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Title:

Study on the potential interactions of the local nervous system and the intestine tissue under physiological and pathological conditions

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Abstract

Introduction: The Gut-Brain-Axis connects the gastrointestinal with the neurological system and this "relationship" seems to be bidirectional, as each of them affects the functions of the other. The neurotrophins and their receptors have been well studied and proven to play a significant role in various neurodegenerative diseases, but their involvement in the intestinal inflammation has not yet been adequately studied.

Aim: Our aim was to investigate the expression of the neurotrophin receptors TrkA, TrkB and p75NTR in *in vitro* and *ex vivo* intestinal inflammation models, stimulated with the pro-inflammatory cytokines IL-1α and TNF-α, in order to identify disease mechanisms and possible new therapeutic targets.

Materials & Methods: Human colonic subepithelial myofibroblasts (cSEMFs) were isolated from endoscopic biopsies of healthy individuals, set to culture and later stimulated with 5ng/ml IL-1α and 50ng/ml TNF-α for 6, 24 and 48 hours. At the end of the 6h incubation period, total RNA was isolated, while at the end of 24 and 48h incubation periods, supernatants were collected, and total cells were harvested in lysis buffer. The mRNA and protein expression of the neurotrophin receptors TrkA, TrkB and p75NTR was analysed using real-time PCR and Western blot analysis, respectively. Human intestinal organoids (HIOs) were developed from the embryonic stem cell line H1. Colonoids were developed from epithelial crypts isolated from endoscopic biopsies. Both 3D culture models were characterized using immunofluorescence and were later stimulated with 5ng/ml IL-1α and 50ng/ml TNF-α for 12 and 24 hours. At the end of the 12h incubation period, total RNA was isolated, while at the end of 24h incubation period, supernatants were collected, and organoids were harvested in lysis buffer. The mRNA expression of several chemokines was analysed using real-time PCR.

Results: Non-stimulated cSEMFs were lacking the expression of all neurotrophin receptors (TrkA, TrkB and p75NTR). Stimulation with IL-1α and TNF-α led to a statistically significantly upregulation of the mRNA levels of TrkB and p75NTR. This mRNA upregulation was also verified at the protein level and specifically, the IL-1α and TNF-α stimulation resulted in a significant and time-dependent increase in the protein expression of TrkB and p75NTR in cSEMFs. Additionally, the 3D *in vitro* and *ex vivo* intestinal models of HIOs and Colonoids were successfully developed and established, as both systems were responsive to the stimulation of IL-1α and TNF-α, resulting in the mRNA upregulation of the chemokines CXCL1, CXCL8, CXCL10, CXCL11, CCL2 and CCL20, and thus rendering them as promising tools for the further investigation of the role of neurotrophin receptors in the intestinal inflammation.

Conclusions: Our results show that the expression of TrkB and p75NTR neurotrophin receptors is induced upon inflammatory challenges in cSEMFs, indicating a significant role on this process. Our *in vitro* approach could mimic the microenvironment of inflamed mucosal tissue of patients with Inflammatory Bowel Diseases or related disorders, and thus to be proven useful as an *in vitro* platform for an initial drug screening of novel or repurposed agents against these diseases. Conclusively, we propose a new experimental approach for the evaluation of pharmacological ligands of these neurotrophin receptors as potential anti-inflammatory compounds.

Τίτλος και Περίληψη

Μελέτη των αλληλεπιδράσεων μεταξύ του τοπικού νευρικού συστήματος και του εντερικού ιστού κάτω από φυσιολογικές και παθολογικές συνθήκες

Εισαγωγή: Ο Άξονας Εντέρου-Εγκέφαλου συνδέει το γαστρεντερικό με το νευρολογικό σύστημα και αυτή η σύνδεση φαίνεται να είναι αμφίδρομη, καθώς το καθένα από αυτά επηρεάζει τις λειτουργίες του άλλου. Οι νευροτροφίνες όπως και οι υποδοχείς τους έχουν μελετηθεί διεξοδικά και έχει αποδειχθεί ότι παίζουν σημαντικό ρόλο σε πολλές Νευροεκφυλιστικές ασθένειες, αλλά η εμπλοκή τους στην εντερική φλεγμονή δεν έχει μελετηθεί επαρκώς.

Σκοπός: Στόχος μας ήταν να διερευνήσουμε την έκφραση των TrkA, TrkB και p75NTR (υποδοχείς νευροτροφινών) σε *in vitro* και *ex vivo* μοντέλα εντερικής φλεγμονής, έπειτα από διέγερση με τις προφλεγμονώδεις κυτταροκίνες IL-1α και TNF-α, με σκοπό να εντοπιστούν πιθανοί παθογενετικοί μηχανισμοί και νέοι θεραπευτικοί στόχοι έναντι της εντερικής φλεγμονής.

Υλικά & Μέθοδοι: Ανθρώπειοι κολονικοί υποεπιθηλιακοί μυοϊνοβλάστες (ΥΕΜ) απομονώθηκαν από ενδοσκοπικές βιοψίες υγιών ατόμων, καλλιεργήθηκαν και διεγέρθηκαν με 5ng/ml IL-1α και 50ng/ml TNF-α για 6, 24 και 48 ώρες. Στο τέλος της 6-ωρης επώασης, απομονώθηκε ολικό RNA, ενώ μετά από τις επωάσεις των 24 και 48 ωρών, συλλέχθηκαν τα κύτταρα και τα υπερκείμενά τους. Η mRNA και πρωτεϊνική έκφραση των υποδοχέων TrkA, TrkB και p75NTR αναλύθηκε χρησιμοποιώντας RT-PCR και Western blot, αντίστοιχα. Τα ανθρώπεια εντερικά οργανίδια (ΑΕΟ) αναπτύχθηκαν από την εμβρυϊκή σειρά βλαστοκυττάρων H1, ενώ τα κολονοειδή αναπτύχθηκαν από επιθηλιακές κρύπτες που απομονώθηκαν από ενδοσκοπικές βιοψίες. Έπειτα, τα δύο αυτά 3D μοντέλα χαρακτηρίστηκαν χρησιμοποιώντας ανοσοφθορισμό και διεγέρθηκαν με 5 ng/ml IL-1α και 50 ng/ml TNF-α για 12 και 24 ώρες. Στο τέλος της 12-ωρης επώασης, απομονώθηκε ολικό RNA, ενώ στο τέλος των 24 ωρών, συλλέχθηκαν τα οργανίδια και τα υπερκείμενα τους. Η mRNA έκφραση χημειοκινών αναλύθηκε χρησιμοποιώντας RT-PCR.

Αποτελέσματα: Οι αδιέγερτοι ΥΕΜ δεν εξέφρασαν κανένα από τους υποδοχείς (TrkA, TrkB και p75NTR). Η διέγερση με IL-1α και TNF-α οδήγησε σε στατιστικά σημαντική αύξηση των mRNA επιπέδων του TrkB και του p75NTR. Η επαγωγή του mRNA επαληθεύτηκε σε επίπεδο πρωτεΐνης και συγκεκριμένα, η διέγερση με IL-1α και TNF-α οδήγησε σε σημαντική και χρόνο-εξαρτώμενη αύξηση της έκφρασης των TrkB και p75NTR στους ΥΕΜ. Επιπλέον, τα 3D *in vitro* και *ex vivo* εντερικά μοντέλα (ΑΕΟ – Κολονοειδή) αναπτύχθηκαν επιτυχώς, καθώς και τα δύο συστήματα ανταποκρίνονταν στη διέγερση με IL-1α και TNF-α. Συγκεκριμένα η διέγερση είχε ως αποτέλεσμα την mRNA επαγωγή των χημειοκινών CXCL1, CXCL8, CXCL10, CXCL11, CCL2 και CCL20, καθιστώντας τα ΑΕΟ και τα Κολονοειδή υποσχόμενα εργαλεία για την περαιτέρω διερεύνηση του ρόλου των υποδοχέων των νευροτροφινών στην εντερική φλεγμονή.

Συμπεράσματα: Τα αποτελέσματά μας αναδεικνύουν ότι οι προφλεγμονώδεις παράγοντες επάγουν την έκφραση των υποδοχέων TrkB και p75NTR στους ΥΕΜ, υπογραμμίζοντας τον σημαντικό ρόλο της φλεγμονής στην έκφραση των υποδοχέων νευροτροφινών. Η *in vitro* προσέγγισή μας προσπαθεί να μιμηθεί το φλεγμονώδες μικρο-περιβάλλον του βλεννογόνου των ασθενών με Ιδιοπαθείς Φλεγμονώδεις Νόσους του Εντέρου ή άλλες σχετικές εντερικές διαταραχές, ώστε να αποτελέσει μία χρήσιμη *in vitro* πλατφόρμα για την αξιολόγηση νέων φαρμάκων ή παραγόντων έναντι αυτών των ασθενειών.

Table of contents

Table of Contents

Acknowledgements 2

Abstract 3

Τίτλος και Περίληψη 4

Table of contents 5

List of figures 7

List of tables 8

Chapter 1: Introduction 9

Chapter 2: Literature review 10

2.1. Neurotrophins 10

2.2 Neurotrophin Receptors 11

2.3 Neurotrophin agonists and antagonists 12

2.4 Involvement of Neurotrophins and their Receptors in Gut 14

2.5 Inflammatory Bowel Diseases 15

2.5.1. Crohn’s Disease 16

2.5.2. Ulcerative Colitis 18

2.5.3. Immune mechanisms 19

2.6. *In vitro* and *ex vivo* disease models 20

2.6.1. Human Intestinal Subepithelial Myofibroblasts 20

2.6.2. Organoids 22

Chapter 3: Research methodology 25

3.1. Aim of the Study 25

3.2. Patients 25

3.3. Isolation and Characterization of Human Colonic Subepithelial Myofibroblasts 25

3.4. Development of Human Intestinal Organoids 26

3.5. Development of Human Colonoids 26

3.6. Immunofluorescence 27

3.7. Treatment with Pro-inflammatory cytokines 28

3.8. Total RNA extraction and purification 28

3.9. cDNA synthesis and real time-PCR 28

3.10. Western Blot 29

3.11. Statistics 30

Chapter 4: Results 31
4.1. cSEMFs express the neurotrophin receptors TrkB and p75NTR upon pro-inflammatory stimulation 31
4.2. Development of Human Intestinal Organoids and Colonoids 32
Chapter 5: Discussion 36
Chapter 6: Conclusion 39
References 40

List of figures

Figure 1. (**A**) Gut-Brain axis. (**B**) Microbiome-Gut-Brain axis.

Figure 2. Neurotrophin receptors.

Figure 3. Stages of hESCs differentiation into Human Intestinal Organoids.

Figure 4. Development of Colonoids from tissue biopsies.

Figure 5. Characterization of cSEMFs. cSEMFs are found positive to α-SMA and Vimentin expression and negative to Desmin expression.

Figure 6. Pro-inflammatory cytokines induce the mRNA levels of TrkB and p75NTR in cSEMFs. (A) TrkB mRNA expression. (B) p75NTR mRNA expression.

Figure 7. cSEMFs express the proteins of TrkB and p75NTR upon pro-inflammatory stimulation. (A) TrkB protein expression. (B) p75NTR protein expression.

Figure 8. Characterization of HIOs. HIOs are found to express CDX2, Cytokeratin, Villin, Chromogranin A, Vimentin, E-cadherin, EpCam, MUC2, SOX9, KLF5 and Desmin.

Figure 9. Development of human Colonoids from epithelial crypts.

Figure 10. Characterization of human Colonoids. Colonoids were found to express E-cadherin, Lgr5, Cytokeratin, EpCam and MUC2.

Figure 11. HIOs and Colonoids respond to pro-inflammatory stimuli by overexpressing various chemokines.

List of tables

Table 1. Genes associated with an increased likelihood of IBD development.

Table 2. Primer sequences used.

Chapter 1: Introduction

The Enteric Nervous System (ENS) acts as a second "Brain" (Gershon, 2019) providing a bridge between the gut, the mucosal immune system, the neuroendocrine system, the Autonomic Nervous System (ANS), the Vagus Nerve and by extension the brain (Furness, 2012). Though initially the brain was considered the sole instigator of Gut-Brain relationship, in an attempt to "regulate" the gut, later studies revealed the bidirectional communication between the Central Nervous System (CNS) and the ENS. Afferent and efferent signals to and from CNS, respectively, are going through the Vagus Nerve, while the Circulatory System offers an alternative route for communication, which is not only responsible for monitoring and regulating several organs functions – and specifically the Gut – but also for linking emotional and cognitive centers of the brain with the intestinal mechanisms. (**Figure 1A**) (Carabotti, Scirocco, Maselli, & Severi, 2015). These observations provided the basis for investigating the Gut-Brain Axis in depth, revealing four distinct signaling pathways, consisting of neural, immune, endocrinological and microbial communication pathways (Holzer & Farzi, 2014).

With the new knowledge of the involvement of the microbiota in human health, the Gut-Brain Axis has been extended to include the microbiome in its schematic, referred to in the literature as the Microbiome-Gut-Brain Axis (Bauer, Huus, & Finlay, 2016; Morais, Schreiber IV, & Mazmanian, 2021). Microbial metabolites interact with the host environment, controlling immune responses through the mucosa, reaching the brain through the circulatory system, and modifying neural responses. It is clear that there is an entire ecosystem that affects homeostasis and pathological conditions, through known and unknown mechanisms (**Figure 1B**) (Mayer, Nance, & Chen, 2022; Sampson & Mazmanian, 2015).

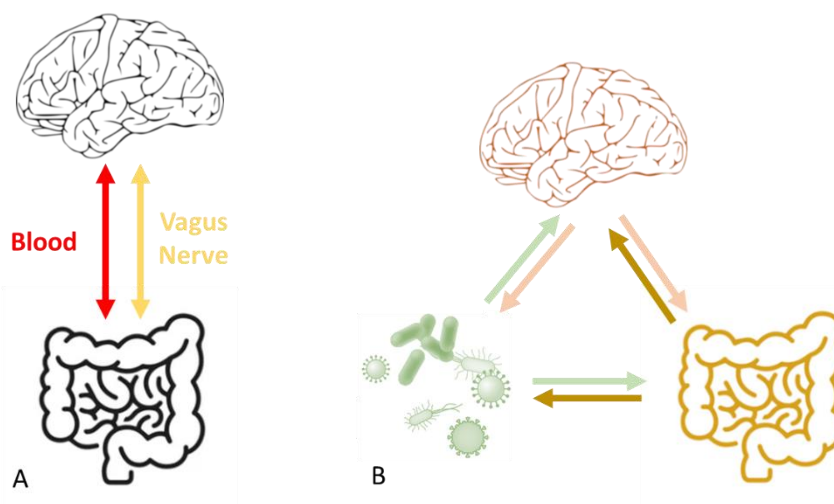

Figure 1. (**A**) Gut-Brain axis. (**B**) Microbiome-Gut-Brain axis.

Chapter 2: Literature review

2.1. Neurotrophins

Neurotrophins are a family of secreted growth factors with a pivotal role in the nervous system, and more specifically, they are mainly involved in neurite outgrowth, neuronal cell differentiation, and survival (Skaper, 2012). They are primarily synthesized as pro-neurotrophins, which upon cleavage, their mature and active forms are released, exerting their effects (Chao, Rajagopal, & Lee, 2006). Neurotrophins can be produced either by non-neuronal cells in the periphery, or by neurons in the central nervous system, and during development, the continuous interaction between neurotrophins and nerve cells is essential for neuronal survival; nerve cells that do not establish a neurotrophin signal transduction are destined to degenerate (Ginty & Segal, 2002).

The most well-known and well-studied growth factor of this family is the Nerve Growth Factor (NGF), which is involved in both the peripheral and the central nervous system. Regarding the peripheral nervous system, NFG is known to have an effect on motor and sensory neurons, while in the central nervous system, it is implicated the survival and functioning of cholinergic neurons in the basal forebrain (Chao et al., 2006). Apart from NGF, three other neurotrophins have been identified in mammals, the brain-derived neurotrophic factor (BDNF), the neurotrophin-3 (NT-3) and the neurotrophin-4 (NT-4), and they act either by forming homodimers or heterodimers. All four seem to have derived from a common ancestral gene, as they present similarities in both their gene sequence and protein structure (E. J. Huang & Reichardt, 2001).

As NGF was the first neurotrophin to be discovered, much research has been done on its possible secretion sources; it is mainly synthesized and secreted by sympathetic and sensory neurons, and upon secretion it can either be endocytosed by other nerve cells and transported to the nerve cell body, and thus exert its effect on neuronal cell survival, or it can act on non-neuronal target cells, such as hair follicles (E. J. Huang & Reichardt, 2001). Except from these sources, previous studies have shown that NGF and other neurotrophins are released upon nerve damage. Macrophages infiltrating the damaged tissue produce pro-inflammatory cytokines, which subsequently induce the production and secretion of NGF from Schwann cells and fibroblasts, and thus promoting nerve regeneration (Korsching, 1993). In addition, during nerve development, many studies have shown that nerve target regions may express neurotrophins in order to guide, attract and support the developing nerve axons that have yet to make contact with their targets (E. J. Huang & Reichardt, 2001).

The protein structure of NGF is characterized by three subunits, the α, β and γ, and many studies have shown that NGF plays a significant role in both embryonic and adult life (Wise, Seidel, & Lane, 2021). During embryonic development, neuroblasts that lack NGF are destined to undergo apoptosis (Ullrich, Gray, Berman, Coussens, & Dull, 1983), while in adulthood, its knockout expression in mice resulted in skeletal muscle dystrophy, reduced number of dorsal root ganglia neurons and impaired learning capacity (Ruberti et al., 2000). BDNF is another well-studied neurotrophin and its mature form consists of 118 amino acids. Its main actions seem to be focused on neuronal differentiation and modulation of the synaptic functions of neurons (B. L. Hempstead, 2015; Sharma, Singh, Kaur, Mannan, & Dhiman, 2023). NT-3 comprises of 119 amino acids (Chalazonitis, 1996), while NT-4 of 60 amino acids, and both these neurotrophins play a significant role in neuronal and synaptic plasticity (Omar, Kumar, & Teoh, 2022).

2.2 Neurotrophin Receptors

The neurotrophin receptor family consists of tropomyosin-related kinase receptor (Trk) A, B, C and p75NTR, and each of them has a different affinity for specific neurotrophins. Although all pro-neurotrophins bind to p75NTR, their mature forms bind to specific receptors; NGF binds to TrkA, BDNF and NT4 to TrkB, and NT3 to TrkC (**Figure 2**) (Sharma et al., 2023).

Regarding the Trk receptors, it has been found that each of them is expressed in specific cell and tissue types. Specifically, cholinergic basal forebrain neurons were found positive for TrkA expression, while cortical and hippocampal pyramidal neurons and cochlear neurons were positive for TrkB and TrkC expression (Cellerino, Maffei, & Domenici, 1996; Sobreviela et al., 1994; Szobota et al., 2019; Xu et al., 2000). Although Trk receptors share many features, they are also characterized by unique structural and expression traits, resulting to different biological functions (Conroy & Coulson, 2022).

Since TrkA, TrkB and TrkC share a significant homology in their gene and protein sequences, their structural forms are similar and consist of three parts: the extracellular part, which comprises of 5 different domains, the α-helical transmembrane part and the intracellular part, which includes a tyrosine kinase, responsible for the signal transduction. Interaction and binding of Trk ligands to the extracellular domains leads to the activation of Trk, and the subsequent autophosphorylation of its intracellular part by its kinase, resulting to the initiation and transduction of further signaling pathways through phosphorylation (BARBACID, 1995; Conroy & Coulson, 2022; Eric J. Huang & Reichardt, 2003).

Regarding p75NTR, although it was initially believed to be an NGF receptor, it was later proved that p75NTR actually belongs to the tumor necrosis factor receptor family (Skeldal, Coulson, Bradshaw, &

Stahl, 2016). Similar to the Trk receptors, the structural form of p75NTR consists of three parts: an extracellular one which has four cysteine-rich domains, an α-helical transmembrane part and an intracellular one (Conroy & Coulson, 2022). In contrast to the Trk receptors, the intracellular domain of p75NTR does not have any catalytic activity, but rather protein binding sites (Gentry, Barker, & Carter, 2004; Tanaka, Kelly, Goh, Lim, & Ibánez, 2016; Verdi et al., 1994). Due to its lack of intercellular catalytic activity, for many years it was believed that p75NTR is just an adaptor protein, unable for signal transduction (M. S. Chang, Arevalo, & Chao, 2004; Conroy & Coulson, 2022; Barbara L Hempstead, 2002). Nonetheless, extensive research revealed that p75NTR is indeed capable for signal transduction, as its intracellular part not only contains several protein binding sites at its N-terminal, but it also has death domain at its C-terminal (Conroy & Coulson, 2022; Liepinsh, Ilag, Otting, & Ibanez, 1997). Although trimerization or polymerization of the death domains of the tumor necrosis factor receptor family members is essential for the induction of apoptotic cascades, the data regarding p75NTR are inconclusive (Conroy & Coulson, 2022). p75NTR may interact with all four neurotrophins, with different affinity, and thus apart from its pro-apoptotic activity, p75NTR might also induce various other signal transductions, such as cell survival and proliferation, growth cone collapse and neurite outgrowth (Barker, 1998; Chen et al., 2009; F. S. Lee, Kim, Khursigara, & Chao, 2001).

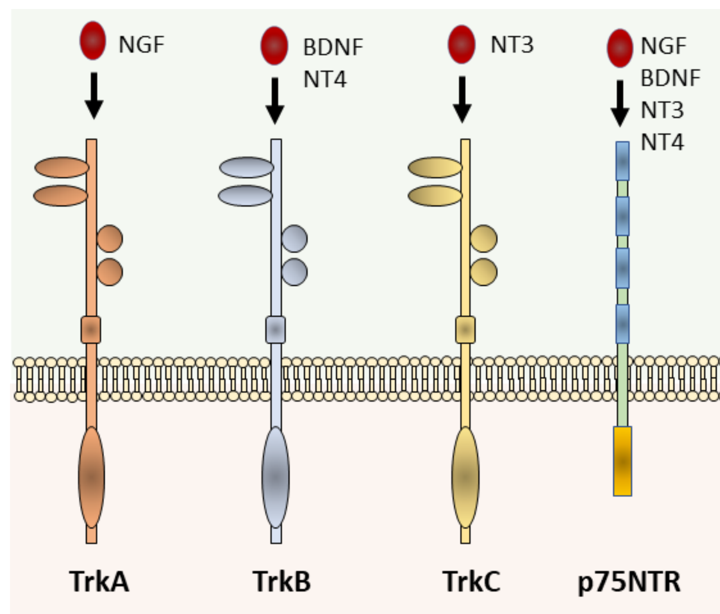

Figure 2. Neurotrophin receptors.

2.3 Neurotrophin agonists and antagonists

The dysregulation expression of both neurotrophins and their receptors is a common characteristic among several neurological disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's

disease, amyotrophic lateral sclerosis and others (Josephy-Hernandez, Jmaeff, Pirvulescu, Aboulkassim, & Saragovi, 2017). Since the interaction of neurotrophins with their Trk receptors results to neuronal survival, development and proliferation, the development of several agonists has been proposed as potential therapeutic strategies for several of the aforementioned disorders (Josephy-Hernandez et al., 2017). On the other hand, since p75NTR activation usually results in apoptosis initiation, the use of antagonists has been also exploited as potential therapy (Saragovi & Gehring, 2000; Saragovi, Hamel, & Di Polo, 2009).

The exogenous administration of neurotrophins was an approach that failed to bring positive results, mainly due to the poor pharmacokinetics and pharmacodynamics of these proteins, but also possibly due to their unintended interaction with the p75NTR. Thus, the development of smaller molecules acting either as agonists or antagonists could be the solution to the aforementioned clinical failures (Peleshok & Saragovi, 2006). Over the years, many small molecules have been developed that could have structural or functional mimicry, modulate downstream pathways, increase the endogenous production of neurotrophins or transactive the Trk receptors through other pathways (Josephy-Hernandez et al., 2017).

Regarding TrkA, Idebenone was a small agonist developed for Alzheimer's disease and although it showed promising results during the preclinical phases (Yamada et al., 1997), it failed to show any improvement during clinical trials (Gutzmann & Hadler, 1998; Orsucci, Mancuso, Ienco, LoGerfo, & Siciliano, 2011; Thal et al., 2003). On the other hand, D3, a partial agonist for TrkA, has been proven to promote dorsal root ganglion neuronal survival and differentiation (Maliartchouk et al., 2000), and to also improve the short- and long-term memory in animal models (Aboulkassim et al., 2011; Bruno et al., 2004). Gambogic acid and its derivatives, gambogic amide, dimethyl-gambogic acid and dihydro-gambogic acid, have also been reported to act as TrkA agonists, and *in vitro* and *in vivo* data suggest that they can promote survival of hippocampal cells and protect them against apoptosis (Jang et al., 2007; Obianyo & Ye, 2013). BNN27 is a small molecule and C17-spiroepoxide derivative of the neurosteroid dehydroepiandrosterone, and previous studies have reported that through its interaction with TrkA, it can protect sympathetic, sensory and cerebellar granule neurons from apoptosis (Pediaditakis, Efstathopoulos, et al., 2016; Pediaditakis, Kourgiantaki, et al., 2016), and it can also have a therapeutic effect in diabetic retinopathy (Ibán-Arias et al., 2017). ENT-A010 and ENT-A013 are two NGF-mimetic molecules, which have been observed to exert their neuroprotective effects through their interaction with TrkA (Rogdakis et al., 2022; Yilmaz et al., 2022).

As for TrkB, several agonists have been developed from various natural products. The Paecilomycine A's derivatives have been reported to interact with TrkB, resulting in the *in vitro* promotion of neuronal differentiation and the *in vivo* induction of neurogenesis (Chakravarty et al., 2015). Similarly, Gedunin's derivative, deoxygedunin has been shown to promote neuronal survival *in vitro* and to enhance memory consolidation *in vivo* (Jang et al., 2010). 7,8-dihydroxyflavone (7,8-DHF) is a flavonoid derivative with promising results, as several studies have reported its neuroprotective effects (Bollen et al., 2013; Chitranshi, Gupta, Kumar, & Graham, 2015; Devi & Ohno, 2012; Z. Zhang et al., 2014). BNN-20, a synthetic microneurotrophin that binds to TrkB, has been shown to have a therapeutic effect in animal models of Parkinson's Disease, as it restored the impaired levels of BDNF, increased the dopamine levels and improved the overall motor activity (Panagiotakopoulou et al., 2020). In another study, BNN-20 was also observed to increase the number of mature oligodendrocytes and to prevent astrocytic accumulation, but this time, its beneficial effects were accomplished through its interaction with TrkA (Kalafatakis, Patellis, Charalampopoulos, Gravanis, & Karagogeos, 2021).

Finally, regarding p75NTR, several small molecules have been synthesized to bind to this receptor and exert possible positive effects. One such is the LM11A-31 that previous studies have reported to inhibit Aβ mediated death *in vitro* and to improve recognition and spatial memory *in vivo* (Knowles et al., 2013; Yang et al., 2008). Similarly, LM11A-24, another small molecule, has been shown to offer neuroprotection and memory improvement *in vivo* (Nguyen et al., 2014).

2.4 Involvement of Neurotrophins and their Receptors in Gut

Although the role and implication of neurotrophins and their receptors have been mainly studied in neurodegenerative disorders, there are a few data suggesting that they may be also involved in gut physiology. Increased expression of NGF and BDNF has been associated with Irritable Bowel Syndrome (IBS), and inhibition of 5-hydroxytryptamine receptor has been shown to reduce visceral hypersensitivity (W. Y. Chang et al., 2022). In another study, increased levels of proBDNF/p75NTR have been associated with depression and gastrointestinal disorders, such as decreased motility, while treatment with fluoxetine improved both neurological and intestinal functions (Yu et al., 2020). Dehydroepiandrosterone and NGF have been shown to promote the survival of colon cancer cells, while inhibition of TrkA abolished the effects of the aforementioned factors (Anagnostopoulou et al., 2013). In the same notion, Genevois et al. showed that reduced expression of TrkC receptor has been observed in colon cancers and its overexpression can lead to the induction of apoptosis in colonic

cancer cell lines (Genevois et al., 2013). Thus, further research on the role of neurotrophins and their receptors in colonic inflammation, fibrosis and cancer development is crucial.

2.5 Inflammatory Bowel Diseases

The Inflammatory Bowel Diseases (IBDs) are characterized by chronic intestinal inflammation of unknown cause, and include Crohn's Disease (CD) and Ulcerative Colitis (UC) (J. T. Chang, 2020). Crohn's Disease can affect the entire gastrointestinal tract, with the most common disease location being the terminal ileum, whereas Ulcerative Colitis is limited to the large intestine. Regarding their clinical manifestations, CD is accompanied by abdominal pain, diarrhea and weight loss, while UC by more generalized symptoms, such as diarrhea and blood loss (Flynn & Eisenstein, 2019). One of the characteristics of CD is the development of transmural intestinal fibrosis which in some cases can lead to the creation of strictures, a serious complication that often requires surgical intervention (Y. Hayashi & Nakase, 2022).

Regarding the prevalence of IBD, it has been observed that during the 21st century there has been a significant increase of their occurrence in industrialized countries (Flynn & Eisenstein, 2019). It is estimated that approximately 2 million people suffer from IBD in Europe and their incidence has increased by 0.3% during the last decades (Ng et al., 2017; Zhao, Gönczi, Lakatos, & Burisch, 2021). Although small, this increase implies an upscale in the cost and time of hospitalization and treatment, as IBDs are chronic diseases that often require long periods of hospitalization, while their treatments are often expensive (Ng et al., 2017). Therefore, it is highly necessary to elucidate their etiopathogenetic mechanisms, in order to achieve more targeted and low-cost treatments.

Regarding their etiology, IBDs are characterized as idiopathic, as many pathogenetic factors are involved and their etiology still remains unknown. Among the various factors that have been correlated with IBD's development are the genetic background, the immune system, the microbiome, as well as the environment (Flynn & Eisenstein, 2019). Regarding the genetic background, specific genes have been associated with an increased likelihood to CD or UC susceptibility, such as NOD2 for CD and NFKB1 for UC (Fritz, Niederreiter, Adolph, Blumberg, & Kaser, 2011; R. Hayashi et al., 2012). Twin studies have shown increased concordance rates for both CD and UC; for CD the rate can be almost at 60% in monozygotic twins (Xavier & Podolsky, 2007), while first-degree relatives of individuals with IBD have a 5-fold increased possibility that they will also develop IBD (Ramos & Papadakis, 2019). Regarding the immune system, CD is characterized by Th1 and Th17 immune

responses, with increased expression of IL-17, IFN-γ and TNF-α, while UC is characterized by Th2 immune response, with increased expression of IL-5 and IL-13 (Flynn & Eisenstein, 2019). Regarding the microbiome, studies so far have shown that specific microbial populations are either increased in IBD, such as Proteobacteria and Bacteroidetes, or reduced and non-existent, such as Firmicutes (Frank et al., 2007). Finally, as far the environment is concerned, the increased intake of saturated fats, the reduced consumption of plant fibers and the excessive, in several cases, use of antibiotics have shown to contribute to an increased likelihood to IBD susceptibility (Flynn & Eisenstein, 2019).

2.5.1. Crohn's Disease

Crohn's Disease, as already mentioned, is characterized by Th1 and Th17 immune responses, with over-secretion of specific cytokines, such as IFN-γ, IL-17 and others (D. H. Kim & Cheon, 2017), and it can affect any part of the gastrointestinal tract. Endoscopically, the CD's affected intestinal tissue may present with ulcerated areas that alternate with healthy mucosa, while histologically, it is characterized by transmural inflammation and accumulation of granulomas and lymphocytes. Initially, CD was named as terminal ileitis, as its more frequent localization is the small intestine, with 30% of patients showing ulcerations mainly in the terminal ileum (Randall et al., 2015).

The main symptoms of CD include abdominal pain, diarrhea, bleeding, weight loss, fever and fatigue (Randall et al., 2015), while, as already mentioned before, another characteristic is the development of transmural fibrosis and intestinal strictures (Strober, Fuss, & Mannon, 2007). In addition, some patients develop fistulas either between segments of the gut, or between the gut and the skin or other organs (Strober et al., 2007).

Previous studies have indicated several genes that may influence the likelihood of CD development (**Table 1**) (Flynn & Eisenstein, 2019), with NOD2 being the most well-studied (Campbell, Williams, Crompton, Cruickshank, & Hardman, 2013; Guerra et al., 2012; Hugot et al., 2001; Ogura et al., 2001). NOD2 encodes the synthesis of a cytoplasmic protein, which is expressed in antigen-presenting cells, macrophages and intestinal epithelial cells and most likely functions as a receptor for bacterial lipopolysaccharide, modulating the pro-inflammatory Nuclear Factor kappa B (NF-κb) and apoptosis in these cells (Abraham & Cho, 2006; Economou, Trikalinos, Loizou, Tsianos, & Ioannidis, 2004).

CD-related Genes	PTPN22, ADAM30, UCN, FASLG, TNFSF18, SP140, ATG16L1, IL6ST, IL31RA , CPEB4, TAGAP, CEB5, JAZF1, RIPK2, LACC1, RASGRP1, SPRED1, NOD2, LGALS9, NOS2, GPX4, FUT2, HMHA1, IFNGR2, IFNAR1
UC-related Genes	TNFRSF14, RFTN2, PLCL1, PRKCD, ITIH4, NFKB1, MANBA, SLC9A3, CARD11, GNA12, DLD, IRF5, JRKL, MAML2, FAM55A, FAM55D, ITGAL, ZFP90, CALM3, ADA, HNF4A
IBD-associated Genes	TNFRSF18, TNFRSF4, TNFRSF9, IL23R, RORC, CD48, FCGR2A/B, FCGR3A, C1orf53, KIF21B, IL10, ADCY3, FOSL2, BRE, REL, SPRED2, IL18RAP, IL1R1, IFIH1, IL18RAP, IL1R1, GPR35, MST1, PFKB4, IL2, IL21, DAP, PTGER4, ERAP2, ERAP1, IBD5 locus, SPRY4, NDFIP1, IRGM, IL12B, DOK3, TRAF3IP2, TNFAIP3, PHACTR2, CCR6, RPS6KA2, ZPBP, IKZF1, SMURF1, EPO, TRIB1, JAK2, NFIL3, TNFSF15, CARD9, IL2RA, IL15RA, MAP3K8, CREM, CISD1, IPMK, TSPAN14, C10orf58, NKX2-3, TNNI2, LSP1, CNTF, LPXN, CD6, RELA, CCDC88B, CXCR5, MUC19, LOH12CR1, VDR, IFNG, SMAD3, GPR183, GPR18, ZFP36L1, FOS, MLH3, GPR65, GALC, CRTC3, SOCS1, LITAF, PRKCB, IL27, IRF8, CCL13,CCL2, ORMDL3, STAT3, TUBD1, RPS6KB1, SMAD7, CD226, TYK2, CEBPG, HCK, CD40, DNMT3B, CEBPB, ZNF831, CTSZ, ICOSLG, TNFRSF6B, LIF, OSM

Table 1. Genes associated with an increased likelihood of IBD development.

Furthermore, CD can be characterized by a dysregulation of the immune system, which may be due to the activation and overproliferation of T effective cell populations (Th1 and Th17) or due to the reduction of T regulatory populations, which aim to suppress immune responses (Strober et al., 2007).

Finally, it has been observed that the disruption of the normal host-microbe relationship, through changes in the composition of the normal microflora and/or through pathological immune responses against it, can ultimately lead to a state of dysbiosis. As already mentioned, previous studies on the composition of the microbiome of patients with CD have highlighted its reduced diversity, with increased percentages of Bacteroidetes and Proteobacteria and decreased Firmicutes (Andoh & Nishida, 2023; Rapozo, Bernardazzi, & de Souza, 2017).

2.5.2. Ulcerative Colitis

One of the characteristics of Ulcerative Colitis, which distinguishes it from CD, is the fact that UC affects only the large intestine and can be limited either to a single part of the large intestine, or extent to the entire colon (Ungaro, Mehandru, Allen, Peyrin-Biroulet, & Colombel, 2017). A second feature of UC is the type of inflammation, which is continuous and limited only to the mucosal layer (Bernstein, Wajda, & Blanchard, 2005; Di, Biancheri, Rovedatti, Macdonald, & Corazza, 2012; I.J. Fuss et al., 2004). Endoscopically, patients suffering from UC may present with erythema and friability of the mucosa, and loss of the normal vascular network (Ungaro et al., 2017), while histologically, characteristic findings are the accumulation of lymphocytes and granulocytes, the loss of goblet cells, as well as the creation of superficial ulcers and cryptic abscesses. As already mentioned, the symptoms of UC are more generalized and these include diarrhea, blood loss, while in some cases, loss of intestinal peristaltic function can be observed, leading to rigidity or even "toxic megacolon" and intestinal perforation (Bouma & Strober, 2003; Ordas, Eckmann, Talamini, Baumgart, & Sandborn, 2012).

Similar with CD, the pathogenetic factors of UC may include the genetic component, the immune system, the gut microbiota, and the environment. As for the genetic component, it seems to have a less important role than CD, as previous studies showed that the percentage of concordance between monozygotic twins reaches only 16% (Ananthakrishnan, 2015). Nevertheless, several genes have been identified that are associated with a possibility of its occurrence (**Table 1**), such as the previously mentioned NFKB1, but also others that may be involved in various mechanisms, such as in the regulation of the immune system (IL-10, ARPC2) and in the function of the intestinal epithelial barrier (HNF4A, CDH1) (Ungaro et al., 2017).

As already mentioned, UC is mainly characterized by a Th2 immune response (Bamias, Kaltsa, & Ladas, 2011; Fuss et al., 1996; Mannon & Reinisch, 2012), while more recent studies mention additional populations of both the effective, such as Th17, Th9 and T regulatory (Treg) immune response (Guan & Zhang, 2017; D. H. Kim & Cheon, 2017; Silva, Rodrigues, Ayrizono, & Leal, 2016), as well as the innate immunity, such as lymphoid cells (innate lymphoid cells - ILCs) (Ungaro et al., 2017), which are involved in its pathogenesis.

Moreover, differences in the composition of intestinal microbiota between normal individuals and UC patients are characteristic, but are nevertheless observed to a lesser extent compared to CD (Rapozo et al., 2017). Finally, the use of non- steroidal anti-inflammatory drugs can modify the intestinal epithelial barrier and lead to an exacerbation of the disease, while on the contrary, some

epidemiological studies have shown that smoking has a beneficial effect in the case of UC, contributing in keeping patients in remission (Bastida & Beltran, 2011; Cosnes, Beaugerie, Carbonnel, & Gendre, 2001; Lindberg, Tysk, Andersson, & Jarnerot, 1988).

2.5.3. Immune mechanisms

Environmental stimuli can often cause disturbances to the immune mechanisms of patients with genetic predisposition to IBD development (Y. Z. Zhang & Li, 2014). IBDs are characterized by insufficient healing of the intestinal mucosal tissue, due to extensive and persistent inflammation and the prolonged presence of pro-inflammatory factors and components of the extracellular matrix (Rieder, Brenmoehl, Leeb, Schölmerich, & Rogler, 2007; Valatas, Filidou, Drygiannakis, & Kolios, 2017). These inflammatory responses are the result of the incorrect overactivation of the immune system of genetically susceptible individuals, usually responding to changes in the balance of the intestinal microflora (Strober et al., 2007). Therefore, in individuals with a genetic predisposition to develop IBD, an infection with a microorganism capable of penetrating the mucosal layer may be sufficient to activate the immune mechanisms of innate immunity at the expense of anti-inflammatory Treg cells (Hansen (Hansen, 2015; Lord, 2015; Segal, 2019).

The pro-inflammatory immune pathways involved in Crohn's Disease and Ulcerative Colitis significantly differ. In CD, there is a distinguished activation of the Th1 and Th17 immune responses, with increased production of cytokines, such as TNF -α, IFN -γ, IL -1β, IL -6, IL -17, IL -12 , IL -23 (Brand, 2009; Petagna et al., 2020; Siakavellas & Bamias, 2012). Also, following an infection, there may be reduced secretion of the cytokines that regulate neutrophil migration (Segal, 2018, 2019), otherwise capable of mitigating the granulomatous inflammation caused by the exogenous pathogens (Kienle & Lämmermann, 2016). On the other hand, the dysfunctional immune mechanisms that characterize UC are Th2 immune responses, with increased leukocyte migration, due to possible defective antigen presentation of macrophages bearing a mutation of the TLR4 gene (Rahman et al., 2010), as well as increased production of IL-5, IL-13 and CXCL10 from Natural Killer cells (NK) and reduced production of IFN-γ (I. J. Fuss et al., 2004).

In addition, new developments in IBD research show that the sub-phenotypes of Crohn 's Disease do not depend exclusively on the differentiation of specific T lymphocyte types, but rather on the balance between Th1, Th2, Th17 and Treg immune responses (Leppkes & Neurath, 2020). The differentiation of T lymphocytes into Th1, Th2, Th17 and Treg is directly dependent on the regulatory capacity of the

secreted proteins of the TGF-β family (Transforming growth factor -β, TGF -β1, -β2, -β3), which can lead to both pro-inflammatory and anti-inflammatory reactions, depending on the environment they are in (Travis & Sheppard, 2014; Zhou et al., 2007). This cytokine has a critical role in pathways of growth, cell migration and differentiation, epithelial-mesenchymal transition, and under normal conditions, it contributes to the healing of the mucous membrane (Leask & Abraham, 2004), while its prolonged secretion leads to inflammatory conditions (Jacob, Targan, & Shih, 2016; McEntee, Gunaltay, & Travis, 2020) and fibrosis, through overproduction of extracellular matrix components, including collagen (Tzavlaki & Moustakas, 2020; Wells, 2000).

2.6. *In vitro* and *ex vivo* disease models

2.6.1. Human Intestinal Subepithelial Myofibroblasts

Depending on the life stage and the microenvironment, different types of SEMFs may be observed. In embryos, myofibroblasts are seen from week 21, and various stem cell types, such as neural crest and serological mesothelium cells, can differentiate into myofibroblasts (Powell, Pinchuk, Saada, Chen, & Mifflin, 2011). In adults, they occur mostly as a result of the differentiation of fibroblasts, which initially express β- and γ-actin, while at their final stage of differentiation they express α-SMA (Koumas, Smith, Feldon, Blumberg, & Phipps, 2003; Pinchuk, Mifflin, Saada, & Powell, 2010). In addition, epithelial and/or endothelial transition may also result in differentiated myofibroblasts (J. M. Lee, Dedhar, Kalluri, & Thompson, 2006; Stone et al., 2016). Lastly, myofibroblasts may also rise from the differentiation of bone marrow stem cells (De Wever, Demetter, Mareel, & Bracke, 2008).

Colonic subepithelial myofibroblasts (SEMFs) are found under the epithelial layer (Mifflin, Pinchuk, Saada, & Powell, 2011; Roulis & Flavell, 2016) and are necessary for the proper development of the intestinal tissue (N. Y. Lei et al., 2014). They are the second most numerous stromal cell after fibroblasts, followed by smooth muscle cells, pericytes and mesenchymal stromal cells (Owens, 2015). These cells are either spindle- or stellate-shaped, they are positive for α-SMA (Alpha Smooth Muscle Actin), non-muscle myosin, fibronectin and vimentin, while negative for smooth muscle myosin and negative or slightly positive for desmin (Eyden, 2008; Valatas et al., 2017).

Under normal conditions, possible injuries to the endothelium or the mucosa of the gastrointestinal tract may activate SEMFs (Badid, Mounier, Costa, & Desmoulière, 2000; Roulis & Flavell, 2016). Having the appropriate receptors to receive pro-fibrotic and pro-inflammatory signals from their environment

(Filidou et al., 2018a), SEMFs start migrating towards the damaged tissue and multiply, while simultaneously producing components of the extracellular matrix (ECM), such as collagen, to temporarily cover the wound surface (Powell et al., 1999; Valatas et al., 2017).

Under abnormal conditions, such as those prevailing in IBD, the extensive inflammation, due to the massive migration of T lymphocytes, neutrophils and macrophages (Rieder & Fiocchi, 2009), may lead to pathological responses by myofibroblasts (Filidou et al., 2018a). In pathological conditions, myofibroblasts are unable to seize proliferating once the damaged tissue is restored, and instead are constantly activated, overproducing collagen, and thus resulting in fibrosis (Latella et al., 2014; Lawrance et al., 2017; Rieder et al., 2007; Roulis & Flavell, 2016). Furthermore, it has also been shown *in vitro* that when SEMFS are stimulated with the cytokines of the Th1 immune responses, IFN-γ and TNF-α, or with the cytokines of the Th17 immune responses, IL-17, IL-22, and IL-23, there is an increased secretion of collagen and fibronectin compared to unstimulated SEMFs (Filidou et al., 2018a). In addition to their inability to deactivate, the inhibition of apoptosis and collagen degradation may also contribute to the creation of fibrosis (Mortensen et al., 2019; Petrey & de la Motte, 2017; Sanders et al., 2015).

Additionally, it has been shown that SEMFs are able to produce various pro-inflammatory cytokines (Reinecker et al., 1993; Valatas et al., 2017). Upon dose-dependent stimulation with IL-1β and TNF-α, SEMFs express increased amounts of IL-8, Monocyte Chemoattractant Protein 1 (MCP-1) and Matrix Metalloproteinase 1 (MMP-1) (Okuno et al., 2002). In the presence of IL-1α and TNF-α, SEMFs produce increased amounts of Tumor Necrosis Factor - like cytokine 1 A (TL1A) and Death - domain receptors Receptor 3 (DR3) and Decoy Receptor 3 (DcR3) (Bamias et al., 2017). After the addition of IL-17, with and without the simultaneous administration of either IL-1β or TNF-α, SEMFs overexpress the proinflammatory cytokines IL-6 and IL-8, as well as MCP-1 and NFkB1 (Hata et al., 2002). Finally, administration of IL-1α, TNF-α, and IFN-γ to epithelial cells causes an increase in TGF-β and TIMP-1 production, and the use of supernatants from these cells to stimulate SEMFs, results in increased production of collagen and MMP-9 (Drygiannakis et al., 2013).

In summary, when SEMFs are in a microenvironment, consistent with the conditions observed in individuals with IBD, the expression of factors that exacerbate these inflammatory conditions is enhanced. Thus, due to the central role SEMFs have in fibrotic processes and inflammatory immune responses, selective inhibition of their differentiation or the production of profibrotic components could be promising drug targets (Cosin-Roger et al., 2019; Mifflin et al., 2011; Osterman, 2013).

2.6.2. Organoids

The need to find either suitable tools for the study of organ development (organ development), or alternative *in vitro* models - beyond classical cell lines - and more accessible sources for transplantation, led the scientific community to the discovery of organoids. Organoids are three-dimensional (3D) biological structures, usually composed of many types of cells, that simulate an organ and some of its functions. The beginning of this technology can be attributed to JG Rheinwald and H.Green, who in the 70s developed stratified squamous epithelial colonies (Rheinwald & Green, 1975), which provided a basis for the creation of three-dimensional *in vitro* structures. In the following years, advances in technology, stem cells, and materials science, contributed significantly to establishing organoids as one of the dominant *in vitro* models. Nature magazine named organoids development as Method of the year 2017 ("Method of the Year 2017: Organoids," 2018). For their development, either pluripotent stem cells (embryonic, iPSC) or pluripotent adult stem cells (Adult Stem Cells) are required (Clevers, 2016; Rookmaaker, Schutgens, Verhaar, & Clevers, 2015). Organoids can be cultured and grown/multiplied rapidly, cryopreserved, genetically modified by the usual methods used in molecular biology (Drost et al., 2017), and used in high-throughput analyses (high-throughput) (Li, Tang, Cai, Peng, & Hua, 2020). Therefore, organoids are one of biomedicine's most successful attempts to bridge the gap between classical cell cultures and animal models, and they are a very useful tool with the potential to overcome many of the hurdles that classical models could not up until now (J. Kim, Koo, & Knoblich, 2020). The process of developing organoids simulates organogenesis and mostly involves using specific culture materials, which contain growth factors and chemical substances. To create the three-dimensional structure and provide the extracellular matrix (ExtraCellular Matrix) organoids need, a specific gel, Matrigel, is usually used, which derives from Englebreth-Holm-Swarm (EHS) muscle cancer cells (Hughes, Postovit, & Lajoie, 2010). Finally, scientists are now able to develop organoids that simulate organs from all three layers of origin, namely the ectoderm, the mesoderm, and the endoderm (He et al., 2020).

2.6.2.1 Human Intestinal Organoids

The development of Human Intestinal Organoids (HIOs) in 2011 was a milestone in intestinal mucosal research (Spence et al., 2011). HIOs are an innovative *in vitro* models for studying epithelial and mesenchymal cells, as well as their interactions (Spence et al., 2011). HIOs are three-dimensional structures developed from pluripotent stem cells, through a process that mimics organogenesis. They have a similar architecture to intestinal tissue, as their lumen is surrounded by epithelial cells that

form villi and crypts, which are further supported by an outer layer of mesenchymal cells. Therefore, the use of HIOs as *in vitro* models for studying intestinal inflammation and fibrosis offers more advantages than classical two-dimensional *in vitro* models, as HIOs are composed of many different types of epithelial and mesenchymal cells that interact with each other. Although HIOs still lack vascular, neurological or immunological structures, compared to animal models of IBD, yet they provide significant advantages in studying both mesenchymal and epithelial physiology in immune responses (Tsuruta, Uchida, & Akutsu, 2020).

Despite their increasing use to study less complex diseases, intestinal organoids have rarely been used to study multifactorial diseases, such as IBD. Recent studies have shown that intestinal organoids continue to mature and change throughout their culture, mimicking the process of development from the embryonic to adult stage (Kraiczy et al., 2019; Lewis et al., 2020; van der Hee, Madsen, Vervoort, Smidt, & Wells, 2020). Therefore, knowing the appropriate time during their culture period to study inflammatory and fibrotic responses that closely mimic the progression of IBD is crucial to use as an effective *in vitro* study model. Indeed, in a recent publication from our research team, we showed that HIOs from early culture passages are ideal for studying intestinal inflammation and fibrosis (Kandilogiannakis et al., 2021).

2.6.2.2. Colonoids

Another category of organoids are Colonoids (or Enteroids) which in recent years, due to certain advantages they possess, are used more and more in research. Colonoids are three-dimensional intestinal structures that are developed from intestinal crypts, which contain adult stem cells, and are composed only of different types of epithelial cells and no mesenchymal cells (Ranganathan, Smith, Foulke-Abel, & Barry, 2020; Zachos et al., 2016).

The development of Colonoids requires the isolation and fragmentation of the intestinal crypts, in order to release the epithelial stem cells located at the base of the crypt, and then culturing in a special gel (Matrigel) in the presence of specific growth factors, such as Wnt3a, R-spondin and Noggin (Sato & Clevers, 2013; Sato et al., 2011; Sato et al., 2009). These growth factors favor the differentiation of epithelial stem cells, which are $Lgr5^+$, into complex structures simulating either the small or large intestine, depending on the site collection of the original tissue (small or large intestine) (Stelzner et al., 2012). During the early stages of their differentiation, 3D spheroidal structures are generated that possess polarity, as their apical surface forms an internal lumen, while their basal surface contacts the

Matrigel. In this phase, the spheroid structures are mainly composed of Paneth cells, enteroendocrine cells, enterocytes and goblet cells, as well as $Lgr5^{+}$ stem cells, which support the self-renewal and further maturation of these structures. During their final differentiation and maturation, spheroid colonoids acquire complex structures composed of villi, with mature enteroendocrine cells, enterocytes and goblet cells, but with the absence of $Lgr5^{+}$ stem cells (Zachos et al., 2016).

Therefore, the main advantage of Colonoids is their comparatively easier, faster and less expensive development, compared to Human Intestinal Organoids, and for these reasons the former are often preferred in research protocols (Zachos et al., 2016). A second advantage is the possibility to develop and study Colonoids derived not only from normal individuals, but also from patients with any intestinal disease, such as Crohn's Disease or Ulcerative Colitis. The development and study of such structures from patients with enteropathies enables researchers to study both the etiopathogenetic mechanisms of the epithelium, as well as the effect of potential new drugs on a specific disease and individual, and therefore, Colonoids could particularly contribute in Personalized Medicine and Precision Medicine (George, Rahman, Connors, & Stadnyk, 2019).

However, the main disadvantage of Colonoids is the fact that they consist of only one type of cell, the epithelial one. In addition, the simulation of an organ requires conditions as close as possible to reality and in this field, Human Intestinal Organoids excel, as in addition to the different types of epithelial cells, they are also composed of mesenchymal cells, offering the possibility for complex studies of interaction between epithelial and mesenchymal cells (Singh, Poling, Spence, Wells, & Helmrath, 2020).

Chapter 3: Research methodology

3.1. Aim of the Study

Our aim was to investigate the expression of the neurotrophin receptors TrkA, TrkB and p75NTR in *in vitro* and ex vivo intestinal inflammation models, stimulated with the pro-inflammatory cytokines IL-1α and TNF-α, in order to identify disease mechanisms and possible new therapeutic targets.

3.2. Patients

Colonic tissue biopsies were endoscopically obtained from healthy individuals, without systematic autoimmune disease or malignancy, who underwent screening colonoscopy and had no pathological findings. The endoscopies were performed at the Endoscopy Department, University Hospital of Alexandroupolis, Greece. The local Research Ethics Committee approved this study, and patients gave their informed written consent before participation (Protocol number 14127/07-04-2021).

3.3. Isolation and Characterization of Human Colonic Subepithelial Myofibroblasts

Colonic subepithelial myofibroblasts (cSEMFs) were isolated from colonic tissue biopsies of healthy individuals as previously described (Filidou et al., 2018a). Briefly, the biopsies were collected in ice cold Hank's Balanced Salt Solution (HBSS; Biosera, Nuaille, France) supplemented with Ca^{2+}/Mg^{2+}, penicillin (P;100 U/mL; Biosera, Nuaille, France), streptomycin (S;100 μg/mL; Biosera, Nuaille, France), amphotericin B (A;2,5 μg/mL; Biosera, Nuaille, France) and gentamycin (G; 50 μg/mL; Biosera, Nuaille, France). After three washes in HBSS with Ca^{2+}/Mg^{2+} and three washes in HBSS without Ca^{2+}/Mg^{2+}, the biopsies were deepithelialized for 15 minutes in 1 mM dithiothreitol (DTT, Sigma-Aldrich, Darmstadt, Germany), followed by 3 half hour incubations with 1 mM Ethylene-Diaminetetraacetic Acid (EDTA, Sigma-Aldrich, Darmstadt, Germany) at 37°C. The tissues were then placed in 75cm^2 flasks containing RPMI 1640 (PAN Biotech, Aidenbach, Germany) supplemented with 10% v/v Fetal Bovine Serum (FBS; Biosera, Nuaille, France) and the aforementioned antibiotics and kept in 5% CO_2 at 37°C for 4 weeks, with the medium being changed every day for 4 days and then twice a week. Once numerous myofibroblast colonies started to form, the biopsies were removed and the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, 1.5 g/l glucose; PAN Biotech, Aidenbach, Germany) plus 10% FBS and P/S/A in 5% CO_2 at 37°C. The myofibroblast phenotype was verified with immunofluorescence microscopy as being α–smooth muscle actin (α-SMA) and vimentin positive and desmin negative using a fluorescent microscope (Leica DM2000, Leica Microsystems GmbH, Wetzlar,

Germany). All experiments were performed with cSEMFs at passages 2–6 cultured in 6-well-plates with FBS- and antibiotics-free DMEM until 95% confluence.

3.4. Development of Human Intestinal Organoids

Human Intestinal Organoids (HIOs) were developed from H1 embryonic stem cell line using the STEMdiff™ Intestinal Organoid Kit (StemCell Technologies, Vancouver, Canada), according to the manufacturer's instructions and as previously described (Kandilogiannakis et al., 2021). Briefly, H1 cells were seeded onto Matrigel-coated 24-well plates and cultured in mTeSR™1 medium (StemCell Technologies, Vancouver, Canada) until they reached the appropriate confluency. H1 cells were then cultured in Endoderm Basal medium containing Activin A and fed daily until day 3, when Definitive Endoderm (DE) was created. DE was subsequently cultured in Endoderm Basal medium containing WNT3A and Fibroblast growth factor 4 (FGF4) for another 5-6 days, until Mid-/Hindgut (MH) spheroids were released into the supernatant. MH spheroids were then collected, counted, seeded into domes made of Matrigel (Corning, New York, United States) and cultured in Intestinal Organoid Basal (IOB) medium containing epidermal growth factor (EGF) and Noggin, until HIOs were finally formed (**Figure 3**). HIOs were continuously cultured in EGF- and Noggin-supplemented IOB medium, fed every 3-4 days and passaged every 10 days at a ratio 1:3. HIOs and their intermediate developmental stages were characterized using immunofluorescence.

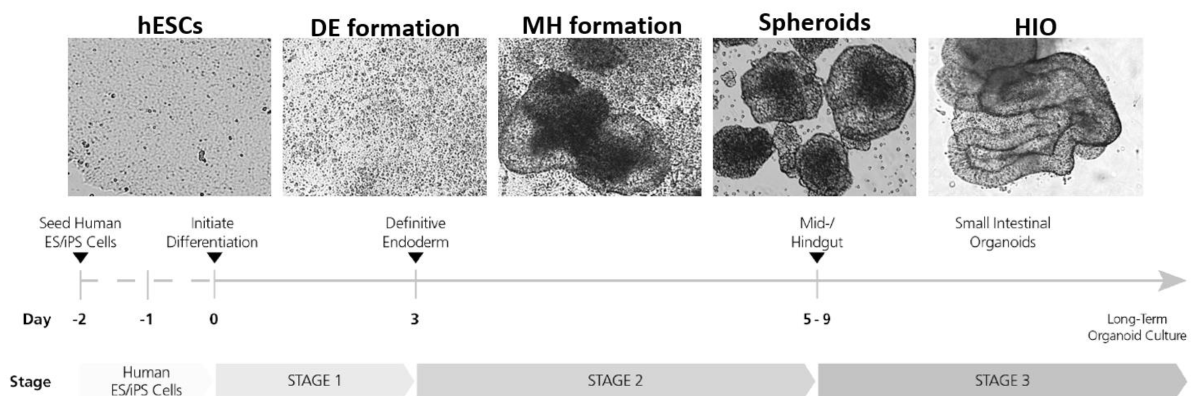

Figure 3. Stages of hESCs differentiation into Human Intestinal Organoids.

3.5. Development of Human Colonoids

Human epithelial crypts were isolated from endoscopic biopsies collected from healthy individuals, without systematic autoimmune disease or malignancy, who underwent screening colonoscopy and had no pathological findings. Human Colonoids were developed using the IntestiCult™-SF Organoid

Growth Medium (Human) (StemCell Technologies, Vancouver, Canada), according to the manufacturer's instructions (**Figure 4**). Briefly, the endoscopic biopsies were collected in ice cold Hank's Balanced Salt Solution (HBSS; Biosera, Nuaille, France) supplemented with Ca^{2+}/Mg^{2+}, penicillin (100 U/mL; Biosera, Nuaille, France), streptomycin (100 μg/mL; Biosera, Nuaille, France), amphotericin B (2,5 μg/mL; Biosera, Nuaille, France) and gentamycin (50 μg/mL; Biosera, Nuaille, France), and then washed two times with ice cold DPBS (PAN Biotech, Aidenbach, Germany). The endoscopic biopsies were then centrifuged, minced tissue into the smallest fragments possible and centrifuged again. Next, the tissue fragments were incubated with Gentle Cell Dissociation Reagent (GCDR; StemCell Technologies, Vancouver, Canada) for 30 minutes, centrifuged and passed through a 70 μm strainer. Finally, the epithelial crypts were counted, around 1000 crypts were plated into Matrigel domes and culture medium was added. Culture medium was changed every two days and by the end of day 10, Colonoids were developed and characterized using immunofluorescence.

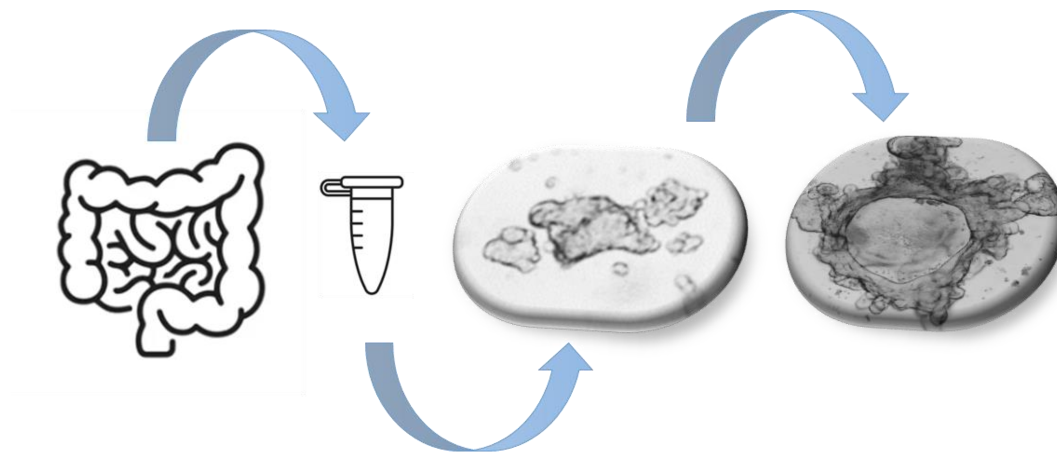

Figure 4. Development of Colonoids from tissue biopsies.

3.6. Immunofluorescence

cSEMFs, HIOs and Colonoids were characterized using immunofluorescence, as previously described (Filidou et al., 2018a). Briefly, samples were first fixed in 4% ice-cold paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, Missouri, United States) for 40 minutes, then washed in phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, Missouri, United States) and treated with 0.1% Triton-X (Sigma-Aldrich, St. Louis, Missouri, United States) for 15 minutes, in order to achieve membrane permeability. Samples were then treated with blocking solution containing 5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, Missouri, United States) for 1 hour and later incubated overnight at 4oC with primary antibodies in 0.5% BSA (Sigma-Aldrich, St. Louis, Missouri, United States). The next day, samples were washed and incubated with secondary fluorochrome-conjugated antibodies in 0.5% BSA (Sigma-

Aldrich, St. Louis, Missouri, United States) for 2 hours. Finally, nuclei were stained either with DAPI (Sigma-Aldrich, St. Louis, Missouri, United States) and observed under a fluorescent microscope (Leica DM2000, Leica Microsystems GmbH, Germany) or DRAQ5 (Novus Biologicals, Abingdon, UK) and observed in 3-dimensions under a light-sheet fluorescent microscope (Ultramicroscope II; LaVision BioTec, Bielefeld, Germany).

3.7. Treatment with Pro-inflammatory cytokines

SEMFs were cultured and prior to cytokine stimulation, they were left with no growth factors for 15h. Next, SEMFs were stimulated with 5ng/ml IL-1α and 50ng/ml TNF-α for 6h, 24h and 48h and at the end of the 6h-incubation period, SEMFs were collected for RNA extraction and mRNA expression analyses through qRT-PCR, whereas at the end of the 24h- and 48h-incumbation period, supernatants were collected and SEMFs were collected in lysis buffer for further protein analyses.

3.8. Total RNA extraction and purification

Total RNA from SEMFs was extracted using Nucleozol (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's instructions and as previously described (Tarapatzi et al., 2022). Briefly, SEMFs were first lysed and H_2O was added to each sample. Samples were vortexed, incubated and centrifuged, and their supernatants were transferred to new tubes. Isopropanol was added and samples were incubated and centrifuged in order for the RNA to be precipitated. RNA was washed twice with 75% Ethanol and later dissolved in H_2O. The concentration and purity of total RNA was measured using a Q5000 UV-Vis spectrophotometer (Quawell, San Jose, California, United States). Any DNA contamination was removed using Deoxyribonuclease I (TaKaRa, Kusatsu, Shiga, Japan) that was later EDTA- and heat-inactivated.

3.9. cDNA synthesis and real time-PCR

cDNA synthesis was performed using the PrimeScript RT Reagent Kit (Perfect Real Time) (TaKaRa, Kusatsu, Shiga, Japan) according to the manufacturer's instructions and as previously described (Kandilogiannakis et al., 2021). In brief, 250ng total RNA were mixed with 5X PrimeScript Buffer, reverse transcriptase, oligo dT primers, random hexamers and RNase Free H_2O, and incubated at 37^oC for 15 minutes. Reverse transcriptase was then inactivated by heat treatment. The gene-specific mRNA expression was quantified by quantitative real-time (qRT)-PCR using the KAPA SYBR FAST qPCR Kit (Kapa Biosystems Ltd, Boston, MA, USA), as previously described (Kandilogiannakis et al., 2021).

Briefly, 10ng of cDNA were mixed with gene-specific primers, described in **Table 2**, and the KAPA SYBR FAST qPCR mastermix, and a two-step amplification protocol was performed. All amplification reaction took place at a StepOne Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) under a cycling program consisted of 20s at 95 °C, followed by 40 cycles of 95 °C for 3s and 60 °C for 30s. The gene expression of each studied gene was normalized against Actin gene expression in the same sample using the $2^{-\Delta\Delta Ct}$ method.

Gene	Forward	Reverse
Ntrk2 (TrkB)	GGGACACCACGAACAGAAGT	CACCACAGCATAGACCGAGA
NGFR (p75)	CACCGACAACCTCATCCCTG	TGCAGCTGTTCCACCTCTTG
Actin F	CCAACCGCGAGAAGATGAC	TAGCACAGCCTGGATAGCAA
CXCL1	GCCCAAACCGAAGTCATAGCC	ATCCGCCAGCCTCTATCACA
CXCL8	TGGGTGCAGAGGGTTGTG	CAGACTAGGGTTGCCAGATTTA
CXCL10	CCTGCTTCAAATATTTCCCT	CCTTCCTGTATGTGTTTGGA
CXCL11	GACGCTGTCTTTGCATAGGC	GGATTTAGGCATCGTTGTCCTTT
CCL2	AGGAAGATCTCAGTGCAGAGG	AGTCTTCGGAGTTTGGGTTTG
CCL20	GCTGCTTTGATGTCAGTGC	GCAGTCAAAGTTGCTTGCTTC

Table 2. Primer sequences used for RT-PCR.

3.10. Western Blot

Western blotting for TrkA, TrkB and p75NTR was performed in total protein samples of lysed cSEMFs as previously described (Delivanoglou et al., 2020). Briefly, SEMFs were lysed in ice-cold lysis buffer consisting of 10 mM Hepes pH 7.4, 10 mM KCL, 0.1 mM EDTA, 10% NP-40, 0.1 mM EGTA, 1 mM DTT, and a mixture of protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin). The total protein concentration was assessed by Bradford method. Twenty micrograms of protein lysates were resolved on SDS-PAGE in 8% and 10% acrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Macherey-Nagel, GmbH&Co, Germany). The membranes were blocked with 5% BSA in TBS supplemented with 0.1% Tween-20 (P1379, Sigma) (TBST) for 1 h at room temperature (RT), followed by overnight incubation at 4°C with primary antibodies such as p75NTR (1:1000 anti-CD271, Biolegend), TrkB (1:1000 anti-TrkB, 07-225-I, Millipore), Actin (1:1000 anti-Actin beta C4, sc-47778, Santa Cruz Biotechnology) and TrkA (1:250, sc-118, Santa Cruz Biotechnology, Dallas, TX, USA). The membranes were washed three times for 10 min with TBST, incubated with the appropriate HRP-conjugated secondary antibodies secondary (anti-mouse 1:5.000, HRP-conjugated anti-mouse IgG

(Cell Signaling Technology, Leiden, The Netherlands) and anti-rabbit 1:5.000,) for 1 h at RT and then developed using the chemiluminescence reaction (ECL, GenScript, NJ, USA). All membranes were stripped and re-probed with β-actin as loading control. Image analysis and quantification of bands' density were performed with the software Fiji.

3.11. Statistics

Results are presented as means with standard error of mean (SEM). Comparison of values among sample groups was performed with ordinary one-way ANOVA. Statistical significance was set at $p < 0.05$.

Chapter 4: Results

4.1. cSEMFs express the neurotrophin receptors TrkB and p75NTR upon pro-inflammatory stimulation

cSEMFs were isolated from colonic endoscopic biopsies, cultured, and characterized using immunofluorescence as described in the Materials and Methods. Specifically, cSEMFs were found to be positive for α-SMA and vimentin expression, while negative for desmin expression (**Figure 5**).

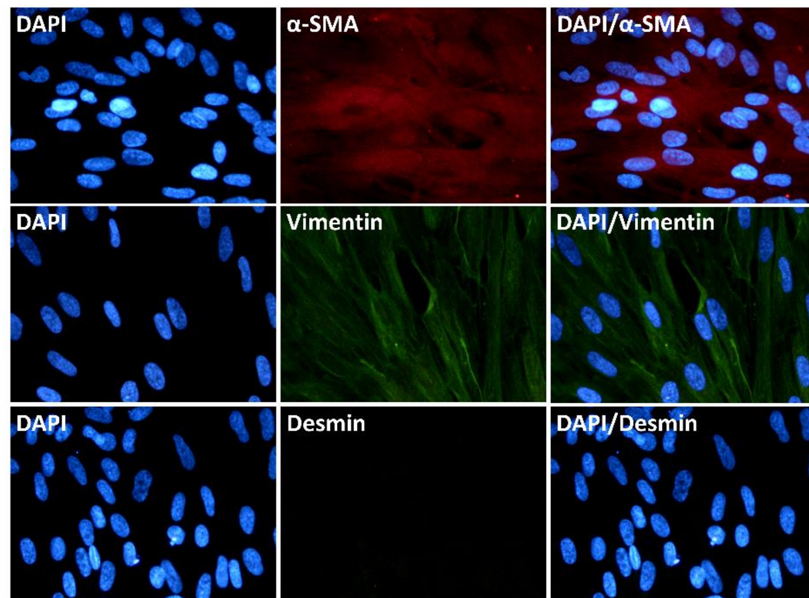

Figure 5. Characterization of cSEMFs. cSEMFs are found positive to α-SMA and Vimentin expression and negative to Desmin expression.

The cSEMFs were next seeded to 6-well plates and upon reaching 90-100% confluency, serum-free culture medium was added and cells were incubated for 15h. After 15h, cSEMFs were either stimulated or not with 5ng/ml IL-1α and 50ng/ml TNF-α for 6h, 24h and 48h; at the end of the 6h-incubation period, total RNA was extracted, while at the end of the 24h- and 48-incubation period, total protein was collected.

Regarding the mRNA expression of TrkB and p75NTR, no mRNA levels were detected in unstimulated cSEMFs. On the contrary, when cSEMFs were stimulated with 5ng/ml IL-1α and 50ng/ml TNF-α, TrkB and p75NTR mRNA expression was statistically significantly upregulated when compared to the unstimulated cells (TrkB: 3.13-fold, ±0.67, $p<0.05$; **Figure 6A**; p75NTR: 6.76-fold, ±0.18, $p<0.0001$; **Figure 6B**), suggesting that inflammatory-related mediators can induce the mRNA expression of these neurotrophin receptors.

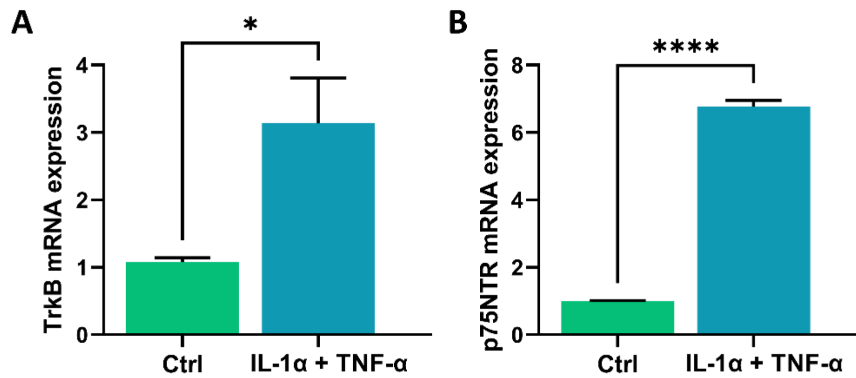

Figure 6. Pro-inflammatory cytokines induce the mRNA levels of TrkB and p75NTR in cSEMFs. (A) TrkB mRNA expression. (B) p75NTR mRNA expression.

Having observed these results on the mRNA level, we proceeded in investigating their protein expression in lysed cSEMFs. Again, unstimulated cells were lacking the expression of all neurotrophin receptors (TrkA, TrkB and p75NTR), while stimulation with 5ng/ml IL-1α and 50ng/ml TNF-α for 24 and 48h led to a significant and time-dependent increase in the protein expression of the TrkB (48h: 278.7%, ±17.0, $p<0.05$; **Figure 7A**) and p75NTR (48h: 155.8%, ±7.4, $p<0.05$; **Figure 7B**) compared to unstimulated cSEMFs.

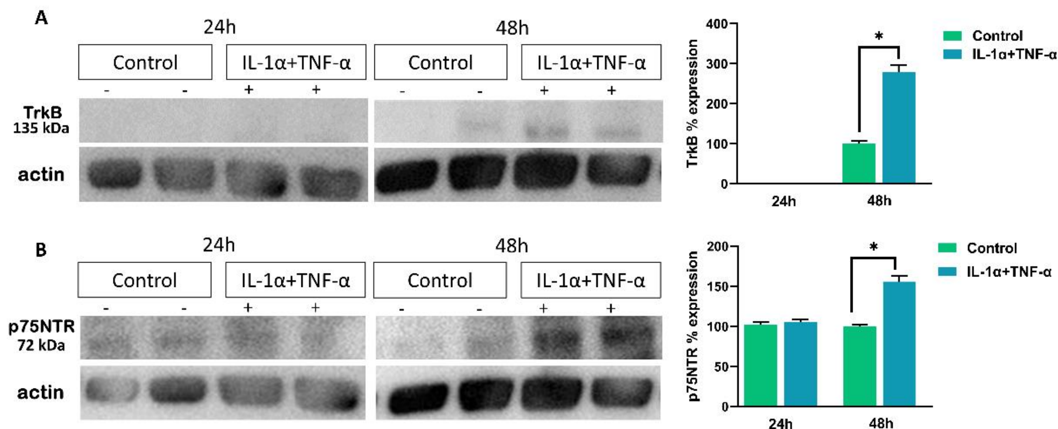

Figure 7. cSEMFs express the proteins of TrkB and p75NTR upon pro-inflammatory stimulation. (A) TrkB protein expression. (B) p75NTR protein expression.

4.2. Development of Human Intestinal Organoids and Colonoids

Having observed that the pro-inflammatory cytokines IL-1α and TNF-α induce the mRNA and protein expression of the neurotrophin receptors TrkB and p75NTR in cSEMFs which are of mesenchymal origin, we proceeded with the development of human Intestinal Organoids (HIOs) in order to further investigate their expression in a 3D system that consists of both epithelial and mesenchymal cells. In

addition, we also developed Colonoids from epithelial crypts in order to examine the expression of the aforementioned neurotrophin receptors in a 3D system that consists only of epithelial cells.

Regarding HIOs, they were developed and characterized as described in the Materials and Methods. Specifically, HIOs were successfully developed as they consisted of both mesenchymal and epithelial cells, as indicated by the positive expression of Vimentin, Desmin and E-cadherin, respectively. Regarding their epithelium, it consisted of intestinal CDX2-expressing epithelial cells, which appeared to form a compact epithelial barrier, as they intensively expressed the cell adhesion molecules Ecadherin and EpCam. In addition, the epithelial cytoskeleton was successfully developed, as abundant cytokeratin expression was observed, and various types of epithelial cells were identified, such as goblet (stained positive for MUC2) and enteroendocrine cells (stained positive for Chromogranin A). The epithelial cells were observed to form villi, as shown by their positivity for Villin, and finally, SOX9 and KLF5 positive staining revealed the presence of intestinal epithelial stem cell niches, possibly supporting the renewal of the intestinal epithelium (**Figure 8**).

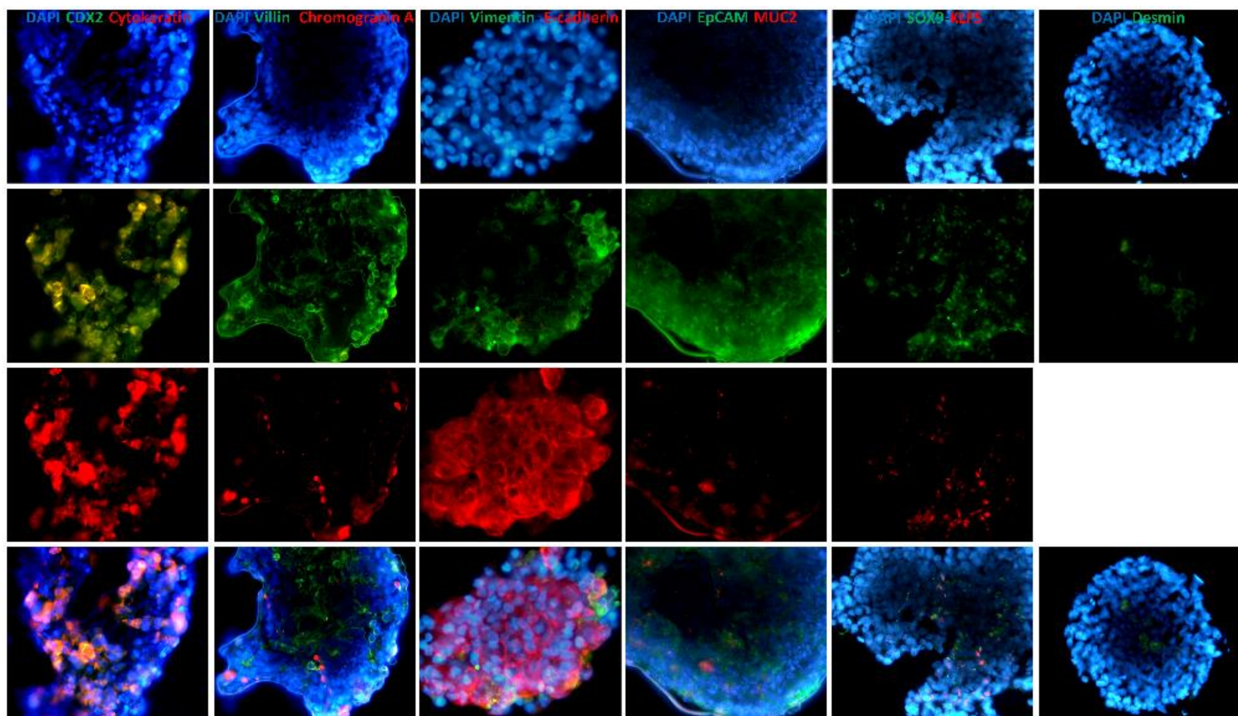

Figure 8. Characterization of HIOs. HIOs are found to express CDX2, Cytokeratin, Villin, Chromogranin A, Vimentin, E-cadherin, EpCam, MUC2, SOX9, KLF5 and Desmin.

Additionally, human Colonoids were developed and characterized as described in the Materials and Methods. Specifically, colonoids were developed from human epithelial crypts after 10 days of culture (**Figure 9**) and were later characterized using immunofluorescence (**Figure 10**). Colonoids were

successfully developed as they expressed the cell adhesion molecules Ecadherin and EpCam, and they consisted of goblet cells expressing MUC2. Their epithelial cytoskeleton was successfully developed as positive cytokeratin expression was observed and Lgr^+ epithelial stem cells were identified.

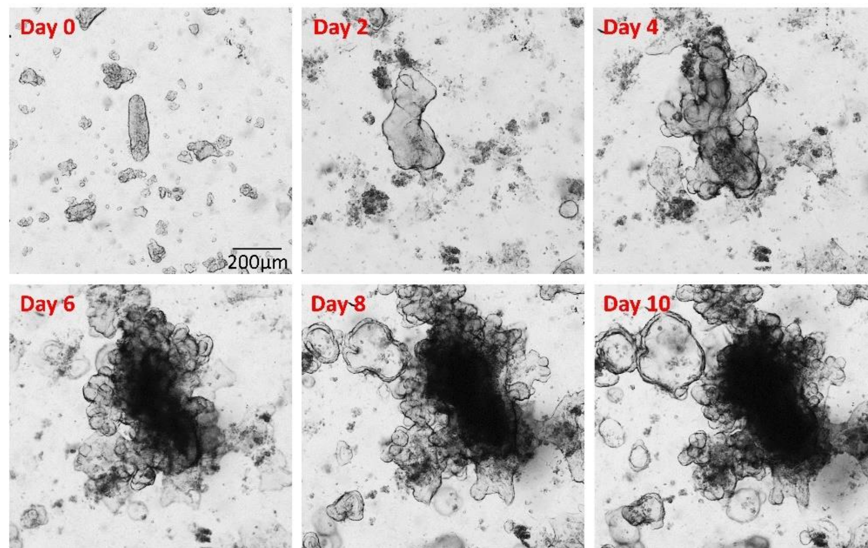

Figure 9. Development of human Colonoids from epithelial crypts.

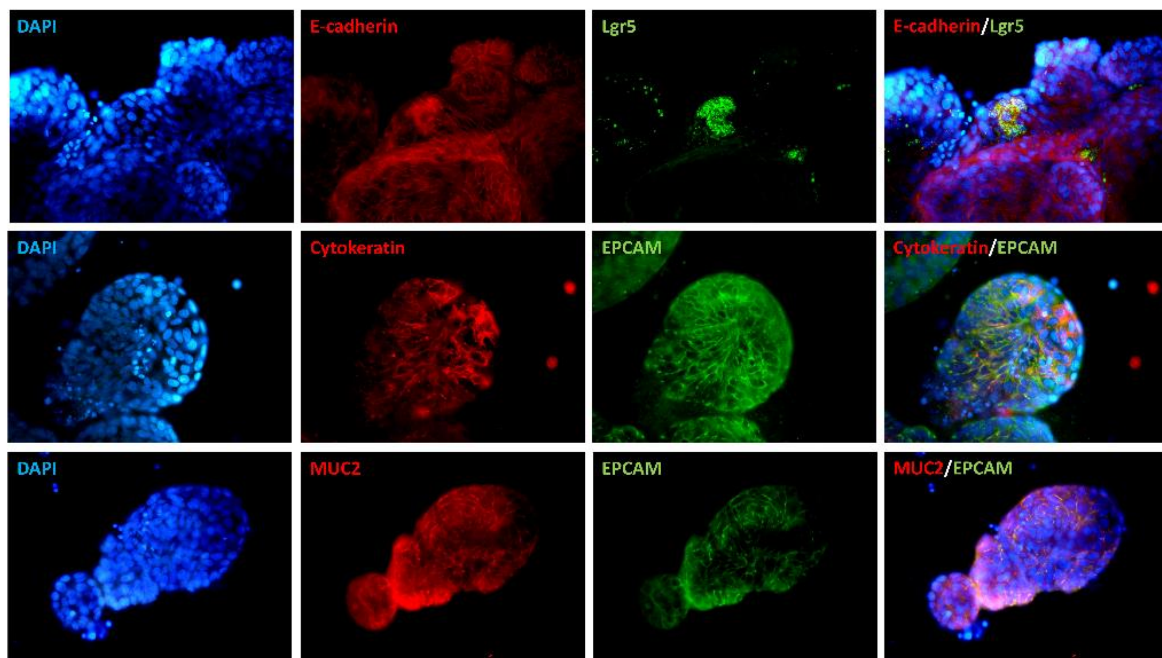

Figure 10. Characterization of human Colonoids. Colonoids were found to express E-cadherin, Lgr5, Cytokeratin, EpCam and MUC2.

After the successful development of both HIOs and Colonoids, we proceeded in performing pilot experiments by stimulating them with IL-1α and TNF-α, in order to establish an *in vitro* model of

intestinal inflammation. As shown in **Figure 11**, both HIOs and Colonoids responded in a similar way to the pro-inflammatory stimuli of IL-1α and TNF-α, by overexpressing the chemokines CXCL1 (HIOs: 22.30-fold, ±1.30, p<0.0001; Colonoids: 13.07-fold, ±2.52, p<0.05), CXCL8 (HIOs: 13.30-fold, ±1.76, p<0.001; Colonoids: 6.59-fold, ±1.20, p<0.05), CXC10 (HIOs: 235.30-fold, ±20.73, p<0.0001; Colonoids: 60.34-fold, ±7.75, p<0.01), CXC11 (HIOs: 14.53-fold, ±1.29, p<0.001; Colonoids: 7.70-fold, ±0.74, p<0.01), CCL2 (HIOs: 52.29-fold, ±2.59, p<0.0001; Colonoids: 39.40-fold, ±5.86, p<0.01) and CCL20 (HIOs: 23.86-fold, ±2.43, p<0.001; Colonoids: 534.80-fold, ±41.73, p<0.01), compared to unstimulated controls. These results indicate that the 3D *in vitro* and *ex vivo* models of intestinal inflammation were successfully developed and could be employed in the investigation of possible pathogenetic mechanisms or pharmacological targets.

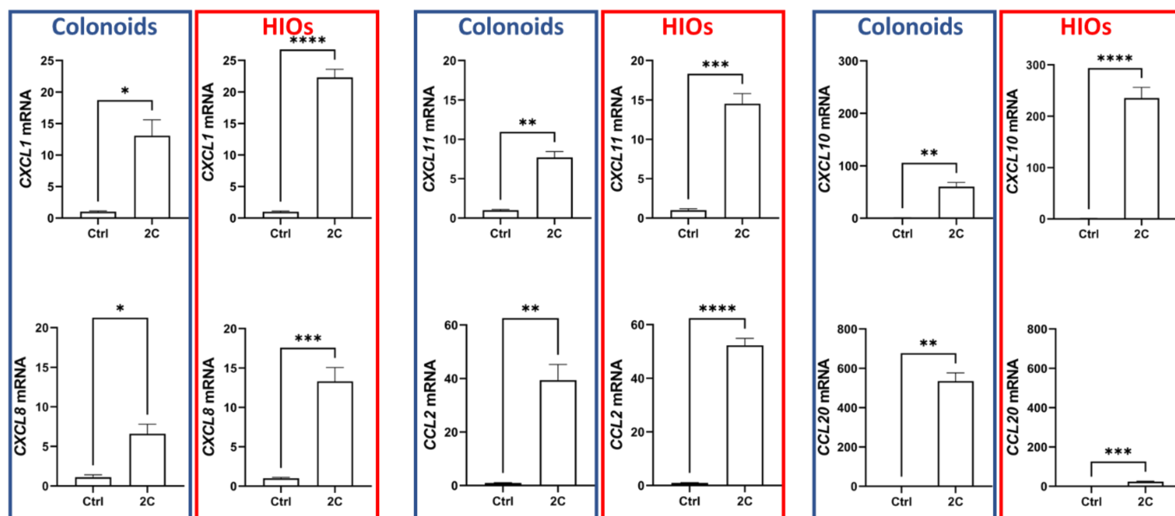

Figure 11. HIOs and Colonoids respond to pro-inflammatory stimuli by overexpressing various chemokines.

Next, HIOs and Colonoids will be stimulated or not with 5ng/ml IL-1α and 50ng/ml TNF-α for 12h, 24h and 48h; at the end of the 12h-incubation period, total RNA will be extracted, while at the end of the 24h- and 48-incubation period, total protein will be collected. The mRNA and protein expression of TrkB and p75NTR will be subsequently studied using real-time PCR and Western Blot, respectively. Due to time limitation of the present thesis, these experiments are still ongoing.

Chapter 5: Discussion

This Master's Thesis reports for the first time that the neurotrophin receptors, TrkB and p75NTR, are expressed in intestinal subepithelial myofibroblasts upon stimulation with the pro-inflammatory cytokines IL-1α and TNF-α. In addition, it is also shown that the development of 3D Human Intestinal Organoids and Colonoids can be used as *in vitro* models of studying intestinal inflammation, and thus further investigate the role of neurotrophin receptors in gut physiology.

Specifically, our results indicate that the pro-inflammatory cytokines IL-1α and TNF-α induce a time-dependent upregulation of TrkB and p75NTR expression in cSEMFs and that the overexpression is more prominent at the 48h stimulation compared to the 24h one. To our knowledge, this is the first report showing that these two neurotrophin receptors are expressed in the mesenchymal cells of the intestinal tissue. Our results agree with a previous study, which shows that differentiated skin and lung myofibroblasts not only secrete various neurotrophins, but also express TrkB and p75NTR in higher levels than undifferentiated fibroblasts (Micera et al., 2001). In a similar study of skin healing, Palazzo et al. identified that the inhibition of either Trk or p75NTR resulted in reduced fibroblast proliferation and migration, suggesting that this signaling pathway plays a critical role in wound healing (Palazzo et al., 2012). In addition, TrkB has also been found overexpressed in myofibroblasts from diabetic retinopathy epiretinal membranes (Abu El-Asrar et al., 2013), suggesting that pathologically related situations may induce the expression of neurotrophin receptors in mesenchymal cells. In another study, treatment of Idiopathic Pulmonary Fibrosis (IPF)-isolated fibroblasts with the ligand of TrkB, Brain-Derived Neurotrophic Factor (BDNF), resulted in enhanced expression of epithelial-to-mesenchymal transition markers, promoting the mesenchymal activity of the fibroblasts (Cherubini et al., 2017). All the aforementioned studies conclude that pathogenetic mechanisms seem to induce the expression of TrkB, and thus validate our results that clearly show induction of the expression of TrkB and p75NTR in cSEMFs after treatment with the pro-inflammatory cytokines IL-1α and TNF-α.

Regarding p75NTR, few studies have reported its expression in cells, which are of mesenchymal origin and isolated from other than the intestinal tissue. Specifically, in conjunctival fibroblasts, after treatment with the Nerve Growth Factor (NGF), the p75NTR's ligand, not only induced the expression of p75NTR, but also enhanced the migratory capacity of these cells, and upregulated the production of α-SMA and TGF-β (Micera et al., 2005). Similar results were reported by Micera et al., where isolated fibroblasts from patients with vernal keratoconjunctivitis expressed the p75NTR, while fibroblasts from healthy controls lacked in its expression (Micera et al., 2007). In addition, although the α-SMA

expression did not change in these cells upon NGF stimulation, the expression of extracellular matrix components (ECM), such as collagen, was indeed induced, suggesting that the NGF/p75NTR axis is implicated in fibrogenesis (Micera et al., 2007). Apart from fibrogenesis, the same research team showed that acute or chronic stimulation with NGF can induce apoptosis to an *in vitro* model of conjunctival myofibroblasts (Micera, Puxeddu, Balzamino, Bonini, & Levi-Schaffer, 2012).

Except from the ocular system, the expression of p75NTR has also been studied in hepatic stellate cells, which are of mesenchymal origin (Kendall et al., 2009; Passino, Adams, Sikorski, & Akassoglou, 2007). Specifically, Passino et al. showed that the depletion of p75NTR from hepatic stellate cells inhibited their differentiation into myofibroblasts, as well as failed to support the proliferation of hepatocytes (Passino et al., 2007). Similar to Micera et al. who showed that p75NTR activation leads to apoptosis in conjunctival myofibroblasts (Micera et al., 2012), Kendall et al. reported that an inactive form of p75NTR in hepatic myofibroblasts may be involved in the reduced resolution of hepatic fibrosis, through a weaker induction of apoptosis (Kendall et al., 2009). These results suggest that although TrkB has been involved in pathological conditions, p75NTR may have a protective effect in the resolution of fibrosis. On the contrary, another research team showed that the NGF/p75NTR axis induces the α-SMA and collagen expression in fibroblasts through the nuclear translocation of MRTF-A (Liu et al., 2019), a significant factor that is implicated in fibrosis (Ge et al., 2018; H. Lei et al., 2015; Luchsinger, Patenaude, Smith, & Layne, 2011; Shiwen et al., 2015), suggesting that p75NTR may have a homeostatic role.

Regarding the development of HIOs and Colonoids as *in vitro* models for studying the intestinal inflammation, these 3D systems may offer many advantages. As far as HIOs are concerned, the results of this Master’s Thesis show that HIOs can accurately mimic the architecture of intestinal tissue, as they develop villi and contain different types of epithelial and non-epithelial cells. On the other hand, although Colonoids consist only of epithelial cells, they also seem to mimic the intestinal tissue, in a lesser extent, as they also develop villi and contain various types of epithelial cells. In addition, it is also shown that both HIOs and Colonoids exhibit inflammatory responses to pro-inflammatory stimuli similar to intestinal tissue. Moreover, the use of HIOs or Colonoids can overcome the difficulties of isolating, culturing and maintaining 2D cultures of primary cells (Pamies et al., 2018), while also can offer the advantage of studying healthy epithelial cells; primary epithelial cells are difficult to isolate and maintain (Grossmann et al., 2003), while the available epithelial immortalized cell lines differ from the healthy epithelium (Kaur & Dufour, 2012). Finally, both HIOs and Colonoids allow for large number of high-throughput screening experiments, but without the need for a large number of experimental animals (according to the Reduce-Replace-Refine principle). Thus, we believe that by investigating the

possible expression of TrkB and p75NTR in 3D intestinal *in vitro* models, such as HIOs and Colonoids, it would further highlight the role of these two neurotrophin receptors in the pathophysiology of intestinal inflammation.

Chapter 6: Conclusion

In conclusion, the aim of this study was to investigate the expression of the neurotrophin receptors TrkA, TrkB and p75NTR in intestinal inflammation models, stimulated with the pro-inflammatory cytokines IL-1α and TNF-α, in order to identify disease mechanisms and possible new therapeutic targets. We demonstrated for the first time that neurotrophin receptors are expressed in colonic subepithelial myofibroblasts - a well-established *in vitro* intestinal inflammation model - after stimulation with pro-inflammatory cytokines. Therefore, our findings suggest that neurotrophin receptors are implicated in intestinal inflammation and may be targets for therapeutic intervention in IBD. In addition, we also report the successful development of both Human Intestinal Organoids and Colonoids that may be used as 3D *in vitro* and *ex vivo* models for studying intestinal inflammation and fibrosis. Thus, our future research plans include the further investigation of the role of neurotrophin receptors in intestinal inflammation and fibrosis using these 3D complex intestinal cultures, in an effort to fully elucidate the pathogenetic mechanisms of Inflammatory Bowel Diseases, discover new associations between the gut and the brain, and identify possible new therapeutic targets.

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