that the mean displacement length and mean speed of IEL movement within enteroids were reduced by treatment with an antibody which inhibits the binding of IL-15 with its receptor. However, tracking the movement of IELs is challenging as organoids have a three-dimension structure and IELs might move out of focus thus the track displaement length might not express the real replacement of IELs. Also, I was not able to separate the data of IEL movement within and outside enteroids. Furthermore, the number of enteroids was approximated and the distribution of organoids and IELs when plating was inevitably uneven. Another caveat of my study was that TM-β1 blocks the IL-2Rβ chain, which is used by both IL-2 and IL-15. There have been no studies of IL-2 effects on IEL migration, however IL-2 was added in both the controls and TM-β1 treatment. Thus, I can conclude that blocking IL-2Rβ affects IEL movement *in vitro* which was consistent with the observation from previous study, which implied that either IL-2 or IL-15 signals are needed for IEL migration.

In addition, I showed that cultured IELs could migrate along a chemotactic gradient of a chemokine, CXCL16. The proteomic data indicated that CXCR6 were upregulated in all three IEL subpopulations after 24 hours exposure to IL-15, and I confirmed these data by flow cytometry. CXCL16 was shown to strongly induce a chemotactic migration of activated CD8 T cells and its chemoattraction was proportional with the amount of the

receptors expressed on the cells (Matloubian, David et al. 2000). It could explain the higher number of IELs migrated to the bottom of the plate with the presence of CXCL16 based on the high expression of CXCR6 on cultured IELs. There was no significant difference in the number of IELs migrated when IL-15 was added to the cells, indicating that IL-15 was not directly affecting IEL chemotaxis, but indirectly through the expression of chemokine receptors, in this case CXCR6. CXCL16 is expressed by intestinal epithelial cells, suggesting that the interaction between CXCR6 on IELs and CXCL16 on epithelial cells might contribute to IEL migrate to and retaining in the intestinal epithelium (Diegelmann, Seiderer et al. 2010). Matsumura et al. showed that ionizing radiation can enhance CXCL16 production by mouse and human breast cancer cells, which can recruit other anti-tumour CXCR6-expressing cells (Matsumura, Wang et al. 2008). A study from Diegelmann showed CXCL16 stimulates the activation of several signaling pathway such as ERK-MAP and Akt kinase (Diegelmann, Seiderer et al. 2010). The mRNA and protein expression of CXCL16 were increased by proinflammatory stimuli and upon intestinal inflammation, suggesting the critical role of CXCL16 in mucosal innate and adaptive immune response regulation (Diegelmann, Seiderer et al. 2010). CXCR6 functions on IELs indeed needs to be investigated as it might suggest novel insights about IEL-IEC interaction, how it contributes to IEL immunosurveillance of the gut and provide new strategies in intestinal cancer treatments.

The functions of chemokine receptors affected by IL-15 in the proteomic data were previously described in the introduction. Among them, CXCR6 was studied in my project and it showed that CXCR6 involved in IELs migration. In addition, with the treatment of Pertussis toxin, which blocks signalling of a major chemokines, strongly inhibited lymphocytes migration (Spangrude, Sacchi et al. 1985). Indeed, treatment of IEL-enteroid co-culture with Pertussis toxin has shown inhibited IEL migration into enteroid. Further, I observed by live imaging that there were less IELs moving in and out of enteroid in co-culture when Pertussis toxin was added in the medium than the control (data not shown). However, I did not have the time to establish whether CXCR6 signaling was important for IEL migration into the enteroids and for IEL intraepithelial movement. Further, it is possible that other chemokines, not just CXCR6, are involved.

Blocking chemokines signaling with Pertussis toxin only partially inhibit IEL migration, indicated that there are other signalling pathways involve. Beside chemokine receptors, the proteomic data has shown the expression of several adhesion molecules on IELs. So

far functions of adhesion molecules on IEL have been shown to relate to IEL recruitment and retention to the epithelial layer. For example, β_2 integrins involve in IEL homing and β_7 integrins play a critical role in interactions between IELs and epithelial cells. Other surface adhesion molecules were upregulated in IELs when pre-treated with high concentration of IL-15 could contribute to the retention of IEL in the epithelium. The adhesion molecules I detected include L1CAM, PECAM-1, ICAM-2 and ALCAM. L1CAM is a neuronal cell adhesion molecule which plays a role in cell migration, cell adhesion and neuronal differentiation (Samatov, Wicklein et al. 2016). This protein is expressed not only on neuronal cells but also non-neuronal cells such as T cells, B cells and monocytes. PECAM-1 is highly expressed at endothelial cell-cell junctions and serves as an adhesive stress-response protein to maintain the endothelium integrity (Lertkiatmongkol, Liao et al. 2016). ICAM-2, intercellular adhesion molecule 2, is an endothelial ligand of LFA-1, together with ICAM-1 (Bargatze, Jutila et al. 1995). LFA-1 (lymphocyte function associated antigen 1) is use by naïve lymphocytes to transmigrate into mucosal sites at the later step of the lymphocyte trafficking to the gut cascade (Springer 1994, Bargatze, Jutila et al. 1995). ICAM-2 is expressed at high levels on endothelium and mediates the LFA-1-dependant adhesion of lymphocytes to endothelial cells (Xu, Bickford et al. 1996, Lehmann, Jablonski-Westrich et al. 2003). Activated leukocyte cell adhesion molecules (ALCAM) interacts with its ligand, CD6 can activate T cells as co-stimulatory molecules (Hassan, Barclay et al. 2004). ALCAM also contributes to murine intestinal stem cell homeostasis by maintaining intestinal stem cell interactions with their niche (Smith, Davies et al. 2017). One noticeble among the upregulated proteins is JAML. JAML (Junctional Adhesion Molecule-Like) belongs to JAM transmembrane protein family that regulate cell-cell interactions and acts in neutrophil chemotaxis (Moog-Lutz, Cave-Riant et al. 2003, Zen, Liu et al. 2005). The interaction between JAML and its ligand, CAR (Coxsackievirus and adenovirus receptor) can mediate epithelial γδ T cell-specific activation and result in cellular proliferation, cytokine and growth factor generation (Witherden, Verdino et al. 2010). Therefore, its upregulation in IELs suggests a potential role for this protein in IEL survival within the epithelium. Vice versa, the downregulated adhesion molecules in pre-treated IELs which could imply the ability of IELs to be less adherent to IECs and move more freely within epithelium layer. Thus, changes in expression of adhesion molecules could also affect IEL interaction, migration to IECs and retention within epithelium. It would be interesting to test the effects of these adhesion molecules on IEL migration in the co-culture.

In addition, there have been several studies about γδ IELs but not αβ IELs migration in intestinal epithelial layer (Hoytema van Konijnenburg, Reis et al. 2017, Hu, Ethridge et al. 2018). From the proteomic data, there were some proteins only upregulated or downregulated in TCRαβ CD8αβ IELs but not in the other two populations. For example, CCR5 were downregulated in TCRγδ CD8αα IELs but strongly upregulated in TCRαβ CD8αβ IELs and TGF-β receptor type 1 expression was not found in *ex vivo* TCRαβ CD8αβ. It suggests that these conventional IELs subpopulation might migrate under different signalling, different movement patterns from TCRγδ IELs. Therefore, it would be interesting to compare TCRαβ IELs and other IEL subpopulations behaviours within the epithelium.

In conclusion, my project has shown a sustainable co-culture between IEL and enteroids and that co-culture was a good model to study IEL intraepithelial movement as IELs were highly motile within enteroids. This movement was attenuated when the binding of IL-15 with its receptors was inhibited, confirming a role for IL-15 in IEL kinetics. IL-15 inhibition via IL-2Rβ blockade reduced IEL migratory patterns, however it is not clear whether it was because of reduce IEL survival or activation, or whether IL-2 contributed to IEL migration. Also it was not clear how much endogenous cytokines produced by enteroids contributed to IEL migration as the movement track was not be able to separate between inside and outside enteroids. One way in which IL-15 can regulate IEL migration is through the upregulation of CXCR6 expression. IELs express a high level of CXCR6 and CXCL16 is the only known ligand for CXCR6, and I showed that IELs cultured with IL-15 migrated in response to the CXCL16 gradient. Treatment of the co-culture with Pertussis toxin showed a reduced migration of IEL into organoids. It suggests CXCR6 and CXCL16 might involve in IEL recruitment and retention to intestinal epithelium. Other newly identified molecules are interesting as they all play important roles in other cell types. The identification of novel molecules that regulate IEL functions will provide targets for improving IEL surveillance of the gut. For example, in cancer or in vaccination strategies against pathogens. Conversly, these targets may also be important for the treatment of intestinal autoimmune diseases, where inhibiting IEL functions may reduce intestinal damage.

Materials and reagents

1. IEL culture media

Component	Cat no.
RPMI medium	31870-025
FBS (10%)	F7524 (Sigma)
Pen/Strep	15140-122
L-Glutamine	25030-024
HEPES 25mM	17-737E
Sodium Pyruvate	11360-070
Non-essential acid amin (NEAA)	11140-035
B-mercaptoethanol (100uM)	

2. IEL isolation media

Component	Cat no.
RPMI medium	31870-025
FBS (10%)	F7524 (Sigma)
Pen/Strep	15140-122
L-Glutamine	25030-024

3. Crypt and organoid culture media

3.1. Crypt media

Component	Cat no.
Advanced DMEM/F12+++	12634-010
Pen/Strep	15140-122
L-Glutamine	25030-024
HEPES	27-737E
B27 supplement 50X	12587-010
N2 supplement 100X	400-163
n-Acetylcysteine 500mM	A9165-5G

3.2. Organoid culture media

Component	Cat no.
Crypt media	
Murine EGF	315-09
Murine Noggin	250-38
Murine R-spondin-1	315-32
CHIR-99021	SML-1046
Valporic acid	P4543-10G
Y-27632	Y-1000

4. L-WRN conditioned media preparation

4.1. L-WRN cells media for thawing and expanding

Component	Cat no.
DMEM High Glucose (Gibco)	11960-044
FBS (10%)	F7524 (Sigma)
Pen/Strep	15140-122
L-Glutamine	25030-024

4.2. L-WRN cells washing media

Component	Cat no.
Advanced DMEM/F12	12634-010
HEPES (15mM)	17-737E
FBS (10%)	F7524 (Sigma)
Pen/Strep	15140-122
L-Glutamine	25030-024

4.3. L-WRN primary media for cell collection and top-up

Component	Cat no.
Advanced DMEM/F12	500mL
FBS (20%)	F7524 (Sigma)
Pen/Strep	15140-122
L-Glutamine	25030-024

Methods

1. IEL isolation from mice

The small intestine was dissected from mice with C57BL/6J background and GranzymeB-Cre-TdTomato and flushed with PBS, then was cut longitudinally and transversely to small 5 – 10mm pieces into warm IEL isolation medium (RPMI medium supplemented with 10% FBS, 5mL Pen/Strep and 5mL L-glutamine) with 1mM DTT (D5545-5G, Sigma-Aldrich). The tissue was incubated for 40 minutes in a shaker following by centrifugation at 500 x g for 5 minutes to remove the supernatant. 10mL warm IEL isolation medium was added and vortexed the tube for 3 minutes. The suspension was passed through a 100µm sieve (EASYstrainer^{TM}, Greiner Bio-One). The tissues were collected and vortexed with another 10mL and further 30mL IEL isolation medium were added to wash the sieve. Subsequently, the content was centrifuged at 500 x g for 5 minutes, and the pellet was resuspended in 5mL of 40% Percoll (diluted in PBS). Then the suspension was carefully added on top of 5mL 75% Percoll, following by centrifugation at 700 x g for 30 minutes with no brake. After centrifugation, IELs at the interface between two layers of Percoll were collected and washed with IEL culture medium (500mL RPMI supplemented with 10% FBS, 5mL Pen/Strep, 5mL L-glutamin, 12.5mL HEPES, 5mL Sodium Pyruvate, 5mL Non-essential acid amin (NEAA), 1mL B-mercaptoethanol (100µM)).

2. Crypt isolation and culture

A part of small intestine, about 5 – 7cm long, was dissected from WT mice (C57BL/6J). Crypts isolated from duodenum, jejunum and ileum can be used for organoid culture. In my study, I used duodenum part. The intestine was washed with cold PBS, cut longitudinally then spread open in a petri dish placed on ice. The villus was scraped off carefully using a coverslip. The intestine was cut into small 3 – 5mm pieces and transferred to 30mL of PBS with 1mM EDTA cooled on ice, then incubated for 20 minutes at 4^{o}C on a tube roller. After incubation, the content was passed through a 100µm sieve following by 30 minutes incubation with 30mL PBS with 5mM EDTA. The content was passed through the sieve again and the intestine was transfer to 10mL cold PBS. The tube was shaken vigorously for one minute. Observed the mixture to see the crypts and take 2mL to a new 15mL tube. The tube was centrifuged at 100 x g for 5 minutes, the supernatant was discarded then resuspended the pellet with 2mL cold Advanced

DMEM/F12 (ADF) (500mL ADF; supplemented with 10% FBS, 5mL Pen/Strep, 5mL L-glutamine and 7.5mL 1M HEPES). 10µL was taken to count the number of crypts while centrifuging again. The pellet was resuspended in ADF to have about 50 – 100 crypts per 50µL. The suspension was added into pre-thawed Matrigel (Corning, Cat no. 356231) with a ratio of 1:3 (suspension versus Matrigel), mixed thoroughly then 20µL was seeded at the centre of each well of a 24-well plate. The plate was incubated at 37^{o}C for 10 – 15 minutes for Matrigel polymerization before adding 500µL of organoid growth medium. Organoids were cultured at 37^{o}C and 10% CO_2 and fresh medium was changed every 2 – 3 days.

Organoids were passaged every 5 – 6 days, 1:2 or 1:3, depending on the number of organoids in each well and how dark the lumens were. 1mL of cold ADF were added in each well to dissociate the organoid and the suspension was centrifuged at 100 x g for 5 mins. The pellet was resuspended in ADF before adding the suspension into Matrigel and plated it out.

Growth medium used:

- ENR: crypts medium supplemented with 50ng/mL EGF (PeproTech, 315-09), 100ng/mL Noggin (PeproTech, 250-38) and 1µg/mL R-spondin-1 (PeproTech, 315-32). For the first two days, 1µM CHIR-99021, Valproic acid and 10µM Y-27632 is necessary to add in the medium (ENR-CVY). Prepared fresh in each experiment. Crypt medium: Advanced DMEM/F12 supplemented with 5mL Pen/Strep, 5 mL HEPES, 5mL L-glutamine,1 mL N-acetylcysteine, 5mL N2 supplement, 10mL B27 supplement.
- OGM: Organoid growth medium, purchased from Stem Cell Technologies (Cat no. #06005), a commercial medium. Aliquots were kept in the freezer, used within 2 weeks after thawing.
- Conditioned L-WRN media: harvested from L-WRN cells (ATCC® CRL-3276™) which are a source of Wnt3a, R-spondin-3 and Noggin. The cells were expanded and selected with Hygromycin B Gold and G418, then let the cells grew overconfluent for 3 to 4 days. Cells were washed then cultured with Primary media (Advanced DMEM/F12 supplemented with 20% FBS, 5mL Pen/Strep and 5mL L-glutamine) for 24 hours. The medium was collected and centrifuged to get rid of dead cells. Conditioned L-WRN media was used at 50% dilution (Miyoshi and Stappenbeck 2013).

3. Cell culture of IEL

IELs were enriched following an EasySep™ Mouse CD8α positive selection kit protocol from Stem Cell Technologies. Enriched IELs were then washed with IEL culture medium. These cells were seeded in a 96-well plate (U version) at 2×10^5 cells per well with 100ng/mL Mouse IL-15R Complex Recombinant Protein (Life Technologies) for migration assays and flow cytometry.

4. IEL-organoid co-culture

IEL were isolated and enriched for CD8α cells following the method above. Two-day-old organoids were collected by adding 1mL of cold ADF media in each well after removing the old medium. The Matrigel was dissociated carefully and collected into 15mL tube. After centrifugation at 100 x g for 5 mins, the supernatant was discarded, and IELs were added into the tube at the ratio 1 crypts versus 500 IELs, 400µL of growth medium was added then incubated the mixture at 37ºC for 30 minutes. After 30 minutes, the mixture was centrifuged at 100 x g for 3 minute. The supernatant was discarded, the pellet was resuspended in cold PBS and added to thawed Matrigel. 20µL of the mixture was seeded in a 24-well plate then the plate was incubated at 37ºC for 10 – 15 minutes before adding the growth medium (supplemented with 10ng/mL soluble IL-15 and 100U IL-2). For confocal microscopy, the co-culture was seeded in a µ-Slide 8 well ibiTreat (Ibidi, Cat no. 80826). TM-β1 (BioLegend, Cat no. 123223) were added at the final concentration of 40µg/mL into medium at 48h and 1h before imaging. Medium was changed every 2 – 3 days.

To block chemokine and G protein-coupled receptor signalling, 10ng/mL Pertussis toxin (Sigma-Aldrich, Cat no. 516560) was added in the medium when plating.

Photos of the co-culture were taken by ZOE fluorescent cell imager (Bio-Rad).

5. Live imaging

Live imaging of co-culture was performed using Zeiss 710 Confocal Microscope system, objective 20X Dry. 20 Z-stacks of each region of interest (ROI) were acquired with an interval between each stack is 3µm. Data was analysed by Imaris and ImageJ (Fiji). For Imaris, IELs were tracked by creating a Spot function that detects cells with diameter from 7.5µm - 8µm. All data of each spot were exported. With ImageJ, TrackMate plug-in was used (Tinevez, JY.; Perry, N. & Schindelin, J. et al., 2017).

6. Migration assay

IELs were isolated and sorted as previously described and put in culture with 100ng/mL IL-15Rα (Life Technologies) for 24 hours. At the day of experiment, the cells were collected and washed twice with IEL culture medium. Approximately 5×10^5 IELs were plated in the Corning® Transwell® polycarbonate membrane cell culture inserts (Sigma-Aldrich, Cat no. CLS3421-48EA) with or without 100ng/mL IL-15Rα. 600µL medium supplemented with 10ng/mL CXCL16 (Novus) was added to the bottom chamber. The plate was incubated for 1.5 hours. Afterwards, Transwell inserts were removed, cells were collected at the bottom chamber and counted with LSR Fortessa by adding CountBright™ Absolute Counting Beads, for flow cytometry (ThermoFisher Scientific).

7. Flow cytometry

Cell were plated at 2×10^5 cells per well to a 96-well plate for staining. Fc block was added to each well for 5 minutes before cells were incubated with monoclonal antibodies against cell surface markers for 15 minutes covered on ice. Cells were stained with the following antibodies: CXCR6 [clone SA051D1 (BioLegend)], TCRβ [clone H57-597 (BioLegend)], TCRγδ [clone GL3 (BioLegend)], CD8α [clone 53-6.7 (BioLegend)], CD8β [clone H35-17.2 (Invitrogen)]. Cells were then collected and resuspended in FACS buffer (PBS + 1% FBS). Data was acquired using a FACS LSR Fortessa flow cytometer with DIVA software (BD Bioscience) and analysed using FlowJo software (TreeStar).

8. Mass spectrometry

Sample preparation for mass spectrometry were previously described in (James, Vanderyken et al. 2020). Briefly, IELs were isolated as previously described and $CD8\alpha^+$ IEL population was enriched using an EasySep™ Release PE positive selection kit (STEMCELL Technologies) with a PE-conjugated anti-mouse CD8α antibody (BioLegend) following instructions from the manufacturer. TCRγδ CD8αα, TCRαβ CD8αα and TCRαβ CD8αα were purified by fluorescent activated cell sorting (FACS). Four biological replicates of each population were generated. IEL cell pellets were lysed in 200µl lysis buffer (4% SDS, 10mM TCEP, 50mM TEAB (pH 8.5)). Lysates were boiled and sonicated (15 cycles of 30s on/30s off) and protein concentrations determined by EZQ® Protein Quantitation Kit (Invitrogen). Lysates were alkylated with iodoacetamide (IAA) for one hour at room temperature in the dark. Proteins and peptide clean-up were performed according to Hughes et al., (2014). Samples were resuspended in 2% DMSO and 5% formic acid and fractionated using an Ultimate 3000 HPLC

(Thermo Scientific). Samples were sent to MRC-PPU Mass Spectrometry facility, University of Dundee, where each fraction was analysed by label-free quantification (LFQ) using an LTQ OrbiTrap Velos Pro (Thermo Scientific) with a 240-minute gradient per fraction.

9. Statistics

All statistics was performed using Graphpad prism 8. Data was presented with mean ± SD or with a 95% confidence interval. Data was analysed using one-way ANOVA with Dunnett's multiple comparisons.

Appendix

Table 1: Upregulated and downregulated proteins by IL-15

	Protein names	Functions
Upregulated	Chemokine (C-X-C motif) receptor 3 (CXCR3)	Chemokine receptor, chemokine signaling pathway
	Chemokine (C-C motif) receptor 5 (CCR5)	
	Chemokine (C-C motif) receptor 9 (CCR9)	
	Chemokine (C-X-C motif) receptor 6 (CXCR6)	
	Adhesion G protein-coupled receptor G5(Adgrg5)	G-protein coupled receptor, GPCR signaling pathway, GPCR activity
	G protein-coupled receptor 132 (Gpr132)	
	G protein-coupled receptor 171 (Gpr171)	
	G protein-coupled receptor 55 (Gpr55)	
	G-protein coupled receptor 65 (Gpr65)	
	G protein-coupled receptor 18 (Gpr18)	
	ATP-binding cassette, sub-family A (ABC1), member 7 (Abca7)	Tight junction, cell junction, cell-cell adhesion, adhesion molecules
	Adhesion molecule, interacts with CXADR antigen 1 (Amica1/JAML)	
	Transforming growth factor, beta receptor I (Tgfbr1)	
	Desmoglein 1 alpha (Dsg1a)	
	Platelet/endothelial cell adhesion molecule 1(Pecam1)	
	Desmoglein 1 beta (Dsg1b)	
	Integrin alpha 3 (Itga3)	
	Embigin(Emb) (only in TCRabCD8ab)	
	Dystroglycan 1 (Dag1)	
	Activated leukocyte cell adhesion molecule (Alcam)	
	L1 cell adhesion molecule(L1cam)	
Downregulated	Desmoglein 1 alpha (Dsg1a)	Cell adhesion, cadherin-binding involved in cell adhesion
	Desmoglein 1 beta (Dsg1b)	
	Claudin 23 (Cldn23)	
	Integrin alpha M (Itgam)	
	Cadherin 17 (Cdh17)	
	G-protein coupled receptor 65(Gpr65)	G-protein coupled receptor, GPCR signaling pathway, GPCR activity
	Chemokine (C-C motif) receptor 5(Ccr5)	Chemokine receptor, chemokine signaling pathway

Table 2: Fold changes of the molecules shown in figure 9 and 10.

	Proteins	TCRγδ CD8αα	TCRαβ CD8αβ	TCRαβ CD8αα
Chemokine receptors	CXCR6	2.67	4.18	4.32
	CXCR3	1.17	3.13	1.13
	CCR5	0.35	4.74	0.00
	CCR9	1.25	5.87	1.50
Adhesion molecules	ALCAM	2.17	3.26	1.25
	PECAM-1	4.69	1.26	1.70
	ICAM-2	2.02	3.39	2.70
	L1CAM	3.98	3.84	4.31
Activating receptors	JAML	8.62	19.60	17.12
	CD226	2.67	2.05	6.34
Down-regulated molecules	Cdh17	0.14	1.93	0.29
	ItgaM	0.45	1.50	0.92
	CCR5	0.35	4.74	0.00

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