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Master thesis

Cytokines influence gut duodenal epithelial cells' differentiation and celiac disease development

Crosstalk between gut epithelium and immune cell secreted cytokines

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Genetics and developmental biology

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Summary

A complex crosstalk network between the epithelium and the immune systems is required to maintain intestinal homeostasis. My master's thesis represents the comprehensive study of duodenal epithelium and immune cell-secreted cytokines crosstalk. We identified how cytokines treatment could mimic the in vivo condition of epithelium and immune cell communication. Furthermore, we found how different cytokines modulate the expression of various genes in duodenal organoids, resulting in different effector responses.

This study describes the comprehensive understanding of the human duodenal intestinal epithelium and cytokine crosstalk. Previous analysis has been performed using different cytokines in ileal and colonic organoids. For example- the impact of IL-22 has been studied on ileal organoids, and they found that IL-22 drives human Paneth cell differentiation. Likewise, we found that IL-22 induces gene signatures specific for Paneth cells in duodenal organoids. Therefore, our study is novel as we have performed in duodenal organoids.

We have dissected the crosstalk between duodenal epithelium and immune cell-secreted cytokines. Using duodenal organoids as a model, we identify the role of IFN-gamma in the induction of celiac disease-associated genes.

Acknowledgements

My time at Rikshospital of Oslo in the Department of Pathology, University of Oslo, has been a year filled with ups and downs. However, it has been the most remarkable year in my life, where I have learned a lot and grown as an individual and scientist. It was hard and challenging to work with many unfamiliar laboratory techniques and write my master's thesis. Still, I would like to appreciate and give my great thanks to those who helped and taught me with their special patience, kindness, and professional guidance. I would only have overcome my challenges with their professional guidance and supportive motivations. They have been inspirations and idols for me and my work, giving me valuable insight into the researcher's life.

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Abstract:

Background: The crosstalk between immune cells and intestinal epithelial cells is essential for gut homeostasis, and alterations can result in gut disease or inflammatory diseases, such as inflammatory bowel disease (IBD), a group of chronic inflammatory diseases of the digestive tract. Both immune and intestinal epithelial cells play a significant role in maintaining our gut homeostasis. Immune cells produce cytokines that play a critical role in controlling inflammation in the body. In contrast, intestinal epithelial cells have mucins and other proteins that build mucous layers to prevent the entrance of pathogens. **Objectives:** The objective of this study is to elucidate the crosstalk between immune cells and intestinal epithelial cells, particularly to study the impact of different types of cytokines, which are the products of immune cells in the proliferation, differentiation, and survival of intestinal epithelial cells such as goblet, Paneth, tuft and enteroendocrine cells (secretory lineage). Furthermore, we associate the impact of those cytokines with the development of celiac disease. In this condition, immune cells attack your tissues when you eat gluten, damaging your small intestine.

Methods: We used the human organoid model to study the crosstalk between immune cells and intestinal epithelial cells. To explore the role of cytokines in intestinal epithelial cells' survival, proliferation, and differentiation and celiac disease development, we chose seven cytokines from the three broad categories of cytokines. We chose five interleukins (IL-4, IL-5, IL-13, IL 17-alpha & IL-22), one Interferon (Interferon gamma) and one TNFs (TNF alpha). We treated the organoids with those cytokines and then bulk-RNA sequenced them. Finally, we validate the few results with immunofluorescence stainings and confocal microscopy.

Results: We found that both type one cytokines IFN- γ and TNF-α affect epithelial cell differentiation. On the other hand, for type two cytokines, IL-13 and IL-4 affect cell differentiation in duodenal organoids, and IL-5 does not affect differentiation. For type 3 cytokines, IL-22 was the most potent inducer of epithelial cell differentiation in duodenal organoids, and IL-17a had no effect. Furthermore, we found that IFN- γ induces genes responsible for celiac disease progression, and we found upregulation of IL-15 and IL-15Rα after IFN- γ stimulation in duodenal organoids.

Conclusion: We dissected the duodenal epithelium – immune cell crosstalk using human organoids as a model. Our study is novel as we have performed comprehensive studies in human duodenal organoids.

List of abbreviations

APC	Adenomatous polyposis coli
BMP	Bone morphogenic protein
BCM	Bone conditioned media
BSA	Bovine serum albumin
CBCS	Crypt base columnar cells
CD	Celiac disease
ChgA	Chromogranin A
CK1	Casein kinase 1
CRS	Cytokine release syndrome
DAPI	4`,6-diamidino-2-phenylindole
DEG	Differentially Expressed Genes
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's Phosphate buffered saline.
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EpCAM	Epithelial cell adhesion molecule
FBS	Fetal bovine serum
GI	Gastrointestinal
GSEA	Gene set enrichment analysis
GSK3	Glycogen synthase kinase 3
IBD	Inflammatory bowel disease

IECs	Intestinal epithelial cells
IgA	Immunoglobulin-A
INF	Interferon
IL	Interleukin
ISCs	Intestinal stem cells
KEGG	Kyoto Encyclopedia of Genes and Genomes
Lgr5	Leucine-rich-repeat-containing G-protein-coupled receptor 5.
LP	Lamina propria
MAPK	Mitogen-activated protein kinase
MUC2	Mucin 2
NES	Normalized enrichment score
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
Pen	Penicillin
REG1A	Regenerating islet-derived protein 1-alpha
RSPO1	Roof plate-specific spondin 1
Strep	Streptomycin
TA	Transit amplifying
TCF	T-cell factor
TEM	Transmission electron microscopy
TGF α	Transforming growth factor- α
TGM2	Transglutaminase 2
TNF	Tumor necrosis factor
TPM	Transcripts per million

Tx100	Triton X-100
UC	ulcerative colitis
UEA 1	Ulex europaeus agglutinin 1

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1.0 Introduction

The crosstalk between intestinal immune cells and intestinal epithelial cells is critical for gut homeostasis, and alterations can cause inflammatory diseases, such as inflammatory bowel disease (IBD), a group of chronic inflammatory diseases of the digestive tract or/and celiac disease. But in this study, I only focused on the small intestine epithelial cells, cytokine-mediated crosstalk between immune cells and epithelial cells in the small intestine, duodenum. The immune system produces small proteins called cytokines and humoral factors to protect the host when threatened by microbial invasion, inflammatory agents, or injury (Picower, 1992). Cytokines are hormonal messengers involved in most biological effects on the immune system, such as cell-mediated immunity and allergic responses (Berger, 2000). In the human gut, several epithelial cells perform different functions. Some of them play an essential role in food digestion and absorption of nutrients, and others play a vital role in body protection from microbial infections.

1.1 The human digestive tract:

The human digestive system consists of the gastrointestinal tract (GI tract) and the accessory organs of digestion. The digestive tract is a tubular organ designed to perform the body's various functional requirements (Van Der Flier & Clevers, 2009). For instance, our digestive tract involves food reception and storage, digestion, peristaltic transport, nutrient absorption, and elimination and acts as a barrier to unwanted and infectious substances. This open-ended tube is about 8-9 meters long and extends from the mouth to the anus (Sensoy, 2021). It comprises the pharynx, oesophagus, stomach, and small and large intestines. The teeth, tongue, salivary glands, liver, gall bladder, and pancreas are accessory organs (Fig 1.1) (Beluffi, 2008). The GI tract and accessory organs break down food into nutrients such as carbohydrates, proteins, and fats. These nutrients are then absorbed into the bloodstream, and the body can use them for energy, growth, and repair of damaged cells. However, food and nutrition can profoundly affect host health and disease (Sánchez et al., 2017). Consumption of various nutrients affects the structure of the microbial community and provides substrates for microbial metabolism (Albenberg & Wu, 2014). This change in the microbial community in the GI tract may cause immune system activation, resulting in inflammation or damage of the bowel.

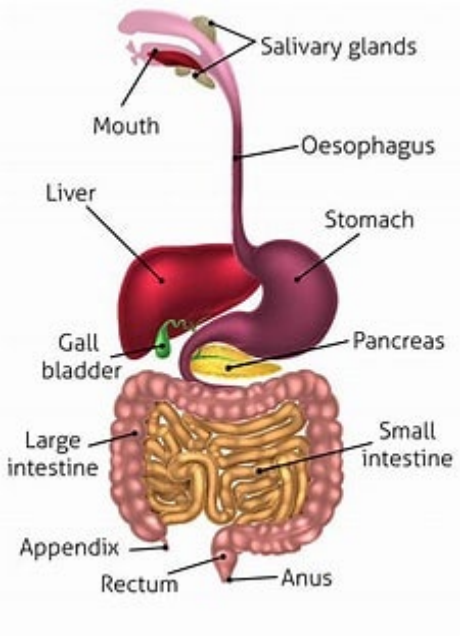

Fig.1.1 The structure of the human gastrointestinal tract with accessory organs. The GI tract consists of the oral cavity, pharynx, oesophagus, stomach, small and large intestine. It also consists of accessory organs such as the teeth, tongue, glandular organs (salivary glands, liver, gallbladder, and pancreas).

1.2 Intestinal epithelial cells and gut homeostasis:

A single layer that renews every 4-5 days covers the human intestine and is called the epithelium. This epithelium comprises proliferative crypts containing intestinal stem cells and villi containing differentiated cell types (Van Der Flier & Clevers, 2009). As I mentioned, this intestinal epithelium is a self-renewing tissue fueled by committed stem cells (Tan & Barker, 2014). The intestinal stem cells reside at the base of the crypt, which poses adjacent epithelial cells, stromal cells, smooth muscle cells, and cell-associated growth and differentiation factors (Fig.1.2) (Hageman et al., 2020; Sailaja et al., 2016). These cells are closely connected to neighboring cells of both epithelial and mesenchymal origin (Hageman et al., 2020). The intestinal cells and their interplay forms the intestinal stem cell niche, which comprises both epithelial cells, specifically Paneth cells and the stromal compartment, in which cell-associated ligands and soluble factors regulate stem cell behavior (Hageman et al., 2020). Therefore, a stem cell niche can be defined as an association of a stem cell with other cells, determining its behavior. An intestinal stem cell marker, Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5, also known as Gpr49), makes it possible to study the crypt-villus units. Lgr5 was selected from a panel of intestinal WNT target genes for its restricted crypt expression (Barker et al., 2007). In over a 60-day, all the epithelial cell lineages are generated by Lgr5-positive crypt base columnar cells (CBCs). This suggests Lgr5-positive represents the stem cell of

the small intestine & colon (Barker et al., 2007). Therefore, Lgr5+ is an accurate marker of CBCs, and Lgr5-EGFP-CreER^{TA} mice are a valuable tool for understanding their regulations (Trentesaux & Romagnolo, 2018). Several signaling factors sustain the proliferative nature of Lgr5+ stem cells. For instance, stem cell maintenance, regeneration, and differentiation are regulated by interleukins, Hippo signaling and metabolic cues, WNT, NOTCH, EGF and bone morphogenetic protein (BMP) (Gehart & Clevers, 2019). As mentioned above, In the small intestine, the continuous renewal of the epithelium is fueled by Lgr5+ intestinal stem cells (ISCs) that give rise to highly proliferative transit amplifying (TA) cells (Barker et al., 2007). Those TA cells differentiated into enterocytes, goblet, Paneth, enteroendocrine, and Tuft cells. Except for Paneth cells, all the differentiated TA cells migrate up to the villus tip, where they are extruded into the lumen (Choo et al., 2022; Hageman et al., 2020). However, the intestinal Paneth cells reside close to the ISCs and have a critical role in establishing the intestinal stem cell niche by producing the above signaling molecules, such as Wnt, EGF and Notch ligands (Choo et al., 2022). Furthermore, the basement membrane affects the stem cell niche or behavior.

Basement membrane support and separate the epithelium from underlying connective tissues and lamina propria (Li & Thompson, 2003). The basement membrane also underlies the basal surface of the intestinal epithelium. This gut epithelial basement membrane is a remarkable structure composed of extracellular matrix (ECM) components such as laminins, collagen IV, proteoglycans and other structural or adhesive proteins (Choo et al., 2022; Li & Thompson, 2003). As I mentioned earlier, the basement membrane influences the behavior by controlling epithelial cells' gene expression, adhesion, migration, proliferation, and apoptosis.

Generally, organ homeostasis is vital for physiology and preventing various infections and diseases. The signaling pathways, as mentioned earlier, are involved in regulating intestinal homeostasis, specifically Wnt, bone morphogenetic protein (BMP), and Notch and epidermal growth factor (EGF) (Hageman et al., 2020; Sailaja et al., 2016). As mentioned, human intestinal epithelium is maintained through constant proliferation in the crypt and apoptosis of differentiated epithelial cells at the villus (Bao et al., 2020). In addition, the gut mucosal barrier is constructed by intestinal epithelial cells and is involved in maintaining gut homeostasis by segregating gut microbiota and host immune cells(Coskun, 2014; Okumura & Takeda, 2018).

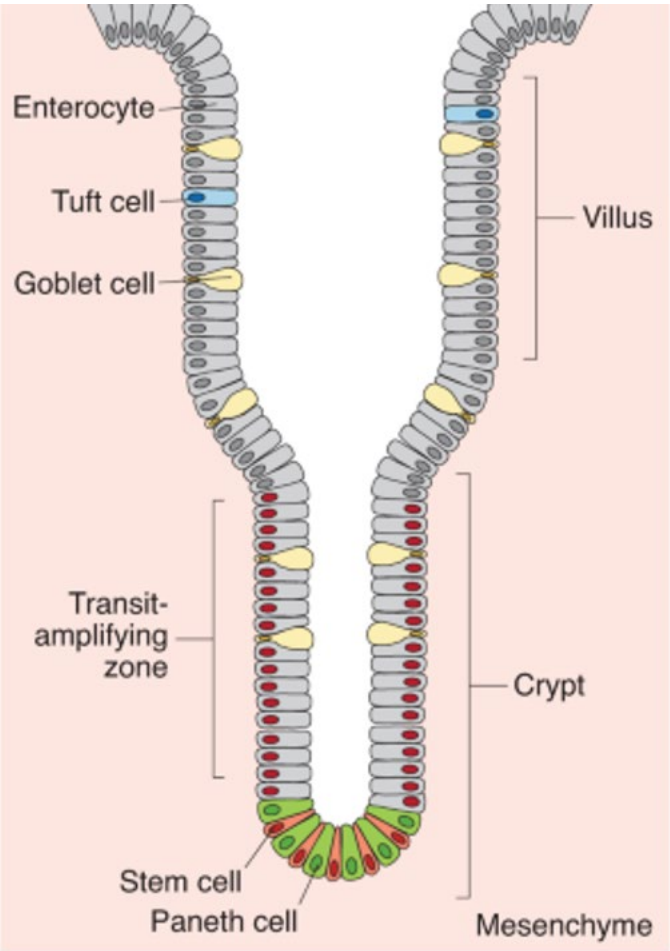

Figure 1.2 Representation of the intestinal epithelium showing the crypt where stem cells and Paneth cells reside and the villi along which differentiated epithelial cells migrate upward toward the tip. The epithelium comprises six different mature cell types, which are separated into absorptive (enterocytes and M cell) and secretory (Paneth, goblet, enteroendocrine and tuft cell) lineages. These different cell types have specialized functions including nutrient uptake (enterocytes), metabolic control or hormone secreting (enteroendocrine cells), immune regulation (Tuft cells), mucin-secreting (Goblet cells) and antimicrobial peptide-secreting (Paneth cells) (Hageman et al., 2020; Tan & Barker, 2014; Trentesaux & Romagnolo, 2018).

1.3 Intestinal epithelium in inflammatory bowel disease (IBD) and Celiac disease.

The abundance of immune cells that reside with many beneficial microorganisms in the gastrointestinal tract requires barrier and regulatory mechanisms that keep host–microbial interactions and tis-

sue homeostasis (Peterson & Artis, 2014). Therefore, intestinal epithelial cells produce a mucosal layer that makes it possible to maintain the symbiotic relationship between the gut microbiota and the host by separating the immune cells and gut microbiota. The intestinal epithelium has an excellent and strategic location as a protective physical barrier to luminal microbiota and contributes to the mucosal immune system (Coskun, 2014). However, for different reasons, chronic inflammatory states, such as IBD and celiac disease, may damage the intestinal mucosa. Furthermore, intestinal mucosa can be degraded because of chemotherapy and radiation therapies. Then the mucosal layer becomes permeable to bacterial entry (see Fig 1.3). Recent studies indicate mucosal barrier dysfunction contributes to developing inflammatory bowel disease (Okumura & Takeda, 2018), a chronic idiopathic inflammation of the gastrointestinal tract with complex etiologies (Coskun, 2014; Dou et al., 2021; Strober et al., 2007). It consists of Crohn's disease (CrD), which affects the entire gastrointestinal tract, and ulcerative colitis (UC), which affects only the mucosa of the large intestine (Coskun, 2014; Maloy & Powrie, 2011). Intestinal wound healing is a complicated process that involves both epithelial and immune cells (Xue & Falcon, 2019). So, the crosstalk between epithelial and immune cells is critical in intestinal wound healing. The wound repair process in the gut involves immune cells, particularly neutrophils, macrophages, regulatory T cells, innate lymphoid cells (ILCs) and cytokines, which are products of these immune cell types such as interleukin-10, tumor necrosis factor-alpha, interleukin-6, and interleukin-22 (Xue & Falcon, 2019). All these cytokines and others, such as interleukin 12 and 23, have a critical role in wound healing, CD and IBD (Figure 1.3)

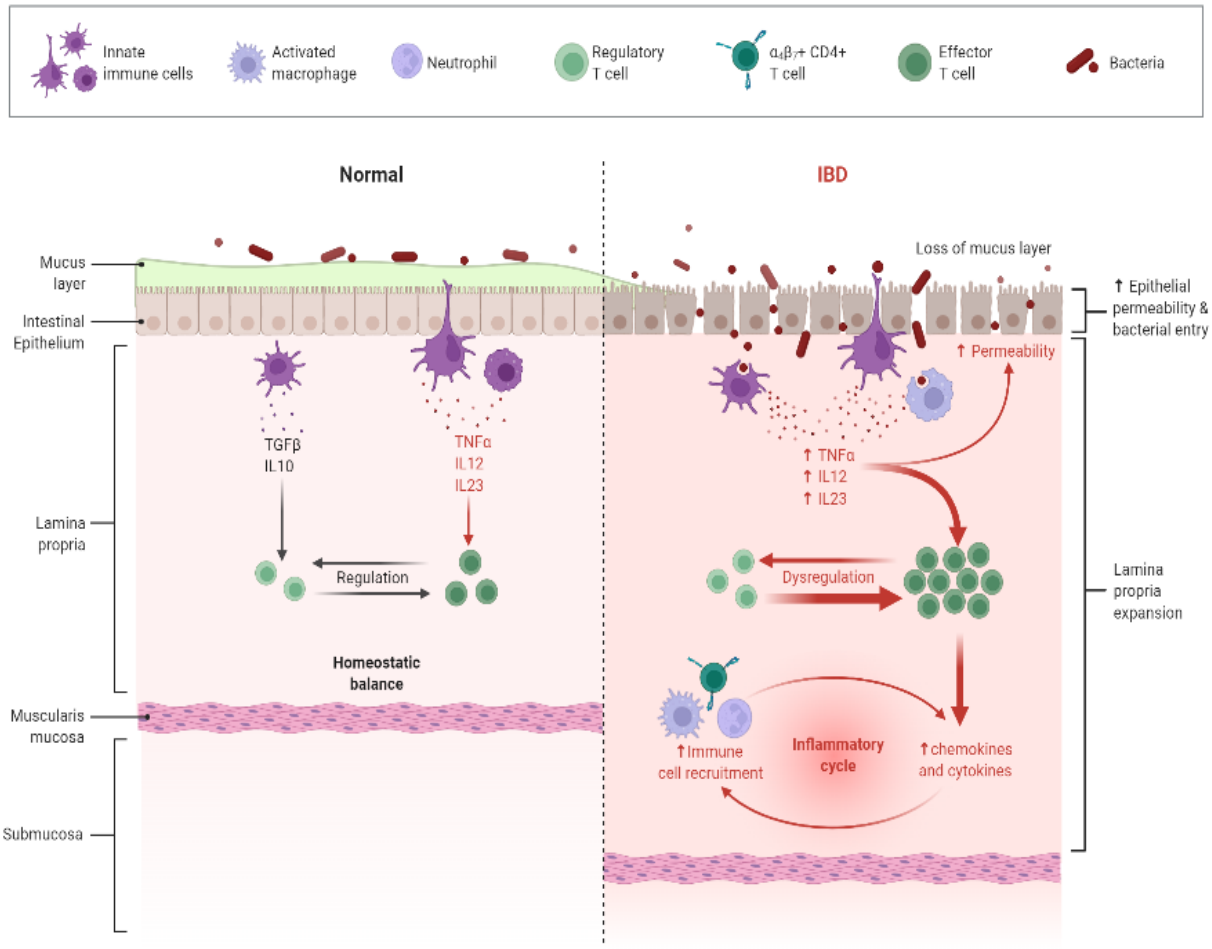

Fig: 1.3. Immune response in normal condition and on the inflammatory bowel disease (IBD). Both figures show the crosstalk between immune and intestinal epithelial cells during homeostatic and inflammatory situations. (Left) normal condition, the innate immune cells produce cytokines such as TNF alpha, IL12, IL23 that activates effector T cells. Furthermore, innate immune cells release TGF beta and IL10 that activate regulatory T cells and as shown on the figure keeps regulated and results homeostatic balance in the LP. (Right) IBD, shows loss of mucus layer and intestinal epithelium becomes permeable to microbes. The entrance of microbes causes the innate immune cells to be activated and increase the production of TNF alpha, IL12 and IL 23 that results in the activation of many effector T cells that causes an increase in chemokines and cytokines that results in the recruitment of neutrophils, activated macrophages, CD4+ T cells. As shown on the figure (right) the lamina propria expanded whereas the submucosa is compressed because of inflammatory cycle. The figure is created from biorender.com.

1.4 Celiac disease (CD):

Celiac disease is an autoimmune disorder caused by dietary gluten in genetically susceptible individuals (Alarida et al., 2011; Canada, 2007; Kagnoff, 2012; Mustalahti et al., 2010; Sharaiha et al., 2012). Gluten is a complex protein that is found in many grains, including wheat, barley, and rye. CD occurs in around 1% of the population worldwide (Canada, 2007; Kagnoff, 2012) with wide regional differences (Mustalahti et al., 2010). For instance, in Europe the prevalence is 0.3% in Germany and 2.4% in Finland (Canada, 2007; Mustalahti et al., 2010). It is also common in developing countries, particularly north Africa and middle east (Alarida et al., 2011; Canada, 2007). CD is also observed mainly in the northwestern part of India, where wheat is the main food of the society. Cases of CD also have been observed in China (X. Q. Wang et al., 2011). The frequency of celiac disease is increasing in many developing countries because of westernization of the diet, changes in wheat production and preparation. CD symptoms vary among different patients. Some common signs and symptoms of celiac disease are diarrhea, anemia, joint pain, headache, depression, weight loss and many other symptoms (Review, 2023). Serologic screening shows that clinically silent CD is increasingly detected (Canada, 2007). Some complications that associated with untreated celiac disease also include osteoporosis, impaired splenic function, neurologic disorders, infertility or abortion, and cancer (Canada, 2007; Sharaiha et al., 2012). Celiac disease is diagnosed by histopathologic examination of duodenal biopsy specimens (Kelly et al., 2015). The only well-known treatment available for CD individuals is a strict life-long gluten free diet (Gujral et al., 2012).

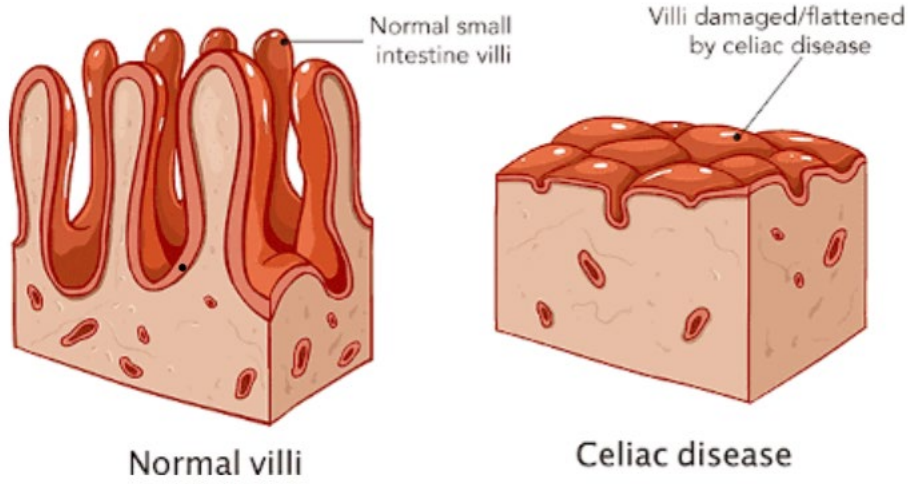

Figure 1.4: The lining of the small intestine. Right figure shows the normal villi which performs proper functioning of the small intestine whereas the left figure shows when an individual with celiac disease feeds upon gluten, the immune system considers gluten as a foreign substance and mounts an attack that damages villi and makes it almost impossible for the body to absorb nutrients that leads to malnourishment. The figure is from free using google.

1.5 Human Intestinal Immune System:

The immune system is a complex system that is critical for the survival of an organism. When the body is exposed to harmful invaders such as viruses, bacteria, fungi, protozoans or injury in body parts, the immune system launches an attack to destroy the pathogens. There are two main types of responses to invading microbes, which are called innate (natural) response and acquired (adaptive) response (Peter J. Delves, 2000). Those two systems work closely together, but they perform different tasks.

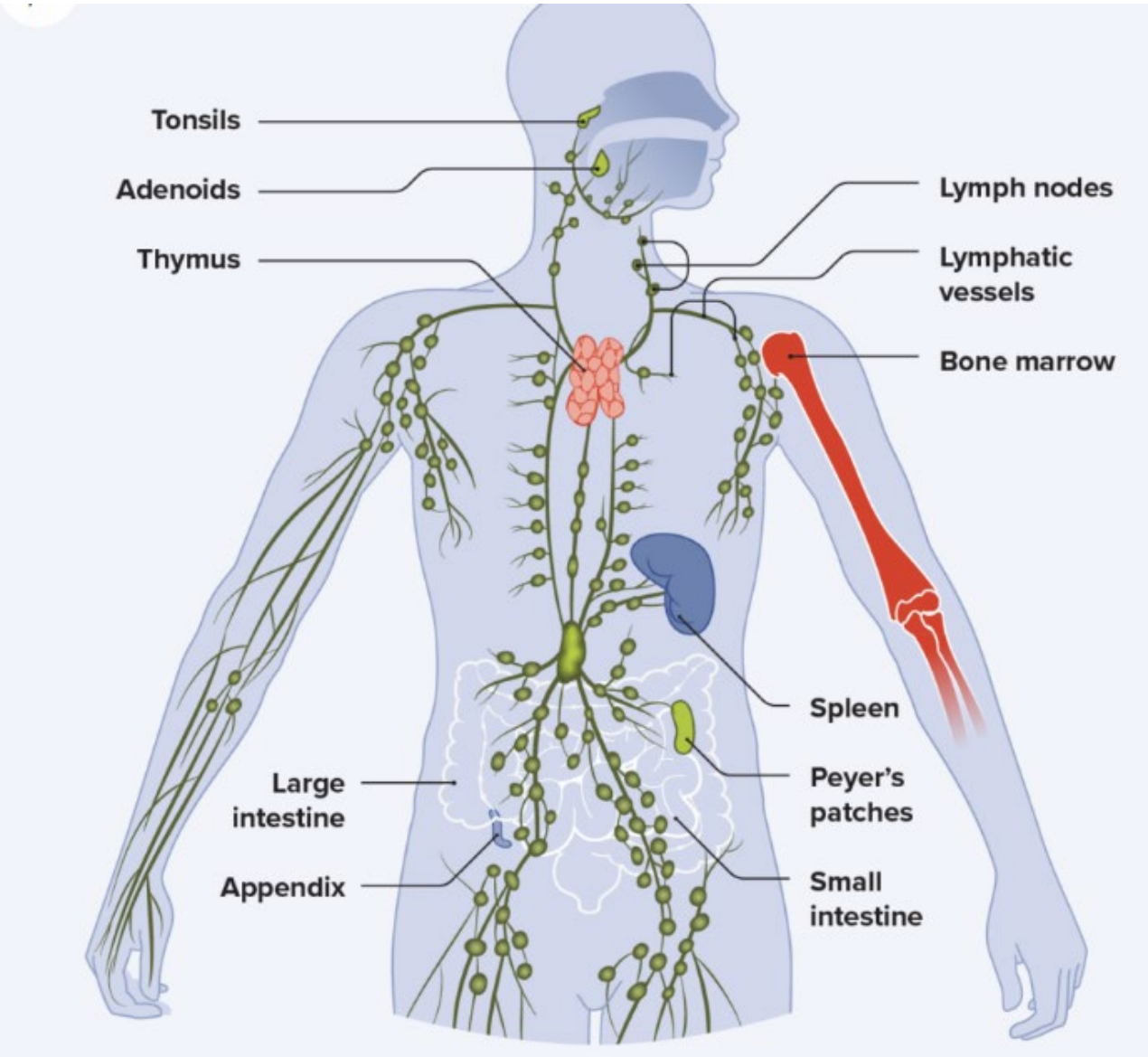

Figure: 1.5 human immune system is a complex network of organs, cells and proteins that protects the body against infection and harmful foreign substances but protecting the body's own cells. The main organs of the immune system are lymphoid organs, lymph nodes, bone marrow, thymus, spleen, tonsils and mucous membranes, more than half of all the body's cells that produce antibodies are found in the bowel wall (large, small intestines and appendix). Peyer's patches are aggregated lymphoid nodules and involves in monitoring intestinal bacteria populations and preventing the growth of pathogenic bacteria in the intestines (Fulton & Walker, 2013).

1.6 Human Innate and adaptive immune system

Innate immunity is the body's first line of defenses that responds in the same way to all germs and foreign substances, and it is also called a nonspecific type of immunity. The innate immune system comprises the skin, mucous membrane, immune cells, and proteins. Therefore, it consists of humoral immunity components and cell-mediated immunity components. All those components have their role in protecting the body from hazardous substances. For instance, the well-protected surface of the skin and mucous membrane forms a physical barrier against germs. Additionally, chemical substances such as enzymes, acid and mucus prevent bacteria and viruses from replicating. The innate immune system also activates special immune system cells and proteins if pathogens pass through the skin and mucous membranes and enter the body.

Adaptive immunity involves a tightly regulated interplay between antigen-presenting cells and T and B lymphocytes, which facilitate pathogen-specific immunologic effector pathways, generation of immunologic memory, and regulation of host immune homeostasis (Bonilla & Oettgen, 2010). Like the innate immune system, the adaptive immune system includes both humoral and cell-mediated immunity components, which play a critical role in destroying invading pathogens. However, unlike innate immunity, it is highly specific to each pathogen the body is exposed.

1.7 Cytokines

The human immune response is regulated by a highly complex and intricate network of control elements (Opal & DePalo, 2000). The most known regulatory components are anti-inflammatory cytokines and specific cytokine inhibitors (Opal & DePalo, 2000). Cytokines are signaling proteins that play a critical role in controlling inflammation in the body. As mentioned earlier, these proteins function as chemical messengers in the human immune system. Our immune system consists of immune cells that fight against invading pathogens, allergens and other harmful substances that enter our body. The small proteins that signal the immune cells to fight invaders are cytokines. Cytokines include different proteins that signal our immune cells where to go and what to do to keep our immune system functioning correctly. For instance, chemokines involve in directing immune cells where they can fight infection. Interferons play a role in controlling immune cells to put their defenses against viruses invading our body. Interleukins are cytokines that release messages between leukocytes (white blood cells). And TNF involves in regulating inflammation in our body furthermore, TNF signals to immune cells that kill tumor cells.

Cytokines, as a chemical messengers tell cells how to behave. They involve in signaling cell activation, cell proliferation and cell differentiation. Cytokines can be divided into pro-inflammatory and anti-inflammatory. Pro-inflammatory cytokines trigger or promote inflammation, whereas the anti-inflammatory cytokines stop or inhibit inflammation. They release messages that prevent an excessive immune response that can damage tissue.

Cytokines are released by immune cells as well as other body cells. Immune cells those release cytokines are macrophages, dendritic cells, lymphocytes (T and B lymphocytes), monocytes, neutrophils, basophils, eosinophils, and mast cells. Other body cells, such as endothelial cells, epithelial cells, fibroblasts, stromal cells, and Schwann cells, also release cytokines.

Cytokines are vital in our immune system. They play a role in most conditions and diseases that may affect us. For instance, if our immune system releases too many cytokines in response to an infection, we may develop cytokine release syndrome (CRS), also called a cytokine storm. CRS can be life-threatening without treatment. Too many cytokines can cause too much inflammatory response. And too much inflammation can damage tissues, which may lead to different diseases and conditions, such as autoimmune diseases, metabolic disorders, cancer etc.

1.8 Essential signaling pathways regulating intestinal epithelial cell differentiation and crypt homeostasis:

An array of interconnected signaling pathways tightly regulate proliferation, differentiation, and migration along the crypt-villus axis (Trentesaux & Romagnolo, 2018). For instance, in mouse the critical signaling path ways that regulate intestinal fate determination are WNT, Notch, EGF and BMP (Gehart & Clevers, 2019).

WNT: Wnt signaling is pivotal for stem cell proliferation; the presence of R-spondin, which amplify Wnt signaling, protects the intestinal architecture from damage. Wnt proteins are cysteine-rich lipid-modified proteins that play a major role in various processes during development including cell proliferation and differentiation, cell fate decisions, apoptosis, migration, and is vital for embryonic develop (Flanagan et al., 2018; Mei et al., 2020; Niehrs, 2012).The Wnt signaling from the stem cell microenvironment (niche) can act as self-renewal factors for stem cells in multiple mammalian tissues (Clevers et al., 2014). For instance, the small intestinal epithelium is the fastest proliferating tissue of adult mammals, being largely made a new every 4 to 5 days as mentioned earlier and is regulated by Wnt-signaling and other factors. Generally, Wnt-signaling involves in early development, maintenance, regeneration of stem cells, and upon deregulation cancer formation (Gehart & Clevers, 2019). In addition, Wnt/β-catenin signaling has been associated with Paneth cell lineage specification, differentiation and maturation (Noah et al., 2011). In humans there are 19 genes that encode WNTs, and pair to various receptors to activate different downstream pathways (Flanagan et al., 2018; Niehrs, 2012). But, in this study we focused only on Wnt-3a that has been revealed to be an essential niche component for the proliferation of Lgr-5 positive stem cells in intestinal epithelial and is essential in the establishment of digestive organoid, human intestinal organoids.

WNT ligands bind to the Frizzled-LRP5/6, a receptor complex which inhibits continuous destruction of β-catenin by APC, destruction complex (Gehart & Clevers, 2019; Nusse & Clevers, 2017). β-catenin accumulates and leads to its translocation to the nucleus, where it binds TCFs and directly regulates gene expression (Nusse & Clevers, 2017) see figure 1.1.3.

Paneth cells involves in the production of epithelial WNT ligand in the form of WNT3 (Gehart & Clevers, 2019; Sato et al., 2011; Tan & Barker, 2014). In addition, the Paneth cell-derived WNT3 has been visualized in intestinal organoids by tagging the secreted protein with haemagglutinin (Alexandre et al., 2014; Gehart & Clevers, 2019). The surfaces of intestinal stem cells around Paneth cells feature WNT3 that is bound to Frizzled receptors (Gehart & Clevers, 2019).

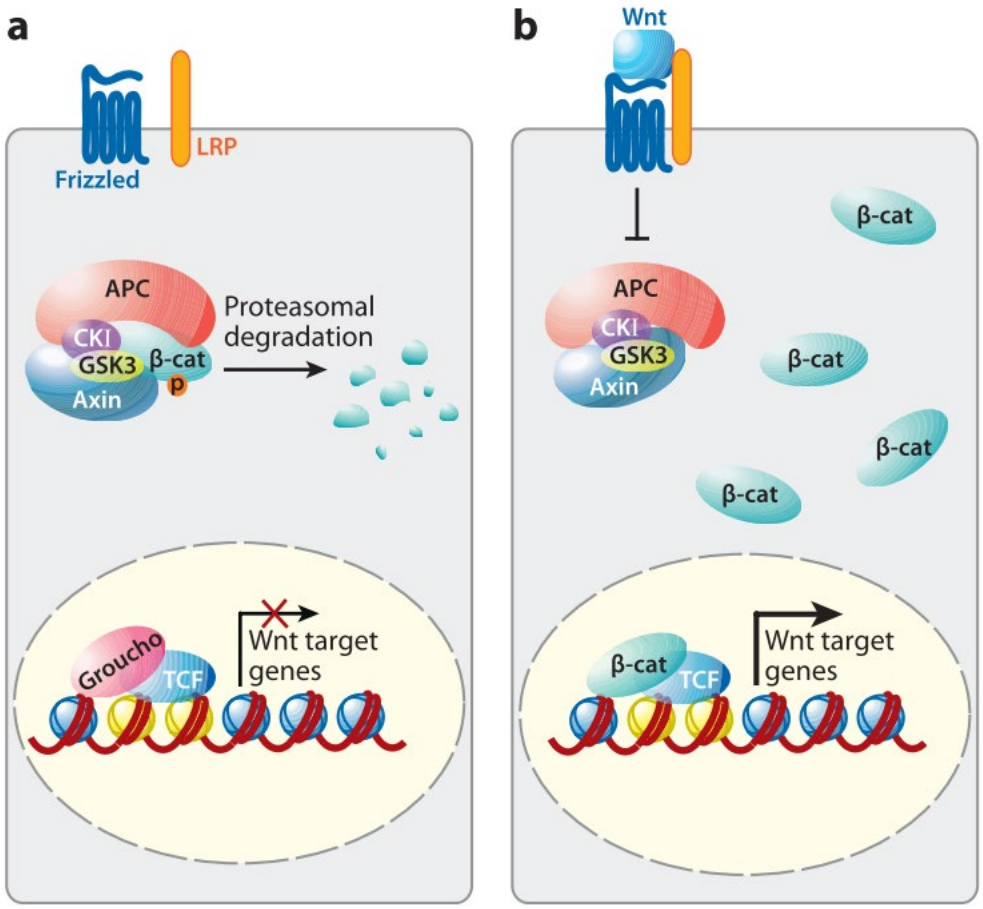

Figure 1.6: The Wnt canonical pathway. (a)In the absence of Wnt stimulation, β -catenin levels are kept at a minimum through the destruction complex composed of APC, Axin, GSK3, β-catenin phosphorylation and CKI causes proteasomal degradation as can be seen on the figure (a). In the

nucleus, Tcf factors associate with transcriptional repressors (Groucho) to block target gene activation. (b) In the presence of Wnt stimulation, the destruction complex is destabilized, and β-catenin accumulates in the nucleus to activate transcription of Tcf target genes (Gregorieff & Clevers, 2005).

Notch: The notch signaling pathway is highly conserved cell signaling that is present in most animals. Notch signaling is initiated by binding of Notch receptors to Notch ligands (Gehart & Clevers, 2019). The Notch receptors are large transmembrane proteins that normally communicate signals between cells when they bind to transmembrane ligands. Mammals have four different notch receptors NOTCH1, NOTCH2, NOTCH3 and NOTCH4, and typical notch ligands such as DLL1, DLL3 and DLL4 (Katoh & Katoh, 2007). The activation of notch signaling pathway is associated with early cell lineages in development (Hori et al., 2013). Active notch signaling depends upon direct contact between two cells that express notch receptor and notch ligand as mentioned. As both ligand and receptor are transmembrane proteins, Notch signaling acts at the shortest range of all instructive cues in the intestinal crypt (Gehart & Clevers, 2019; Hori et al., 2013). The Notch pathway plays a critical role in intestinal epithelial cell fate by regulating the choice of absorptive versus secretory lineages (Noah et al., 2011). In addition, it involves in later sub-specifications such as goblet cell versus enteroendocrine cell (Gehart & Clevers, 2019). Gehart and Clevers, 2019 shows that when notch signaling is inhibited CBC cells were differentiated towards the secretory fate whereas notch signaling is promoted CBC cells differentiated towards absorptive fate (figure 1.7).

EGF: EGF is one of the critical components of intestinal organoid culture medium (Gehart & Clevers, 2019; Sato et al., 2009). The EGF receptors such as ERBB1 are highly expressed in CBC cells (Gehart & Clevers, 2019). The Paneth cells and probably the underlying mesenchymal cells involves in the production of ligands activating the receptor, EGF and TGFα. For instance, EGF is released by enteric glia cells to support mucosal healing (van Landeghem et al., 2011). If the EGF signaling pathway is highly activated the proliferation of stem cells in the crypt also increased and may give rise to mutated cells. An overactive EGF signaling is a step towards the development of a neoplastic growth that may lead to cancer development. However, this signaling pathway is tightly controlled, for example, intestinal stem cells co-express ERBB receptors with their negative regulator LRIG1 (Gehart & Clevers, 2019). Inhibition of EGF signaling pathway in intestinal organoids abolishes proliferation of Lgr5+ stem cells and induces their quiescence and also blocks organoids growth (Basak et al., 2017). The EGF signaling can control the division rate of intestinal stem cells, but it cannot maintain stem cell identity. Therefore, EGF is distinguished from WNT or Notch signaling, which are both required to maintain stem cell identity (Gehart & Clevers, 2019).

BMP: BMP signaling pathway is conserved from Drosophila to vertebrates (S. Wang & Chen, 2018). BMP signaling pathway involves in gut development, homeostasis and pathology (Auclair et al., 2007; Trentesaux & Romagnolo, 2018; S. Wang & Chen, 2018). The BMPs are morphogens belonging to the transforming growth factor beta (TGFβ) - superfamily (Auclair et al., 2007), and play an essential role in many cellular functions in adult animals (Auclair et al., 2007). It signals through the serine/threonine kinase receptor subtypes I and II. The main BMP ligands found in the small intestine are BMP2 and BMP4 (Gehart & Clevers, 2019) that perform their signaling through the type of IA receptor. The intestinal BMP ligands (BMP2 and BMP4) are secreted by intercrypt and intervillus mesenchymal cells (Hardwick et al., 2004). The main receptor for BMPs in the intestinal epithelium is BMPR1A. When BMPR1A is conditionally eliminated in mouse, both stem cell and transit amplifying zones expand and grow to benign polyps (X. C. He et al., 2004). Thus, BMPs inhibits cell proliferation in intestinal epithelium, but it promotes differentiation. Therefore, the stem cell zone is protected from BMPs-by-BMPs inhibitors such as Gremlin 1, Gremlin 2 or Noggin which are secreted by myofibroblasts and smooth muscle cells underneath the crypt (Gehart & Clevers, 2019). Generally, decreasing WNT and increasing BMP signals guide the intestinal epithelial cells from an undifferentiated to a differentiated state during their progression along the crypt–villus axis.

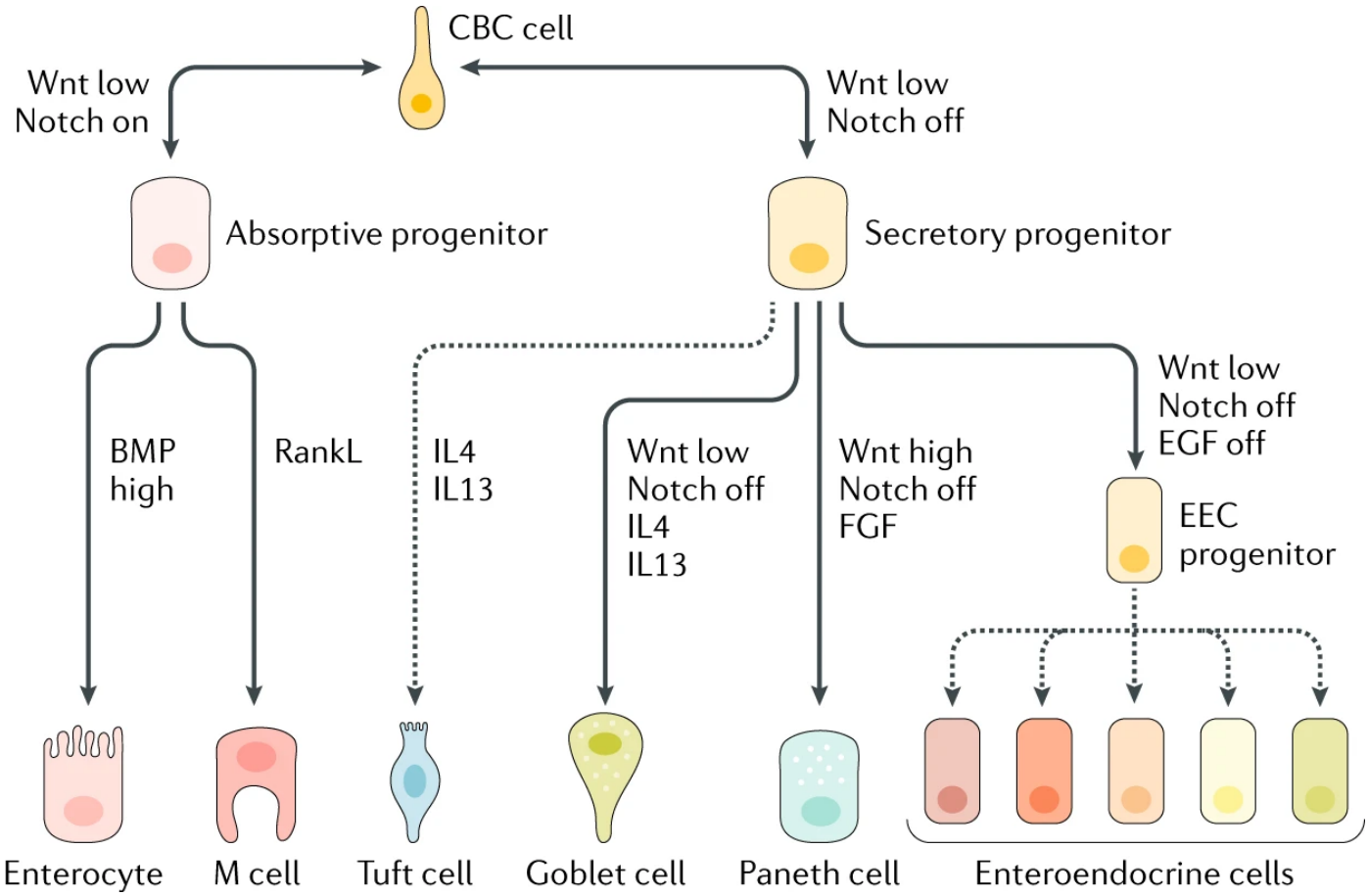

Figure 1.7: Cell specification in the intestinal epithelium. Showing the involvement of interleukins and signaling pathways in the differentiation of secretory and absorptive progenitors, and furthermore cell specification in the intestinal epithelium (Gehart & Clevers, 2019).

1.9 RNA sequencing

RNA sequencing (RNA-seq) is a genomic approach for detecting and quantitatively analysing messenger RNA (mRNA) molecules in a biological sample and is valid for studying cellular responses. Several R packages can detect differentially expressed genes from RNA-Seq data (Varet et al., 2016). Differential analysis of RNA-seq data is vital to identify aberrant transcriptions, and limma, EdgeR and DESeq2 are efficient tools for differential analysis (Liu et al., 2021). Analysing the whole process requires normalisation, dispersion estimation and testing for differential expression (Varet et al., 2016).

DESeq2 and EDGE R are popular Bioconductor packages for differential expression analysis of RNA-Seq, ChIP-Seq, or HiC count data (Varet et al., 2016; Liu et al., 2021). They are well-

documented and easy to use. In this project, we used DESeq2 to detect differentially expressed genes between different experimental conditions. Because DESeq2 is a widely used method in bioinformatics and computational biology for analysing high-throughput RNA-seq data. Moreover, DESeq2 is one of the most commonly used differential gene expression analysis methods in bulk RNA-seq data. It has gained popularity due to its robustness, flexibility, and ability to handle various sources of variability in the data. DESeq2 is known for its statistical rigour and ability to model and normalize count data effectively. It is suitable for detecting differential expression in RNA-seq experiments with many conditions.

1.10 Bulk RNA sequencing (bulk RNA-seq)

Significant innovations in next-generation sequencing techniques and bioinformatics tools have impacted our understanding of RNA (Thind et al., 2021). Bulk RNA-seq is a high-throughput sequencing technique that analyses gene expression profiles globally. It provides a snapshot of the average gene expression levels within a population of cells or tissue samples. Generally, in bulk RNA-seq, total RNA is extracted from a sample containing a cell mixture. Then RNA is processed to isolate and sequence the messenger RNA (mRNA) molecules because mRNA represents the actively transcribed genes in the sample.

Bulk RNA-seq involves RNA extraction, RNA purification and quality control, mRNA enrichment, cDNA synthesis and library preparation, high-throughput sequencing and finally, data analysis.

RNA extraction: Total RNA is extracted from the biological sample. For instance, it can be a tissue sample, cell culture, or a collection of cells.

RNA purification and quality control: The extracted RNA is purified to remove contaminants and checked for quality and integrity using spectrophotometry or capillary electrophoresis.

mRNA enrichment: The extracted RNA is treated with enzymes or oligo-dT beads to enrich the mRNA molecules specifically. This step removes other RNA species, such as ribosomal RNA (rRNA) and transfers RNA (tRNA), which are abundant but do not contribute to gene expression analysis.

cDNA synthesis and library preparation: The enriched mRNA is reverse transcribed into complementary DNA (cDNA) using reverse transcriptase. The cDNA is then processed to create a sequencing library involving fragmentation, adapter ligation, and PCR amplification.

High-throughput sequencing: The prepared library is loaded onto a sequencing instrument, such as an Illumina sequencer. The cDNA fragments are sequenced, generating millions of short reads that represent the RNA molecules present in the sample.

Data analysis: The sequenced reads are aligned to a reference genome or transcriptome, and the expression levels of individual genes are quantified. This involves mapping the reads, counting the number of reads that align with each gene, and normalizing the read counts to account for library

size and gene length variations. Differential gene expression analysis can then be performed to identify genes that show significant changes in expression between different conditions or groups.

Bulk RNA-seq provides valuable insights into the gene expression patterns in a population of cells or tissue samples. It allows researchers to study various biological processes, such as development, disease progression, and treatment response, by comparing gene expression profiles across different conditions.

1.11 Tissue staining techniques

Tissue staining is used in biological research and pathology to enhance the visualization and identification of cells, tissues, and specific cellular components. It involves the application of dyes or markers to biological samples, such as tissue sections or cells, to highlight specific structures, cellular components, or molecular markers of interest.

Different types of tissue staining techniques are available, and each serves a specific purpose. For instance, the following are some of the standard staining techniques:

Hematoxylin and eosin (H&E) staining: This is one of the most widely used staining techniques in histology. Hematoxylin stains the nuclei blue-purple, while eosin stains the cytoplasm and extracellular components pink. H&E staining provides contrast between different tissue components and helps researches to visualize tissue architecture.

Immunohistochemistry (IHC): This technique uses specific antibodies that bind to target proteins of interest in the tissue. The antibodies are typically conjugated with enzymes or fluorophores that generate a visible or fluorescent signal upon binding to the target protein. Immunohistochemistry allows for the visualization and localization of specific proteins within tissue sections.

Immunofluorescence (IF): Similar to IHC, immunofluorescence utilizes specific antibodies to detect and localize target proteins. However, the antibodies are conjugated with fluorescent dyes instead of enzymes in IF. This technique enables the detection of multiple proteins simultaneously using different fluorescent colors.

Nucleic acid staining: Stains like DAPI (4',6-diamidino-2-phenylindole) are used to label DNA or RNA within cells. These stains help visualize cell nuclei or detect specific cellular processes such as apoptosis.

2. Objectives:

The main goal of this study is to elucidate the impact of different cytokines in the proliferation and differentiation of epithelial cells. As we know, cytokines are enormous in number so we couldn't study the effect of all cytokines. Still, we chose some interleukins, interferons and TNFs and elucidated their impact on the differentiation of intestinal epithelial cells such as goblet, tuft and Paneth cells. Furthermore, using the human intestinal organoid culture and bulk RNA sequencing and immunofluorescence staining, we want to study the role of cytokines in gut diseases. In short, the main objectives are:

- To establish and maintain human duodenal organoids.
- To study the crosstalk between gut epithelium and immune cell secreted cytokines.
- To mimic gut associated diseases using human duodenal organoids as a model.

3. MATERIALS AND METHODS

3.1 Organoids, Spheroids & 3D Cell Culture

Organoids, spheroids and studying cells as 3D models show great potential in many applications, including disease modelling and regenerative medicine (thermofisher.com). The organoid model is excellent for studying intestinal epithelium and its proliferative capacity (Noben et al., 2017). Therefore, using an organoid model in this study is vital. It is a current and more applicable model than an animal model.

Ex vivo technologies, especially primary cell cultures, have proven promising for understanding intestinal epithelial functions (Mahe et al., 2015). A culture system for mouse intestinal crypts is established to generate three-dimensional epithelial organoids which contain crypt and villus-like domains. These epithelial organoids mimic normal epithelium gut. The organoids derived from the small intestine and colon are named enteroids and colonoids, respectively. These enteroids and colonoids continuously produce all cell types within the intestinal epithelium. Therefore, this model is straightforward and realistic for studying intestinal stem cell biology and epithelial cell physiology.

3.2 Culture preparation:

Zeocin™ selection in the mammalian cell culture:

Mammalian cells have a wide range of susceptibility to Zeocin. Concentrations of Zeocin™ used to select stable cell lines may range from 50 to 1000 µg/ml, with an average between 250 to 400 µg/ml (ThermoFisher.no). Therefore, in our laboratory, we selected 125 µg/ml depending on the cell lines we used, the Wnt-3A cell line. 1.25 µg/ml medium from 100 mg/ml stock Invitrogen R25001 was prepared.

Preparing a complete human organoid medium and a differentiation medium for one week or more was essential. Because I needed to change the medium for the cells in culture to the first 3 to 4 days proliferation medium and the next 3 to 4 days differentiation medium, this was necessary to check the cell's capability of proliferation and differentiation under different conditions. First, the Wnt-3A-conditioned medium was prepared as follows: According to the manufacturer's instruction (ATCC, CRL, 2647), the Wnt-3A-conditioned medium was made in-house using the cell line L Wnt-3A. Then the media was supplemented with 2 mM glutamine, 10 mM HEPES, 100 µg/ml penicillin, 100 g/ml streptomycin, 1 N2 supplement, 1 B27 supplement, 1% BSA and filtered with sterilized 0.22-µm filter. Then complete human organoid medium was prepared.

Complete human organoid medium (proliferation medium) was prepared as follows: 50% Wnt-3A-conditioned medium was supplemented with 20% R-spondin 1, 100 ng/ml Noggin (1:1,000 dilution of 100 g/mL stock), 50 ng/ml EGF (1:10,000 dilution of 500 µg/ml stock), 500 nM A-83-01 (1:1,000 dilution of 500 M stock), 10 µM SB202190 (1:3,000 dilution of 30 mM stock), 10 nM [Leu]15-Gastrin 1 (1:10,000 dilution of 100 µM stock), 10 mM Nicotinamide (1:100 dilution of 1 M stock) and 1 mM N-Acetylcysteine (1:1,000 dilution of 1 M stock). See (table 3.1) how I prepared human organoid complete media and table 3.2. human organoids differentiation medium. We also grew and maintained organoids in L-WRN conditioned media during my master project.

Table 3.1: shows how a complete human organoid medium for 20 milli liter (ml) volume was prepared**.** In the table, 50% L-WRN conditioned medium is already prepared as mentioned above (Wnt-3A-conditioned medium is supplemented with 1µg/ml R-spondin and 100 ng/ml Noggin). L-WRN refers to cell lines Wnt-3A, R-spondin1 and Noggin (L-WRN).

Components	Volume 20 ml
BCM basal culture media	10 ml
50% L- WRN conditioned	10 ml

medium	
50ng/mL EGF	2 µl
500nM A- 83- 01	0.33 µl
10µM SB202190	6.66 µl
10nM [Leu]15-Gastrin1	2 µl
10 mM Nicotinamide	200 µl
1mM N-Acetylcysteine	40 µl
N2 supplement	200 µl
B27 supplement	400 µl
Plasmocin	2 µl after filtration

Growth medium:

DMEM (Dulbecco's Modified Eagle Medium) is a widely used basal medium supporting the growth of many mammalian cells. We used DMEM 31966 Gibco 500 ml, which contains high glucose, GlutaMAX Supplement, and pyruvate. In addition, 60 ml FBS Sigma F7524 (Fetal Bovine Serum) was used as a growth medium. This medium promotes non-defined growth and provides survival-enhancing factors to cells in culture. The antibiotics penicillin and streptomycin (5 ml pen/strep 15140 Gibco) were used to prevent any bacterial contamination of cell cultures. Tryple Express 12605 Gibco, highly purified cell dissociation enzyme that replace porcine trypsin was used to dissociate cells in culture.

The chelating buffer was prepared as follows: 2% sorbitol, 1% sucrose, 1% bovine serum albumin fraction V (BSA) and 1x Gentamicin/ Amphotericin solution in Dulbecco's Phosphate buffered saline (DPBS) without Ca 2+ and Mg2+ (PBSO) were mixed and filtered with 0.22 µm filter. Ca &Mg ions promote cell adhesion, so ca2+ & mg 2+ free PBS is always recommended when dissociating cells. Incubating cells in Ca & Mg free PBS for 1-2 hours results in cells detaching from the substrate.

Table 3.2: shows how human organoids differentiation media is prepared for 10 ml and 20 ml.

Components	Volume for 10 ml	Volume for 20 ml
BCM	9 ml	18 ml
5% Wnt-3A conditioned medium	0.5 ml	1 ml
5% R-spondin	0.5 ml	1 ml

100 ng/ml Noggin	5 µl	10 µl
50 ng/ml EGF	1 µl	2 µl
500 nM A-83-01	0.17 µl	0.33 µl
10 nM [Leu]15-Gastrin1	1 µl	2 µl
1 mM N-Acetylcysteine	20 µl	40 µl
N2 supplement	100 µl	200 µl
B27 supplement	200 µl	400 µl
Plasmocin	1 µl	2 µl after filtration

3.3 Duodenal crypt Isolation from Whole Tissue

Duodenum tissue was taken from the donor patients in Oslo University Hospital. The whole tissue was washed a few times with a sterile PBS. Duodenum was opened longitudinally, and the tissue was rewashed with PBS. After this step I started to cut the mucosa. 3 to 5 long stripes of mucosa were taken and added into a 30 ml PBS in 50 ml falcon. These mucosa stripes were washed with PBS until PBS was cleared. The muscle's mucosal side or opposite side was scraped with cover slip and villi and mucus was released. PBS with EDTA is used for releasing the crypts. Therefore, 5 mM EDTA was used and then rotated the falcon tube in cold room for 30 minutes. After rotation the mucosal stripes were added into the 50 ml falcon coated with FBS and 20 ml PBS was added. Then it was shaken vigorously to release the crypts. This step was repeated 5 times to have 50 ml crypts in PBS. Next, the soup was filtered through 150 µm mesh into a 50 ml falcon coated with FBS

The crypts were centrifuged at 300 x g for five minutes at four degrees centigrade. Then the supernatant was discarded and a 5 ml cold BCM was added to the crypts. The crypts were kept in falcon on ice. 500 µl dissolved crypts in cold BCM were taken in a 24 well plate. Next, the crypts were observed under a microscope and the crypts were counted. Per well, 200 to 400 crypts were taken in an Eppendorf and then spun at 300 x g for 5 minutes at 4°C. Then the crypts were resuspended in Matrigel, and 30 µl Matrigel dome was seeded in a warm 24 well plate. About 200 to 400 crypts per well were taken and Matrigel was solidify in the 24 well plate at 37 °C incubator for 10 minutes. Finally,

500 µl of WNT-ENR media was added to each well with 2.5 µM CHIR99021 and 10 µM Y-27632 inhibitor.

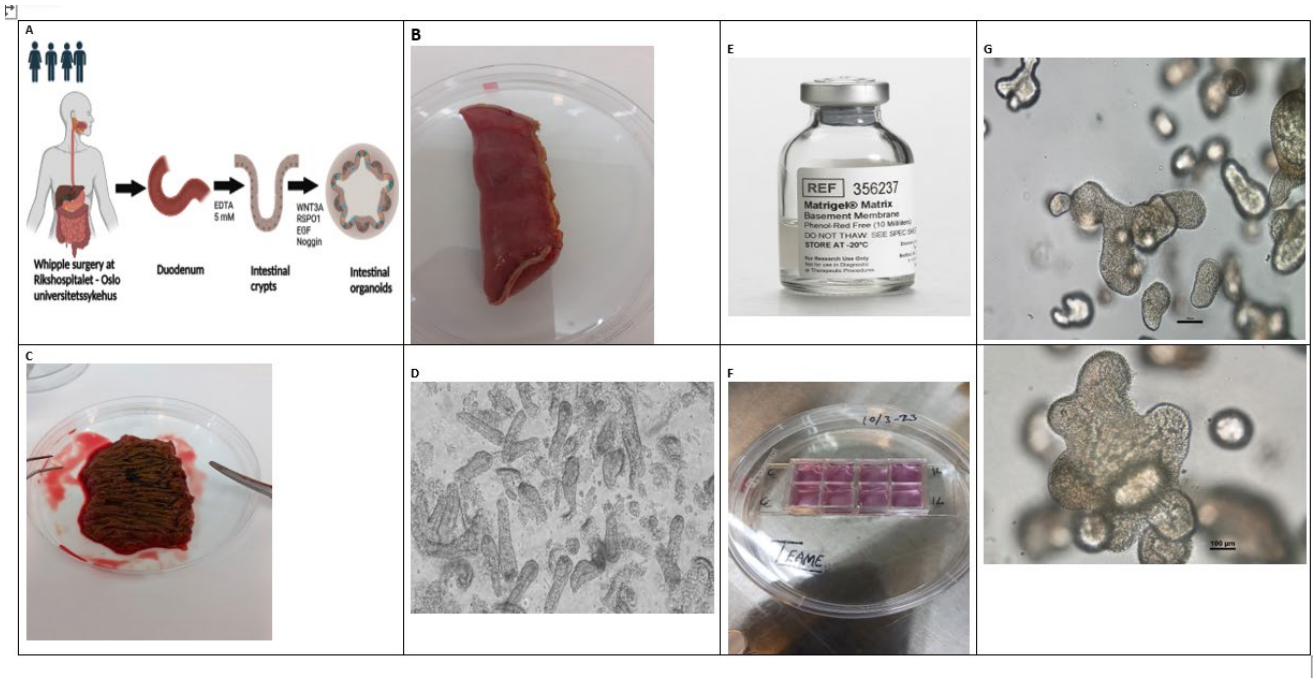

Figure 3.1: Duodenal crypt Isolation from Whole Tissue and organoid establishment. (A) Whipple procedure at Rikshospital Oslo, an operation to remove the head of the pancreas, the first part of the small intestine (duodenum), gallbladder and bile duct and isolation of crypts using 5 mM EDTA. (B) The duodenum is taken after operation at Rikshospital to establish duodenal organoids. (C) When duodenum is opened longitudinally and taken 3 to 5 long stripes mucosa. (D) The duodenal crypts under the microscope after isolation of the crypts (200-500) from the whole tissue. (E) The Matrigel, the crypts cultured in basement membrane matrix. The components of Matrigel are laminin, collagen IV, entactin and heparin sulfate proteoglycan perlecan. (F) 500 µl of 50% WNT-ENR media to each well with CHIR and 10 µM rock inhibitor, and already plasmocin is added to 50% WNT-ENR complete media to prevent mycoplasma contamination. (G) 9 days old organoids established from duodenum.

3.4 Staining of duodenal crypts

Crypts were isolated from duodenum. Next, cytopsin was used to concentrate crypts on glass slide at 400 rpm for 3 minutes. Next, crypts were fixed in 10 % formalin for 15 minutes at room temperature. After fixation, slides were washed with PBS two times. After fixation, permeabilization was performed using 0.2 % Triton x 100. After this, regular Immunofluorescence (IF) protocol was followed.

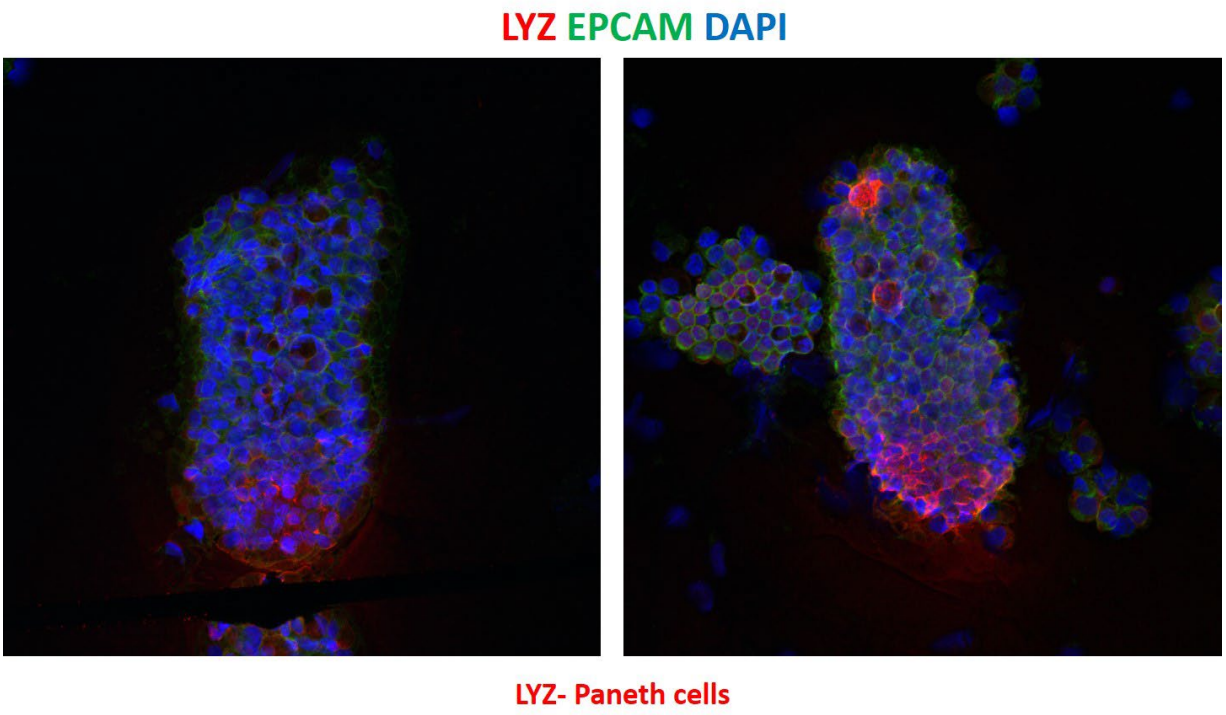

Figure 3.2: Confocal images of duodenal crypts stained for LYZ (red color- paneth cell marker), EPCAM, epithelial cell adhesion molecule - in green color and DAPI in blue color.

3.5 Passaging of Cultured human Enteroids

Passaging in cell culture involves harvesting cells from culture, transferring them to one or more culture vessels with fresh growth medium, and using these cells to start new cultures. Enteroids were passaged every 7 to 10 days after initial plating. Generally, one well was split into 2 to 3 wells. Before the beginning of the experiment (splitting) all the reagents were prepared. The basement membrane matrix (Matrigel) was thawed on ice, and I pre-incubate a 24-well plate in a 5% CO_2 incubator at 37°C. Then the splitting of cultured human enteroids was done as follows.

The old media was discarded and started to break the organoids with TrypLE Express Enzyme, dissociating reagent (500 µl in each well). This recombinant enzyme replaces animal trypsin and is vital to dissociate adherent cells from plastic. TrypLE protects the cell`s surface proteins. After disrupting the organoids into cells with TrypLE ^{TM} about 100 µl and 250 µl was taken in an Eppendorf for maintenance and experiment, respectively. The cells were warmed in 37 °C water-bath for 4 minutes and diluted by 500 µl cold BCM. Then, the cells were spun in a centrifuge with 500 x g 4 °C for 5 minutes. Then the supernatant was discarded, and the cells were in the pellet. 60 µl and 240 µl Matrigel was added to the Eppendorf containing cells for maintenance and experiment, respectively. 30 µl of the samples containing Matrigel were added to each well of the 24 wells plate and 8 wells of ibidi for maintenance and experiment, respectively. Then 24-well plate was placed in a 37°C, 5%

CO2 incubator for 10 minutes to allow a complete polymerization of the basement membrane matrix, Matrigel.

A 500 µl complete organoid media was added in a new Eppendorf tube and 10 µM Y27632 (rock, rho-associated coil kinase) inhibitor protein. ROCK inhibitor Y-27632 is a cell-permeable, highly potent, and selective inhibitor. Rock inhibitor is essential for cell culture practice. It is critical to limit cell death and dedifferentiation. Therefore, it is widely used for induced pluripotent and embryonic stem cell cultures. In addition, 2.5 µM CHIR was added. CHIR is a potent, selective GSK3 inhibitor and WNT signaling activator. They are commonly used in organoid production. GSK3(glycogen synthase kinase 3) is a serine /threonine kinase that is a crucial inhibitor of the WNT pathway. Therefore, the critical use of CHIR is to activate the WNT pathway. Finally, the basement membrane matrix was overlayed with 500 µl of complete human organoid medium which was supplemented with Y-27632 and CHIR as I mentioned on (Figure 3.1).

3.6 RNA isolation and bulk-sequencing:

RNA isolation consists of buffer preparation, sample preparation and total RNA purification. First, old media was discarded and organoids were pooled from two wells in Eppendorf tube in PBS. Next, organoids were centrifuged at 300 x g for 5 minutes. Next PBS was discarded and organoids were resuspended in cell lysis buffer for 5 minutes at room temperature. All the steps for RNA purification were performed at room temperature at 12000 x g for 30 seconds unless it was specified. One volume[1] ethanol (95-100 %) was added to one volume sample lysed in RNA, lysis buffer was 1:1, and it was mixed well. The mixture was transferred into a Zymo-spin™ IC Column[2] in a collection tube, and it was centrifuged. The flow-through was discarded.

DNase treatment: The column was washed with 400 µl RNA Wash Buffer and centrifuged. The flow-through was discarded. In nuclease-free tube, 5 µl DNase I (1 µ/µl) and 35 µl DNA Digestion Buffer was added and mixed well. The mixture was added directly into the column matrix. Then the column was incubated at room temperature for 15 minutes. 400 µl RNA Prep Buffer was added to the column and it was centrifuged. The flow-through was discarded. Next, 700 µl RNA Wash Buffer was added to the column and after centrifuged, the flow-through was discarded. 400 µl RNA Wash Buffer was added and centrifuged the column for one minute to ensure complete removal of the wash buffer. Then, the column was transferred carefully into a nuclease-free tube. Finally, a 20 µl DNase/RNase- Free Water was directly added to the column matrix and it was centrifuged. The eluted RNA was stored at -80 ° C and sent for bulk RNA-sequencing (RNA-seq) to Novogene (UK) Company Limited.

3.7 Bioinformatics analysis:

Our bioinformatics workflow starts with processing RAW FASTQ reads from the sequencer using nf-core-RNA sequencing (nf-core/rnaseq) pipeline (v3.9). Nf-core/rnaseq (Ewels et al., 2016) is a bioinformatics pipeline for analyzing RNA sequencing data from organisms using a reference genome (human genome GRCh38) and annotation. It accepts a samplesheet and FASTQ files as input, does quality control, adapter trimming, and alignment, and generates a gene expression matrix as well as a detailed quality control report. In short, this pipeline utilizes fastqc (Andrews and Others, 2010) and multiqc (Ewels et al., 2016) tools for initial quality control. The reads were then trimmed using cutadapt and then mapped and counted by Salmon (Patro et al., 2017) to generate the expression matrix of raw counts and TPM normalized values. After the pipeline successfully concluded, the differential expression analysis of the count tables was done using DESeq2 (Love et al., 2014) and our own scripts in the R environment. The volcano plots were visualised using enhancedvolcano R package (https://github.com/kevinblighe/EnhancedVolcano). We used in-house R scripts and the cluster Profiler package (v4.7.0) (Yu et al., 2012) for KEGG enrichment analysis of differentially expressed genes. The results were visualized using ggplot2(Wickham, 2011).

3.8 Transmission electron microscopy (TEM) in duodenal organoids:

Organoids were cultured for 11 days in organoids growth media. After that, organoids were centrifuged very gently and fixed in 2% glutaraldehyde in cacodylate buffer (pH 7.4) for 1 hour at room temperature. After that, organoids were centrifuged at 6000RPM (3099rcf) for 5 min. Next, organoids were washed with 0,1M cacodylat buffer pH 7,4, 3 X 5 min at Room temperature. After this, post fixation of organoids was performed with 2 % osmiumtetroksid in cacodylatbuffer, 2 hours at 4^0C. Next, organoids were washed again with 0,1M cacodylat-buffer pH 7,4, 3 X 5min at Room temperature. Next, dehydration of organoids was performed using increasing concentration of ethanol (50 % ethanol-30 min, 70 % ethanol-30 min, 90 % ethanol-30 min, 100 % ethanol-30 mins, 2 % Uranylacetate absolute ethanol 30 min, Acetone- 2X 20 minutes. Next organoids were embedded using EPON and dimethylaminomethyl phenol (DMP30) at Room temperature and then hardened overnight at 60^0C. After this, blocks were sectioned (2um thick) with a Leica microtome (RM2265) and stain the sections with Toluidinblau staining. Ultrathin sections of 60 nm were cut using a diamond knife on a Leica UC7 ultramicrotome and sections were post-stained with uranyl acetate and lead citrate. Next, imaging was performed using Hitachi's Transmission Electron Microscope HT7800 Series.

3.9 Immunofluorescence staining of human intestinal organoids:

Organoids were seeded in a Matrigel which was mixed with 25% BCM in eight well slides (Ibidi, 80821). Before immunostaining cells must be fixed. This is because you need to permeabilize cells to allow antibodies access intracellular structures. To start fixation the old media was removed after completion of the experiment. Then organoids were fixed in a 300 µl fixation solution, 4% PFA in PBS with 2% sucrose for 30 minutes. Total 3 ml fixation solution was prepared see table 3.3. After 30 minutes the fixation solution was removed and 300 µl PBS was added for 5 minutes and this step was done two times. After this step the PBS was discarded, and I stored the Ibidi slide in 300 µl PBS at 4 °C for two days or longer.

Table 3.3: shows the total fixation solution, but for one well only 300 µl fixation solution was used.

PFA	750 µl-4%
Sucrose-30%	200 µl-2%
PBS	2050 µl
Total	3 ml

Staining's was started after complete fixation. A 300 µl 0.2% Triton X-100 in PBS was added for 30 minutes at room temperature to permeabilize the cell membrane. The wells were washed with PBS 3 times 5 minutes each at room temperature. See table 3.4 for calculations for 0.2% Triton X-100.

Table 3.4: shows the calculation for 0.2% Triton X-100.

PBS without calcium and magnesium	500 ml
Triton X-100 stock	1 ml

Free aldehydes have been blocked in 100 mM Glycine for 1 hour at room temperature. Glycine is generally used to block unreacted aldehydes after fixation which can cause an increase in back-ground fluorescence. See table 3.5 calculations for one Ibidi- 100 mM Glycine.

Table 3.5: Calculations for one Ibidi- 100 mM Glycine.

1 M Glycine stock	500 µl
PBS	4500 µl

After free aldehydes have been blocked in 100 mM Glycine, the wells have been incubated in 200 µl blocking buffer consisting of 5-10% human serum and 1.25% BSA in PBS for one hour at room temperature. See table 3.6 calculation for one Ibidi for blocking buffer and primary antibody.

Table 3.6: Calculation for one Ibidi – for blocking buffer and primary antibody.

100% human serum	150 µl
1.25% BSA in PBS	2850 µl

Primary antibodies were diluted in blocking buffer, and it was incubated overnight at 4 °C. See table below (3.7) for how primary antibody was diluted in blocking buffer. The next day, the primary antibody was removed, and samples were washed in cold PBS three times 10 minutes each.

Table 3.7: one example of primary antibody calculations for four wells.

Ki67 dilution is 1:100	6 µl
Blocking buffer	600 µl

The appropriate Alexa fluor secondary antibody (1:500), DAPI (1:1000) and UEA1 (Ulex Europaeus Agglutinin I Rhodamine -labeled Dilution 1:500, vector laboratories RL1062) was added in blocking buffer which consists of 2.5% human serum and 1.25% BSA, and it was incubated at 4 °C with agitation overnight. Calculations was done as shown on table 3.7 and table 3.8 is an example of secondary antibody calculation. The next day, the samples were washed in 300 µl cold PBS. This step was repeated three times 10 minutes each time. Then 250 µl Fluoromount G medium (Thermofisher Scientific, 00-4958-02) was added to the wells. The ibidi slides were stored in cold room with moisture. Finally, I captured images with Zeiss LSM 880 confocal laser scanning microscope.

Table 3.8: calculations for one Ibidi- blocking buffer for secondary antibody:

100% human serum	60 µl
1.25% BSA in PBS	1140 µl

Table 3.9: shows one example of secondary antibody calculations for four wells.

Alexa fluor secondary antibody 488 dilution is 1:500	1.2 µl
UEA1- 1:500	1.2 µl
DAPI – 1:1000	0.6 µl
Blocking buffer	600 µl

To check MUC2 and Ki67 expression in differentiation and proliferation conditions, duodenal human organoids which were established from Whipple-320 (W320) were seeded in 75% Matrigel and 25 % BCM on eight-chamber µ-slides (ibidi, 80826). The four wells were switched to 5% WNT without CHIR. Then the first three to four days only complete human organoid media (proliferation media) was changed whereas the last five to nine days differentiation media was changed. Finally, as mentioned above, staining's were done, and images taken on confocal microscopy at University in Oslo. MUC2+ cells were quantified per organoid area.

Table 3.10: eight-chamber µ-slides (ibidi, 80826). To check MUC2 and Ki67 expression in differentiation and proliferation conditions.

50% WNT Ki67	50% WNT MUC2	5% WNT Ki67	5% WNT MUC2
50% WNT Ki67	50% WNT MUC2	5% WNT Ki67	5% WNT MUC2

Figure 3.3: Eight-chamber µ-slides (ibidi, 80826). Organoids that are established from passage 22 with differentiation media (left), and passage 25 after fixation and then incubated in PBS (right).

3.10 Immunofluorescence staining of human Intestinal Tissue:

Segments of human intestinal were dissected and fixed with 4% formaldehyde for 24 h at 4 degrees temperature. Next day, tissue was put in the cassettes and tissue was subsequently dehydrated through a series of ethanol graded and then embedded in the paraffin wax blocks. 5 µm thick sections were prepared using a microtome and tissue was placed on glass slides. The tissue slides were then deparaffinized and rehydrated, followed by antigen retrieval (Citrate buffer pH-6.0 for Lyzozyme C (1:1,000, A0099Lysozyme EC 3.2.1.17), Anti-EpCAM antibody [Ber-EP4] (ab7504, 1: 100) and Tris EDTA buffer pH-9.0 for MUC2). Sections were blocked in blocking buffer (1,25% BSA and 10% normal human serum) for 1 h at room temperature in a humidified chamber. Tissues were incubated with diluted primary antibody (MUC2, (Cat. No. GTX100664)1:500 overnight at 4°C. Next day, slides were washed with PBS for 10 min. Tissue was incubated with secondary antibody coupled to fluorochromes, anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, Catalog # A-21206 ,1: 500, Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor 647, A-21240 and dilution is 1:500, UEA-1 (1:500 Vector Laboratories RL-1062) and counterstained with DAPI for 1 h at room temperature in the dark. After incubation, slides were washed 3 times with PBS for 10 mins each and then mounted in Fluoromount G. Finally, images were acquired with Zeiss LSM 880 Confocal Laser Scanning Microscope at UiO (see figure 4.1 and 4.2)

3.10 Bulk RNA sequencing experiment:

The composition of culture media for human duodenal organoids is listed in the table. The concentration for expansion media are: 50% Wnt-3A-conditioned medium, 20 % R-spondin conditioned medium and recombinant noggin (100 ng/ml) was used. Other growth factors were used at concentrations: 50 ng/mL EGF, 500 nM A-83-01, 10 µM SB202190- p38 MAPK inhibitor, 10 nM [Leu]15-Gastrin 1, 10 mM Nicotinamide, 1 mM N-Acetylcysteine, 1x N2 supplement, 1x B27 supplement supplemented in basic culture medium consisting of Advanced Dulbecco's modified Eagle's medium (DMEM)/F12 with, Glutamax, HEPES and penicillin/streptomycin. To prevent mycoplasma contamination, Plasmocin was added after syringe 0.2µm filtration.

To induce differentiation in organoids, differentiation media was used. The concentration for expansion media is: 5% Wnt-3A-conditioned medium, 5 % R-spondin conditioned medium and recombinant noggin (100 ng/ml) was used. Other growth factors were used at concentrations: 50 ng/mL EGF,

500 nM A-83-01, 10 nM [Leu]15-Gastrin 1, 1 mM N-Acetylcysteine, 1x N2 supplement, 1x B27 supplement supplemented in basic culture medium consisting of Advanced Dulbecco's modified Eagle's medium (DMEM)/F12 with, Glutamax, HEPES and penicillin/streptomycin. Thus, Nicotinamide and SB202190 were absent in the differentiation media. To prevent mycoplasma contamination, Plasmocin was added after syringe 0.2µm filtration. See figure 3.3for the whole plan and method.

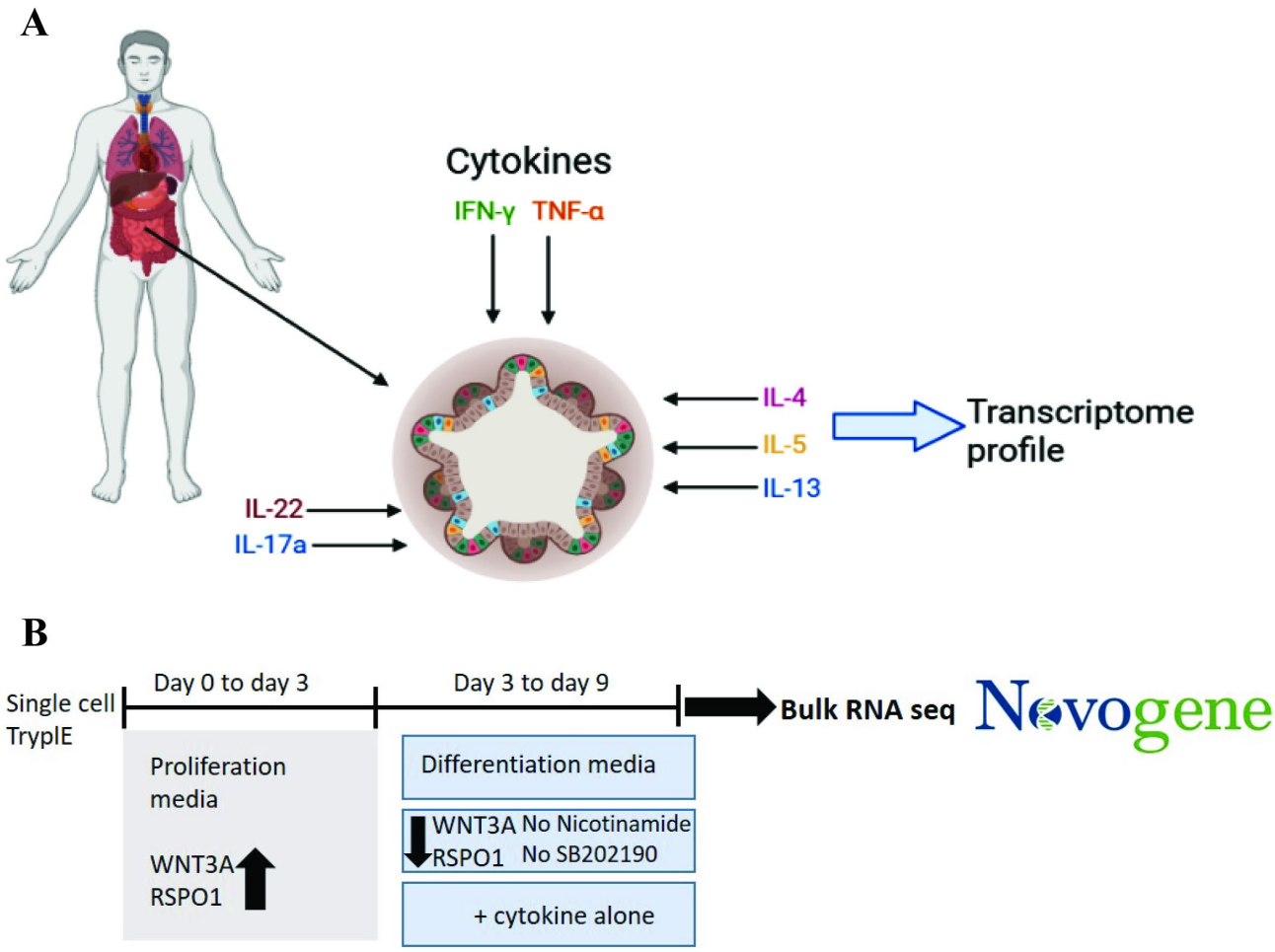

Figure 3.4 (A and B): Cytokine treatment for human duodenal organoid and applying transcriptome profile (A) and supplying proliferation media (day 0 to day 3) with a high concentration of Wnt3a and Rspo1 and then differentiation media (day 3 to day 9) with a low concentration of Wnt3a and Rspo 1 without Nicotinamide and SB2021190 plus cytokine and performing Bulk RNA seq (B).

3.11 Ethical considerations

All experiments described were performed within closed laboratories with virtually no effect on the external environment. This project conforms to EEC Directives concerning medical research and the corresponding Norwegian legislation. All biopsy and surgical resection specimens of human origin were taken after informed consent of the study subjects. The experiments and biobanks are approved by the Regional Committee for Medical Research Ethics (REK 2015/946).

3.12 Statistical analysis

The statistical analysis was performed using the Graph Pad Prism for graphs. Unpaired two-tailed Student's t test was used to determine the Statistically significant differences between the different experimental groups and P value of <0.05 was considered significant. The Statistical tests used are described in the figure legends. For heat maps, statistical analysis was performed in the R programming software.

4. RESULTS

4.1 Tissue staining

Tissue staining techniques are essential for visualizing cellular and molecular features in biological samples, providing valuable information about tissue structure, protein expression, and cellular localization. These techniques are commonly used in research, clinical diagnostics, and pathology to study normal and diseased tissues, characterise cellular abnormalities, and understand various biological processes.

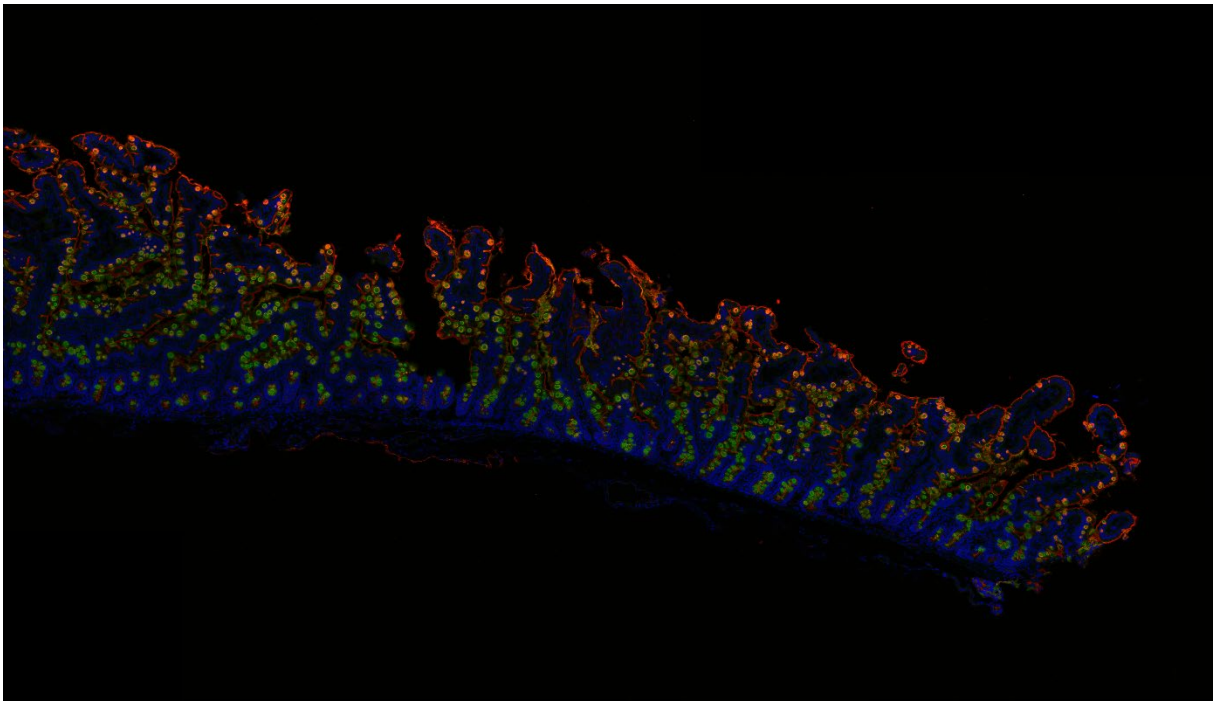

Figure 4.1: Human duodenal tissue section stained for goblet cells (MUC2), UEA1 (red color) binds secretory cells and DAPI binds to DNA (blue color).

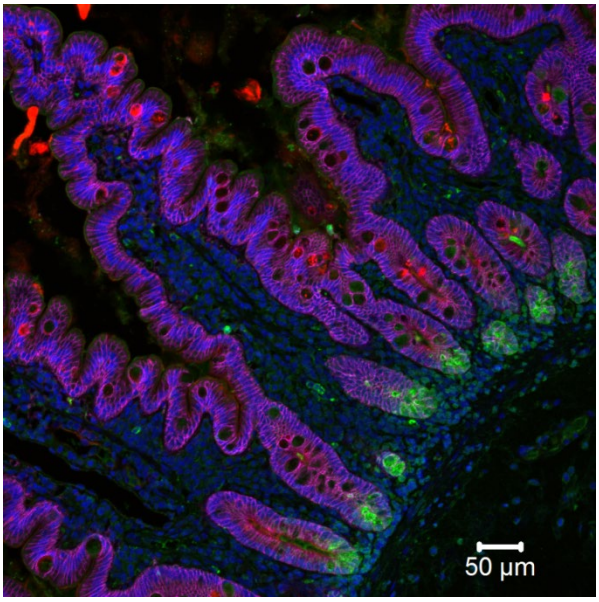

Figure 4.2: Human duodenal tissue section stained for Paneth cells (lysozyme), UEA1 (red color) binds secretory cells, EPCAM marker for the epithelia is shown in purple color and DAPI binds to

DNA (blue color). Paneth cells are specialized intestinal epithelial cells that secrete abundant antimicrobial proteins, including lysozyme.

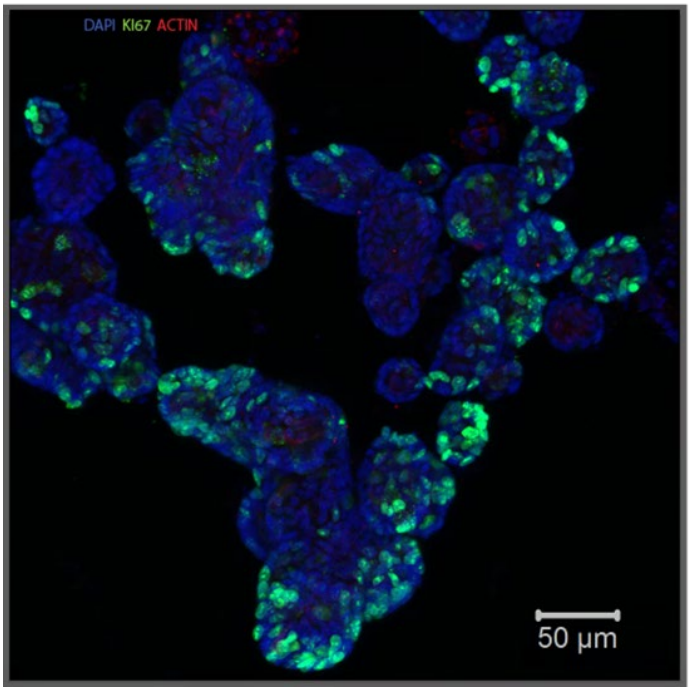

Figure 4.3: Proliferation of cells in organoids. Confocal images of duodenal organoids stained for Ki67 in a light green color (a proliferation marker), DAPI (blue color) binds to DNA and the red color is ACTIN, a protein that provides mechanical support and determines cell shape.

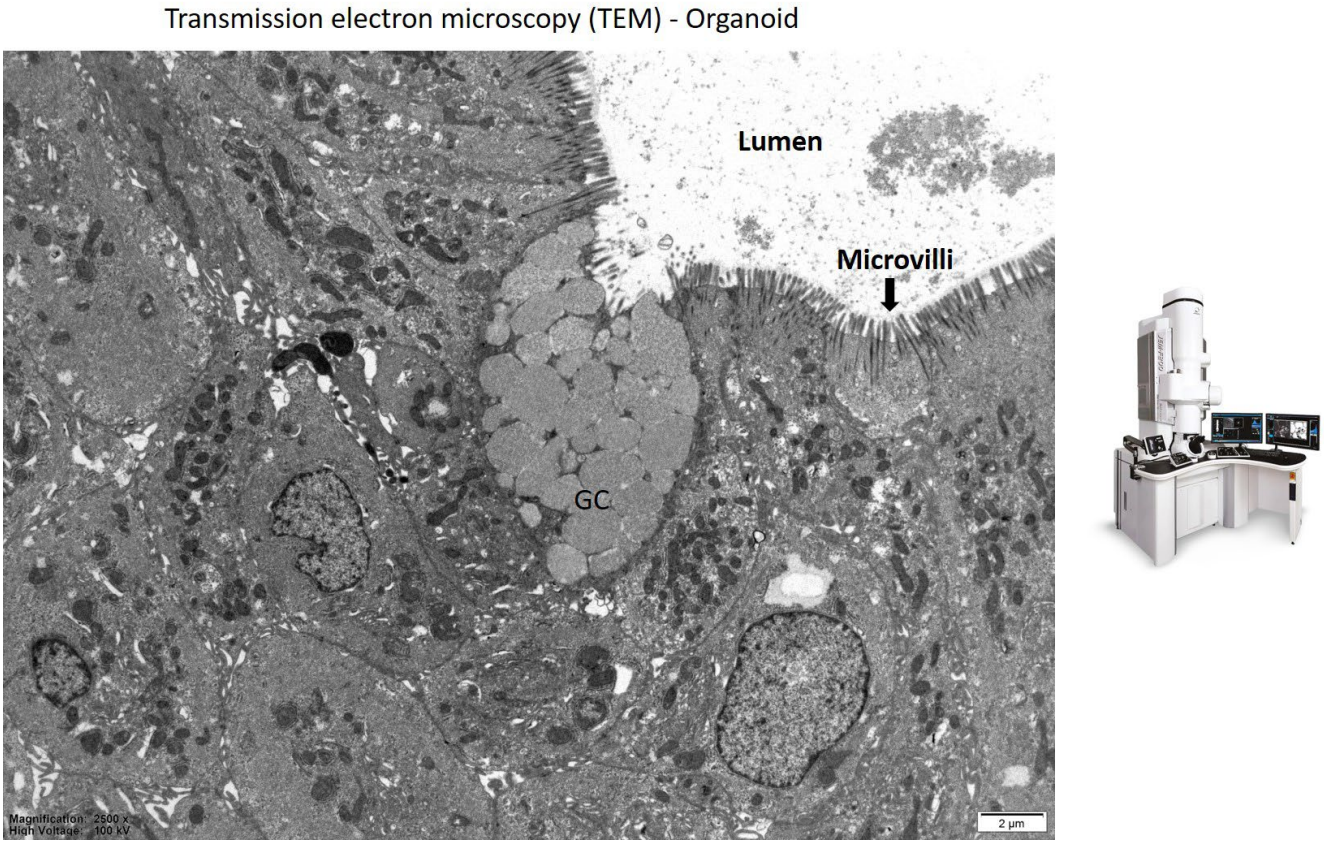

Figure 4.4: TEM image of duodenal organoid showing goblet cell (GC), microvilli and lumen. Scale bars, 2 µm.

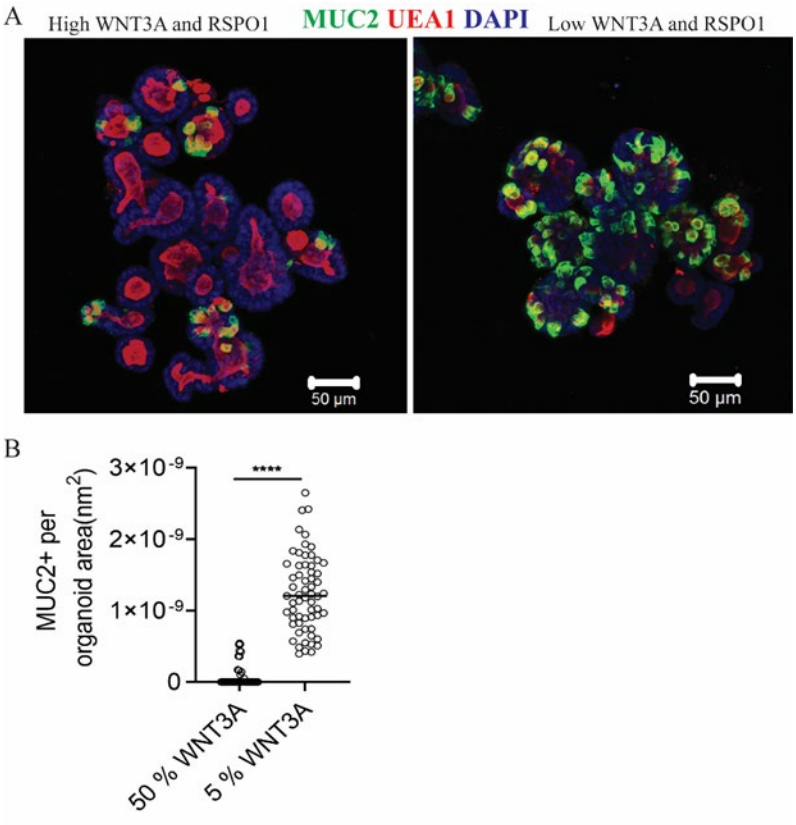

Figure 4.5: Differentiation of goblet cells in low concentration of Wnt3a and RSPO1. A) Confocal images of duodenal organoids stained for MUC2 in green color (a goblet cell marker), UEA-1(UEA1, a marker for secretory cells) and counterstained with DAPI (blue color). B) Quantification of MUC2+ cells per organoids area in duodenal organoids. Each dot represents one organoid and n =one donor.

RSPO1 is a secreted protein known to induce the proliferation of ISCs through activation of the Wnt pathway, with effects on differentiated cell migration and differentiation along the crypt-villus axis. We observed that proliferation and differentiation could be controlled by high (50 % Wnt3a and 20 % R-Spondin 1) and low (5 % Wnt3a and 5 % R-Spondin 1) concentrations of growth factors. Furthermore, we observed a MUC2+ Goblet cell number increase by decreasing the concentration of Wnt3a & R-Spondin 1 and in the complete absence of Nicotinamide and SB202190- a p38 MAPK inhibitor.

4.2 Transcriptome profile of duodenal organoids after cytokine stimulation:

To dissect the immune-duodenal epithelial transcriptome, we treated human duodenal organoids with main cytokines responsible for mediating innate and adaptive cell-mediated effector immunity like Type 1 response (IFN-y and TNF- α, Type 2 response (IL-13, IL-4 and IL-5 and Type 3 immune responses (IL-17a and IL-22). We generated biobank of duodenal organoids (n= 7) from patients undergoing Whipple surgery at Rikshospitalet. However, we used organoids from five donors for bulk RNA sequencing. Duodenal organoids were treated with alone with seven different cytokines. Cytokines play a key role in gut by promoting or preventing inflammation. Epithelial function is regulated by mucosal cytokines produced by myeloid and lymphoid immune cells of tissue. For example, cytokines like IL-22, TNF-α impact both epithelial specific pathological process like apoptosis, impaired barrier function, inducing LGR5+ stem cells. In addition, cytokines also affect other immune cell specific process for example IL-22 induces recruitment of neutrophils and thus impaired therapeutic effects of monoclonal antibody therapies like ustekinumab.

Two research groups have explored the transcriptional landscape of the intestinal epithelium. One group based in the Netherlands found that IL-22 is required to induce paneth cell granules in 3 ileal and one duodenal organoids donor. They found that IL-22 induces differentiation of human Paneth cells in ileal organoids. They claimed that IL-22 was the only Paneth cell inducer amongst a large panel of cytokines examined. A second research group at Imperial College London, London, UK, mapped the IL-22 responsive transcriptional network in colonic epithelial organoids. They found that IL-22-mediated regulation of neutrophils is an essential pathogenic pathway in ulcerative colitis. Therefore, the detailed transcriptional landscape of duodenal organoids after cytokine treatment has yet to be explored. In this study, we tried to map the transcriptional landscape of the human duodenal region using organoids as a model. We have used seven cytokines to study immune-epithelial interactions. These cytokines are responsible for mediating canonical immune responses. For example, IFN-gamma and TNF-α induce type 1 immune responses. Interleukin-4 and IL-13 are the signature cytokines of type II inflammatory response. They are critical players in the inflammatory response triggered by an invading parasite or allergen. The cellular sources of IL-4 and IL-13 have been studied extensively. Along with CD4 T cells, basophils, eosinophils, mast cells, NK T cells, and stimulated type 2 innate lymphoid cells to have the ability to produce IL-4 and IL-13.

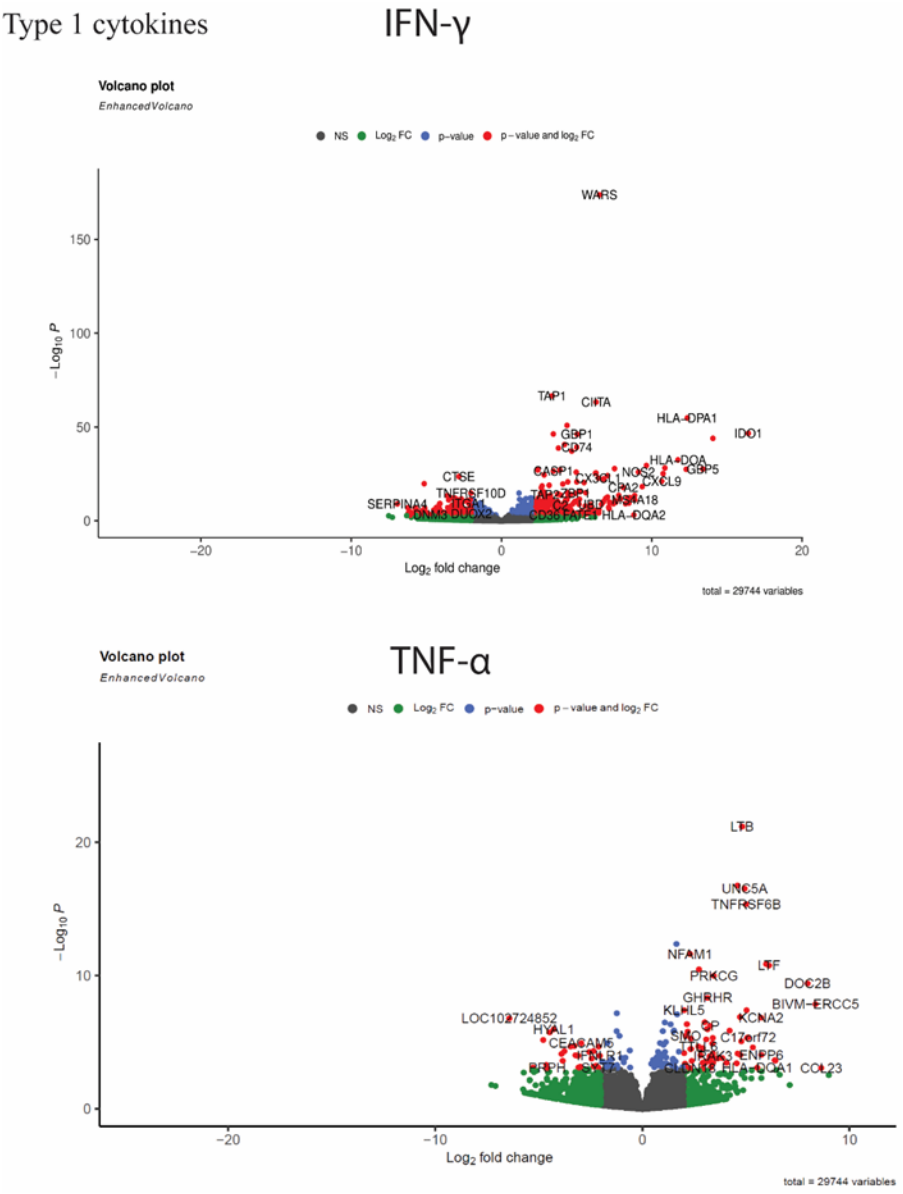

Figure 4.6: A and B) Volcano plots showing significantly expressed genes after IFN- γ (A) and TNF- α (B) treatment in duodenal organoids. Number of donors n= 5.

To compare the gene expression profiles of untreated and treated duodenal organoids, Principal component analysis (PCA) of samples based on the normalized RNA-seq counts showed that untreated organoids from the duodenum clustered together and cytokine treated organoids clustered far from the untreated duodenal organoids. In addition, the comparison of untreated and cytokine-treated duodenal organoids detected differentially expressed genes (FDR-adjusted $p < 0.05$) downregulated and upregulated genes.

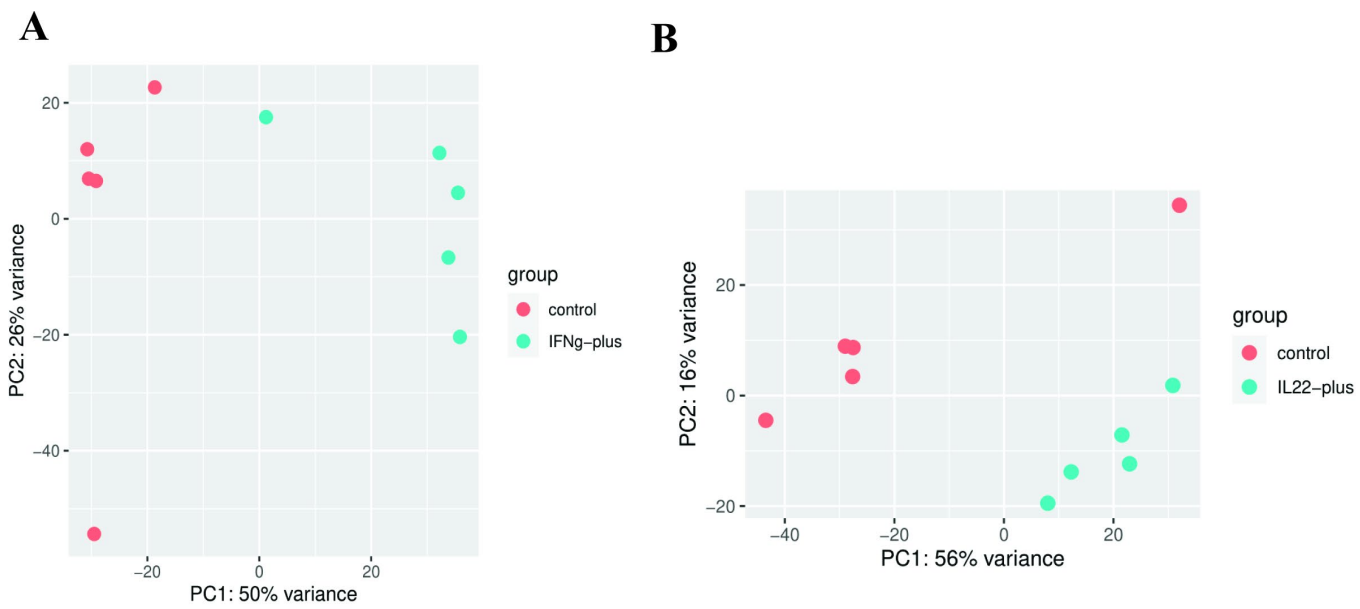

Figure 4.7: (A and B) PCA plots showing the far away clustering of non-treated and treated IFN-γ (A) and IL-22 (B) human duodenal organoids.

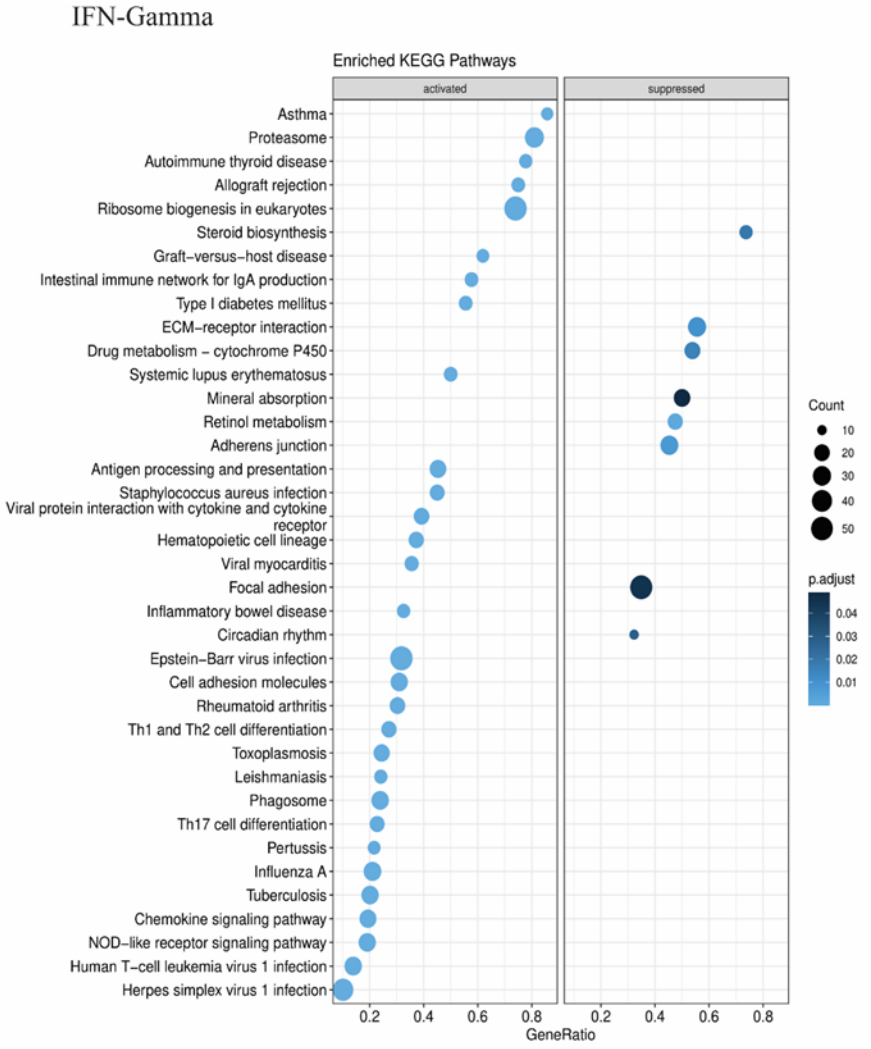

Figure 4.8: Dot plot showing significantly over-represented KEGG pathway terms using the DEGs with $p < 0.05$.

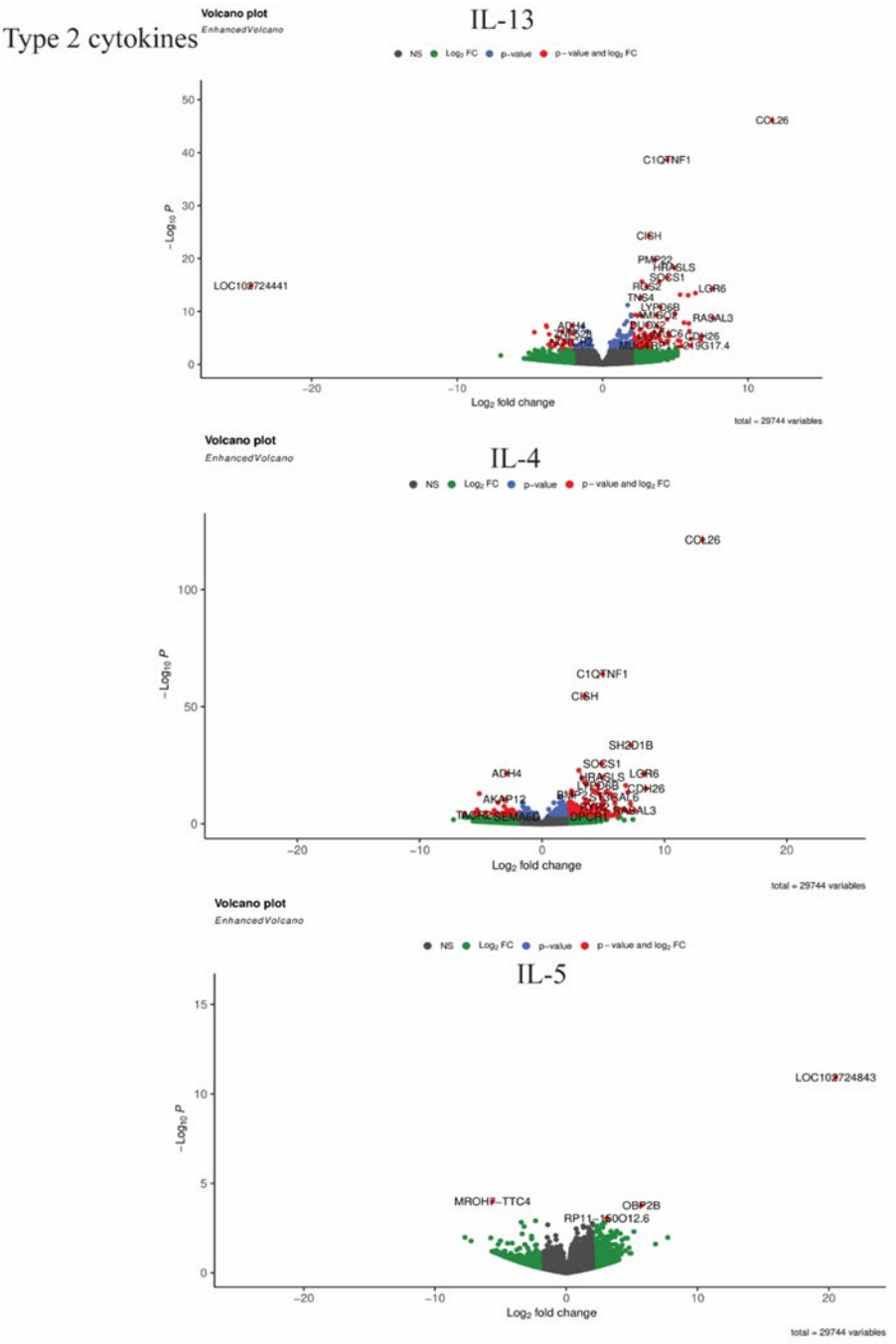

Figure 4.9: A and B) Volcano plots showing significantly expressed genes after IL-13 (A) and IL-4 (B) and IL-5 (C) treatment in duodenal organoids. Number of donors n= 5.

Kyoto Encyclopedia of Genes and Genomes analysis:

To better understand the function of Differentially Expressed Genes (DEG) in cytokine-treated duodenal organoids, we investigated the KEGG pathway enrichment of DEGs using cluster Profiler

(R package). We found the activation of gene expression in well-defined IFN- γ related pathways, including the intestinal immune network for IgA production and inflammatory bowel disease. In addition, KEGG pathway analysis identified the enrichment of the intestinal immune network for IgA production after IL-22 treatment in organoids.

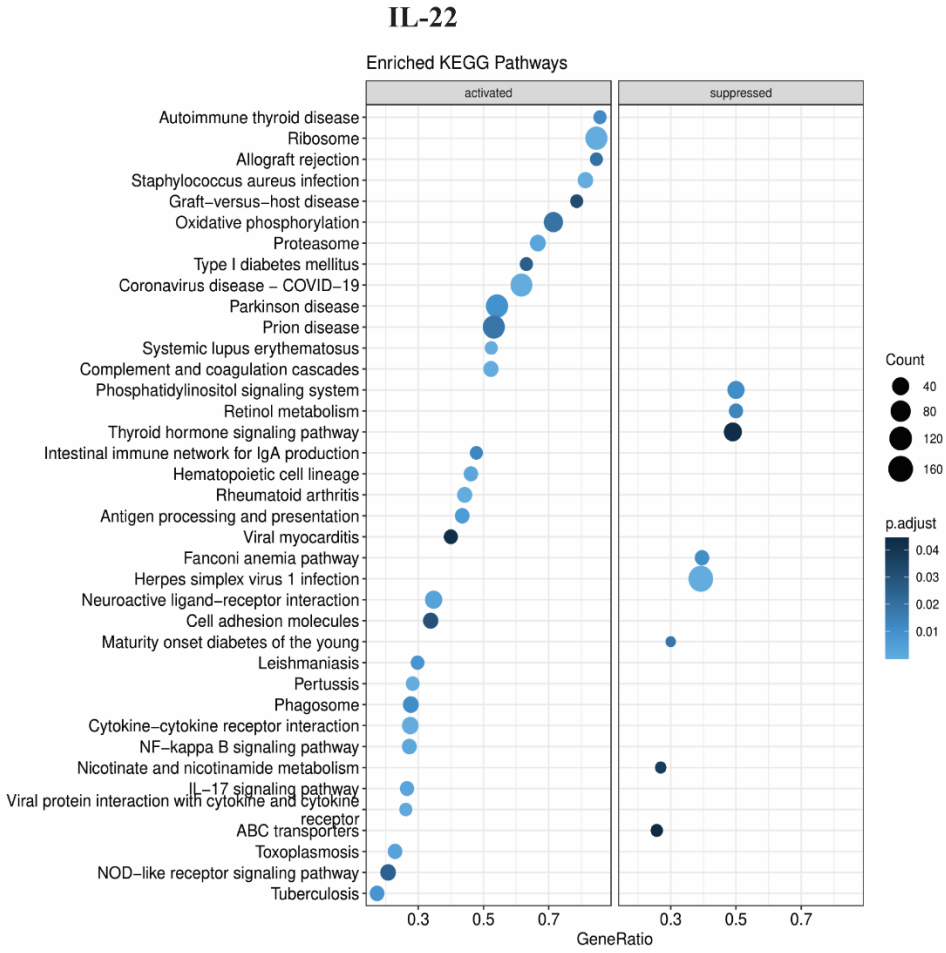

Figure 4.10: Dot plot showing significantly over-represented KEGG pathway terms using the DEGs with $p < 0.05$.

Type 3 cytokines

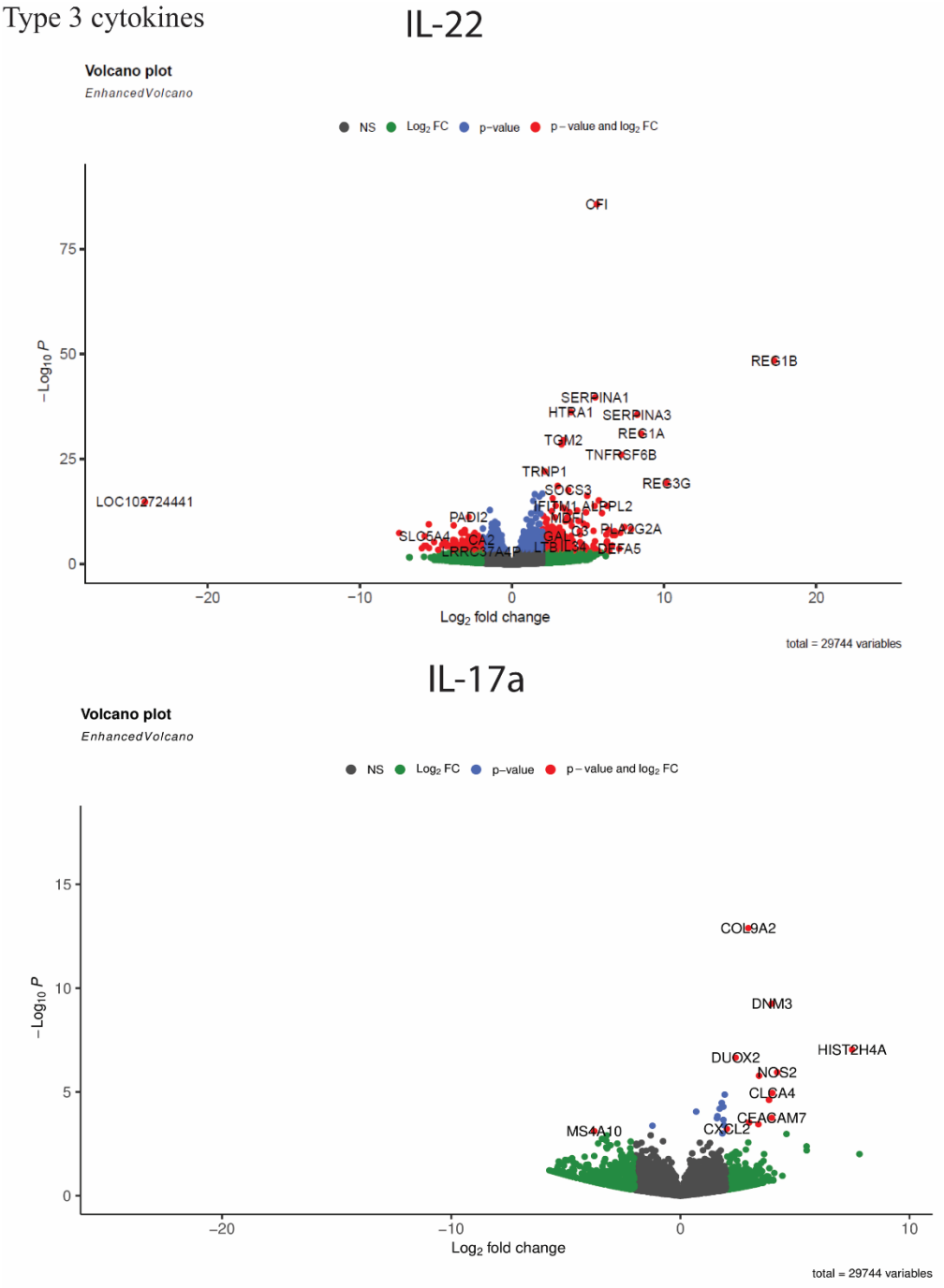

Figure 4.11: **(A and B)** Volcano plots showing significantly expressed genes after IL-22 (A) and IL-17a (B) treatment in duodenal organoids. Number of donors n= 5.

4.3 Effect of cytokines on MUCINS in duodenal organoids:

The intestinal mucus layer is composed of high molecular weight glycoproteins called mucins, which play a role in physical protection and regulate the concentration and passage of water, ions, antimicrobial peptides, and immunoglobulin-A (IgA) within the gut. Goblet cells are chiefly responsible for producing and preserving the mucus layer via mucin production and are influenced by interactions with the immune system. Thus far, more than 20 mucin genes have been identified. Though the corresponding glycoproteins associated with each of these genes have distinct differences, mucins generally share conserved structural features. For example, MUC1 is mostly expressed in the stomach, but now single-cell analysis revealed that it upregulated in non-inflamed terminal ileum samples in Crohn's disease and was further increased during inflammation across epithelial cell types in both terminal ileum and colon.

Our bulk RNA sequencing results revealed that MUC1 was fold increased after IL-22 stimulation. We also observed differential expression of other mucins after cytokine stimulation. Surprisingly, MUC6 was upregulated after IL-13 and IL-4 stimulation. According to these reports, MUC6 is highly expressed in gastric mucosa, duodenal Brunner's glands, gall bladder, seminal vesicle, pancreatic centroacinar cells and ducts, and periductal glands of the common bile duct. However, increased expression of MUC6 was unexpected in duodenal organoids. We found significant 4.6 and 4.4 fold upregulation of MUC6 after IL-13 and IL-4 stimulation in duodenal organoids compared to the non-treated organoids.

On the other hand, IL-22 specifically induces upregulation of MUC1 (3.8 fold) in duodenal organoids. MUC1 expression at the epithelial surface limits the gastrointestinal and systemic spread of *Campylobacter jejuni* and reduces intestinal inflammation in mice. Next, immunofluorescence will confirm MUC6 expression in duodenal organoids after IL-13 stimulation.

MUC2 is the major gel-forming mucin of the colon, forming a protective gel barrier; thus, MUC2 expression is only slightly upregulated after IL-13, IL-22, and IL-4 stimulation. We observed 1, 27 fold after IL-13 stimulation in duodenal organoids.

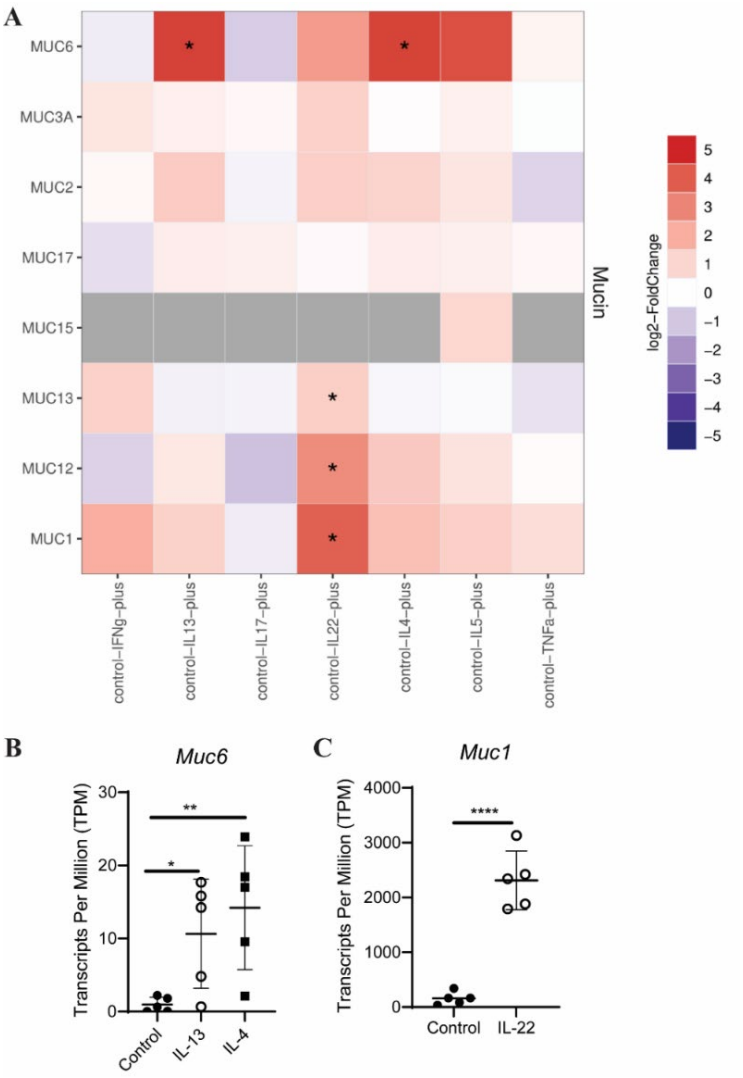

Figure 4.12: Effect of cytokines on Mucins expression. A) RNA heatmap of the differentially expressed (DE) genes in cytokine-treated duodenal organoids. Upregulated expression is red, and blue represents suppressed expression. B) *Muc6* mRNA expression in duodenal intestinal organoids treated with IL-13 and IL-4 for six days determined with RNA-seq. Each dot is one biological donor. C) *Muc1* mRNA expression in duodenal intestinal organoids treated with IL-22 for six days determined with RNA-seq. Each dot is one biological donor. TPM, transcripts per million. Unpaired Student t-test was used to determine the statistically significant differences between the experimental groups (Figures B and C), and a P value of <0.05 was considered significant.

4.4 Effect of cytokines on antimicrobials in duodenal organoids:

Mammalian intestines are exposed to various pathogens and commensal microorganisms. Therefore, antimicrobial proteins produced by epithelial cells are essential for the host against microbial challenges. Thus, we checked the expression of different antimicrobial proteins in duodenal organoids at the transcriptome level. We found that IL-22 was the most potent inducer of different anti-microbials. Reg1b, Reg3g, Reg1a, and Defa5 were significantly upregulated in IL-22-treated duodenal organoids. Our observations are consistent with the other research group, where they reported upregulation of Defa5 and Defa6 in human ileal organoids after IL-22 stimulation.

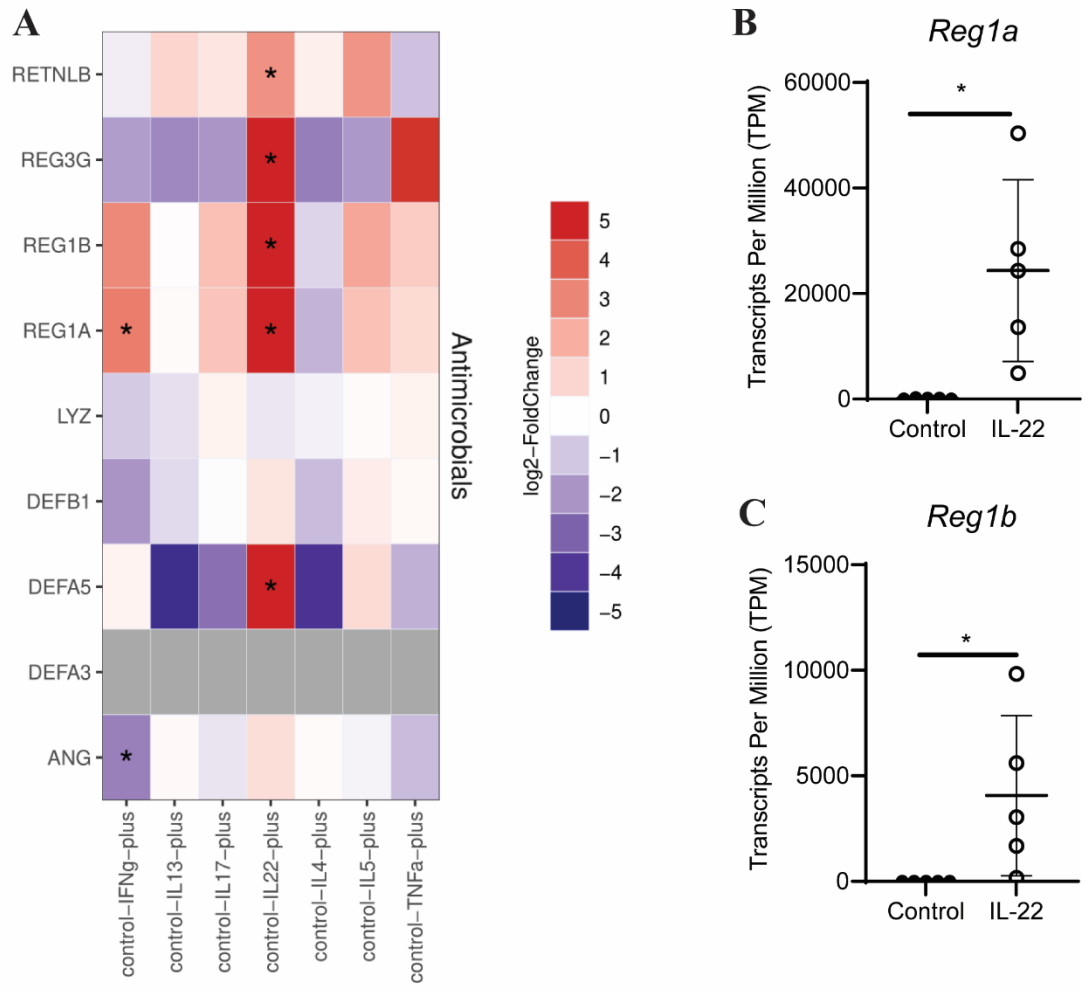

Figure 4.13: Effect of cytokines on antimicrobial expression in duodenal organoids. A) RNA heatmap of the differentially expressed (DE) genes in cytokine-treated duodenal organoids. Upregulated expression is red, and blue represents suppressed expression. B) *Reg1a* mRNA expression in duodenal intestinal organoids treated with IL-22 for six days determined with RNA-seq. Each dot is

one biological donor. C) *Reg1B* mRNA expression in duodenal intestinal organoids treated with IL-22 for six days determined with RNA-seq. Each dot is one biological donor. TPM, transcripts per million.

Unpaired Student's t-test was used to determine the significant differences between the experimental groups (Figures B and C), and a P value of <0.05 was considered significant.

4.5 Cytokine modulates differentiation of epithelial cell types:

We used cell type-specific gene signatures to test the effects of these seven cytokines on cell lineage differentiation. GSEA (Gene Set Enrichment Analysis) revealed that these cytokines affect intestinal cell lineage differentiation. For example, IL-22 strongly induces a paneth cell gene signature. We found increased *Pla2g2a*, *Defa5*, *and Defa6* expression in IL-22-treated duodenal organoids. We found very interesting observations after IFN- γ treatment in duodenal organoids. We discovered that IFN- γ treatment increased gene signatures specific for Enteroendocrine cells. We found significant upregulation of Chromogranin A (ChgA), Chromogranin B (ChgB), and Gcg gene, which encodes glucagon-like peptide-1 (GLP-1). However, TNF-α treatment resulted in downregulating genes specific to Enteroendocrine cells. In addition, TNF-α treatment also reduced the gene signatures for goblet cells, as shown in (Fig. 4.13 A). This group has recently published that IL-13 induces DCLK1+ tuft cell numbers in mouse organoids, and we proposed a model in which activation of the bone morphogenetic protein (BMP) pathways limit IL-13– induced tuft cell differentiation, thus providing a feedback loop to limit tuft cell expansion during immune responses. In this direction, we found that type 2 cytokines IL-13 and IL-4 induce slight upregulation of tuft cell genes, especially *Trpm5*, in human duodenal organoids.

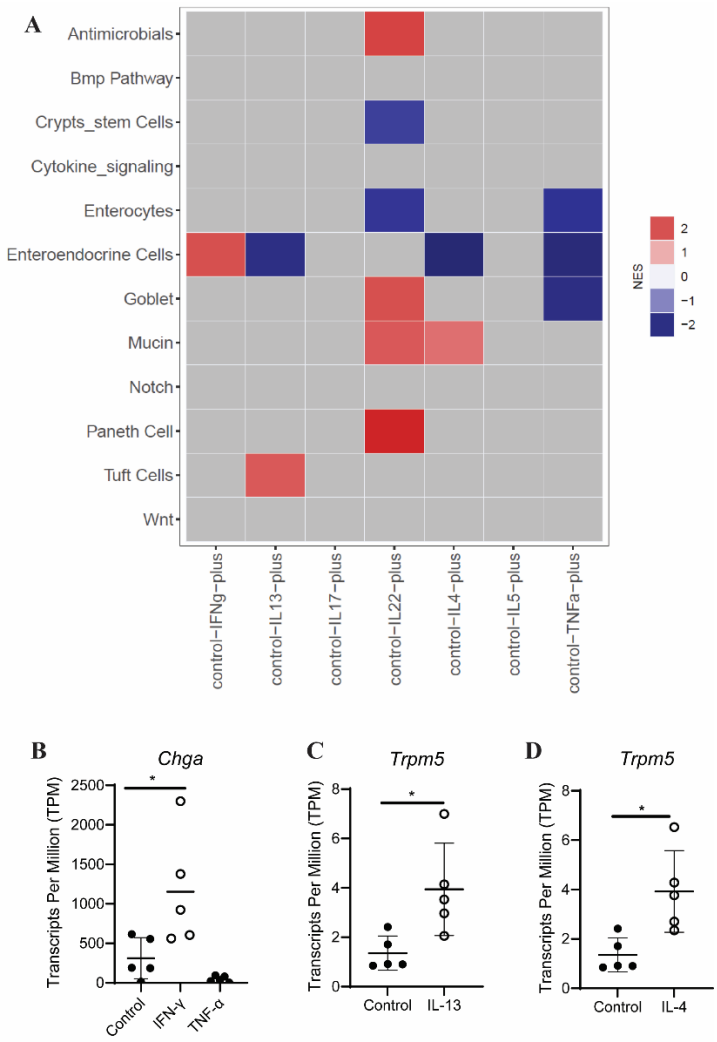

Figure 4.14: Effect of cytokines on epithelial cell differentiation genesets. A) Heatmap of NES values from GSEA analysis of gene sets representing different cell types in duodenal organoids treated for six days with cytokines. B) Chromogranin A (Chga- an Enteroendocrine cell marker) mRNA expression in duodenal intestinal organoids treated with IFN- γ and TNF-α for six days determined with RNA-seq. Each dot is one biological donor. TPM, transcripts per million. **(C and D)** And Trpm5 (transient receptor potential cation channel subfamily M member 5 - is a specific tuft cell marker) mRNA expression in duodenal intestinal organoids treated with IL-13 (C) and IL-4 (D) for six days determined with RNA-seq. Each dot is one biological donor. TPM, transcripts per million. Unpaired two-tailed Student's t-test was used to determine the statistically significant differences between the experimental groups (Figures B, C, and D), and a P value of <0.05 was considered significant.

4.6 Interferon- γ treatment results in upregulation of genes linked with celiac disease in duodenal organoids

The cytokine network in celiac disease is characterized by abundant Interferon- γ in the intestinal mucosa. In addition, the production of interleukin (IL)-15, IL-18, and IL-21 is linked to gluten intake, which can drive the inflammatory response probably sustained by IL-18, IL-21, and IL-27 through STAT1 and STAT5 pathways. The cytokine interferon-gamma (IFN-γ) plays a pivotal role in the subsequent T cell activation and concomitant tissue restructuring. In patients, the IFN-γ gene (IFNG) expression level and the number of IFN-γ producing cells are increased, as observed for both the intestinal mucosa and peripheral blood lymphocytes. This section followed how IFN-γ induced celiac disease-associated gene expression in duodenal organoids. We found that IFN-γ treatment resulted in 4.19-fold induction in duodenal organoids. The enzyme transglutaminase 2 (TGM2) plays a crucial role in celiac disease (CD) pathogenesis by generating immunogenic, deamidated gluten T-cell epitopes and as a target of autoantibodies.

Interestingly, we also showed significant upregulation of the Ccl25 transcript. CCR9 has a sole known ligand CCL25 and promotes the migration of leukocytes into the gut. IECs are known to have increased expression of CCL25. CCR9 and CCL25 are members of the CC subfamily of chemokines involved in various inflammatory diseases and induce inflammatory responses. It has been reported that CCR9–/– and CCL25–/– mice are more susceptible to acute DSS colitis than WT controls. In addition, it has been reported by immunohistochemistry HLA-E was found to be most highly expressed in the enterocytes lining the atrophied villi of celiac patients. In line with this, we also found significant upregulation of HLA-E transcripts in IFN-γ treated duodenal organoids. The chronic upregulation of IL-15 in the epithelium and the intestinal lamina propria (LP) is a hallmark of the disease and correlates with the degree of mucosal damage. We found that *Il15* and *Il-15Rα* expression was significantly upregulated after IFN- γ treatment in duodenal organoids.

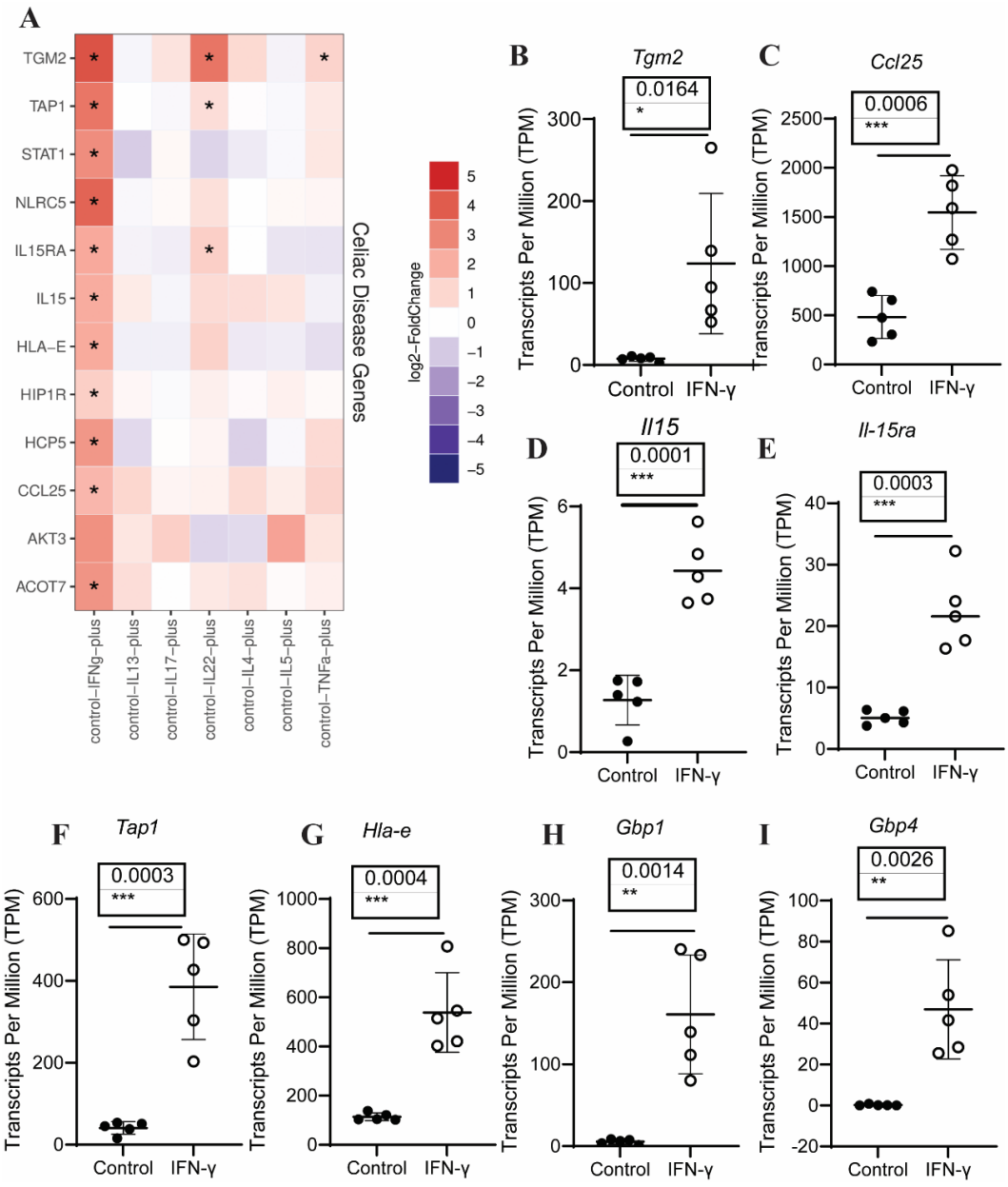

Figure 4.15: IFN-γ specifically upregulates the expression of genes associated with Celiac disease. A) Heatmap of gene expression of Celiac disease. **B- G)** Tgm2, Ccl25, Il15, Il15ra, Tap1, Hla-e, Gbp1, and Gbp4 mRNA expression in duodenal intestinal organoids treated with IFN-γ for six days determined with RNA-seq. Each dot is one biological donor. TPM, transcripts per million. Unpaired two-tailed Student's t-test was used to determine the statistically significant differences between the experimental groups (figure B- I), and the P value of <0.05 was considered significant.

5. DISCUSSION

5.1 Effect of WNT3A and RSPO1 on goblet cell differentiation in duodenal organoids:

Myofibroblasts are stromal cells in the intestinal LP differentiated from fibroblasts that play important regulatory roles in the intestinal stem cell niche by secreting local factors like Wnt and R-Spondin (Snyder et al., 2020). To explore the roles of WNT3A and RSPO1 on goblet cell differentiation in human duodenal organoids, we tried at different concentrations. Thus, we observed that proliferation and differentiation could be controlled by high and low concentrations of growth factors (WNT3A and RSPO 1). In our experiment high (50 % Wnt3a and 20 % R-Spondin 1) and low (5 % Wnt3a and 5 % R-Spondin 1) concentrations of growth factors were supplied to the organoids in the complete absence of Nicotinamide and SB202190. Then as the concentration of WNT3A and RSPO 1 decreases the number of goblet cells in duodenal organoids increases (Fig. 4.5 A& B). For example, we quantified the number of goblet cells per organoid area at higher and lower concentration of WNT3A and RAPO 1, and the number of goblet cells at lower concentrations of WNT3A and RSPO 1 were much higher than the number of goblet cells in higher concentrations of WNT3A and RSPO1 (Fig 4.5 B). Therefore, we concluded that proliferation and differentiation of goblet cells could be controlled by high (50 % Wnt3a and 20 % R-Spondin 1) and low (5 % Wnt3a and 5 % R-Spondin 1) concentrations of growth factors.

5.2 Impact of cytokines on human duodenal epithelial cell differentiation:

As mentioned in the results part, GSEA showed that these cytokines modulate the differentiation of epithelial cell types. For instance, to explore the impact of these cytokines in the proliferation and differentiation of IEC, we treated the human duodenal organoids for six days with different cytokines. When the human duodenal organoids are treated with INF- γ, only enteroendocrine cells are upregulated, whereas, with IL-22 goblet and Paneth cells and their products, mucins and antimicrobial peptides were upregulated. And tuft cells associated genes were upregulated when we treated them with IL-13. The enteroendocrine cells associated genes increase after exposure to INF-γ. In contrast, goblet and Paneth cells increase their response to IL-22, tuft cells increase their response to IL-13 (Fig.4.14 A), and IL-4 induces slight upregulation of Trpm5 (Tuft cells gene) in human duodenal organoids (Fig.4.14 D).

IL-22 inhibits the expansion of crypt stem cells (Fig. 4.14 A); however, it is required for the formation of Paneth cells, the prime producers of intestinal antimicrobial peptides (G. W. He et al.,

2022). (Fig. 4.13 A) showed that IL-22 induced expression of antimicrobials (host defense genes) such as REG1A, REG1B, REG3G, DEFA5 and RETNLB. Particularly, REG1A and REG1B were highly expressed in duodenal intestinal organoids after IL-22 treatment for six days as it was determined with RNA-seq (Fig.4.13 B and C).

Regenerating islet-derived protein 1-alpha (REG1A) is a member of secreted protein family containing a C-type lectin domain, and plays a role in proliferation, differentiation, inflammation, and carcinogenesis of cells of the digestive system (Van Ba et al., 2012), and is abnormally upregulated in a series of gastrointestinal inflammatory disorders (Mao et al., 2021). In our work, REG1A and REG1B were similarly upregulated and downregulated in duodenal intestinal organoids after treatment with those cytokines. For instance, both REG1A and REG1B were highly upregulated when we treated them with IL-22 and INF- γ, and slightly upregulated when we treated them with IL-17a, IL-5 and TNF-α (Fig.4.13 A). However, slightly downregulated when we treated them with IL-4 and IL-13 but, DEFA5 was only highly upregulated in response to IL-22 as we have shown in RNA heatmap of the DE genes in cytokine treated duodenal intestinal organoids (Fig.4.13 A). Therefore, from these observations and results we concluded that IL-22 induces gene signatures specific for Paneth cells in duodenal organoids. And our discovery is novel because it was not studied before in human duodenal organoids.

6. CONCLUSION:

We found that both type one cytokines IFN- γ and TNF-α affect epithelial cell differentiation. On the other hand, for type two cytokines, IL-13 and IL-4 affect cell differentiation in duodenal organoids, and IL-5 does not affect differentiation. For type 3 cytokines, IL-22 was the most potent inducer of epithelial cell differentiation in duodenal organoids, and IL-17a had no effect. A prolonged homeostatic imbalance can result in chronic inflammation. A deeper understanding of the contribution of immune cell-secreted cytokines will likely unveil novel potential strategies to manage gut diseases therapeutically. Cytokines play an essential role in the progression and healing of the celiac disease. For example, The Th1 immune response, with a critical position for interferon-gamma (IFN- γ), is an essential determinant of intestinal remodeling in celiac disease. The cytokine interferon-gamma (IFN-γ) plays a pivotal role in the subsequent T cell activation and concomitant tissue restructuring. In patients, the level of IFN-γ gene expression and the number of IFN-γ producing cells are increased, as observed for both the intestinal mucosa and peripheral blood lymphocytes. In this direction, we also found that IFN- γ induces genes responsible for celiac disease progression. IL-15 is a pro-inflammatory cytokine presented by its private chain IL-15Rα on the cell surface under conditions of stress and inflammation. In active coeliac disease, IL-15 is upregulated in the lamina propria

and intestinal epithelial cells (IECs). We also found upregulation of IL-15 and IL-15Rα after IFN- γ stimulation in duodenal organoids.

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Appendix

Appendix 1: Reagents list with manufacturer and catalog number.

Reagent name	Company	Catalog number	Solvent	Stock concentration	Final concentration
Dulbecco's Phosphate buffered saline Ca2+, Mg2+ free (DPBS)	Life technologies; Gibco	14190-144 431788	-	-	1x
Bovine serum albumin (BSA) Fraction V	Fischer Scientific Life	BP1600-100	DPBS	Powder	1x
Fluoromount G medium	Thermofisher Scientific	00-4958-02			
Advanced DMEM/ F12	Life technologies; Gibco	12634-028			
Wnt-3A condi-	In house				

tioned medium					
HEPES 1M	Life technologies; Gibco	15630-080	-	1M	10mM
Penicillin-Streptomycin (10,000 U/mL)	Life technologies Gibco	15140-148	-	100x	1x
GlutaMAX (glutamine)	Life technologies Gibco	35050-061	-	100x	1x
N-Acetylcysteine	Sigma-Aldrich	A9165-5G	DPBS	1M	10mM
Nicotidamide	Sigma-Aldrich	N0636	DPBS	1M	10mM
B27 Supplement	Life technologies; Gibco	17504-044	-	50x	1x
N2 Supplement	Life technologies; Gibco	17502-048	-	100x	1x
Human recombinant EGF	Sigma-Aldrich	E9644-.2MG	DBPS	500 µg/ml	50 ng/ml
Human recombinant Noggin	R and D	6057-NG/CF	DBPS	100 µg /ml	100 ng/ml
Y-27632	Sigma-Aldrich	Y0503-1MG	DPBS	10 mM	10 µM
CHIR99021	Stemgent	04-0004	DMSO	10 mM	2.5 µM
SB202190	Sigma-Aldrich	S7067-5MG	DMSO	30 mM	10 µM
A-83-01	Tocris	2939	DMSO	500 µM	500 nM
Human [Leu]15-Gastrin 1	Sigma-Aldrich	G9145-.1MG	DPBS	100 µM	10 nM
Fetal Bovine Serum (FBS)	Life technologies; Gibco	10082-147	-	-	-
L Wnt-3A cell line	ATCC	CRL-2647	-	-	
TrypLE Express Enzyme (1x)	Gibco	12605-010			
Matrigel (base-	CORNING	356237			

ment Membrane matrix)					
Plasmocin	Invivogen				

Appendix 2: Detailed consumables, tools and equipment used in crypt isolation and culture.

Equipment	Consumable	Tools
Laminar flow hood	15- and 50 conical tubes	Standard forceps
Centrifuge	24-well plates	Fine scissors
CO2 Incubator	Micropipette tips	
Freezing containers	0.22 µm filters (Sartorius)	
Micropipettes	150 µm mesh openings, nylon screening	
Confocal microscopy	Eight-chamber µ-slides (ibidi, 80826)	
Orbital shaker		