UNIVERSITÀ DEGLI STUDI DI MILANO

DOCTORAL PROGRAMME IN NUTRITIONAL SCIENCE

Dysregulation of Intestinal stem cells in Crohn's disease

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2015-18 XXXI ciclo

Abstract

Introduction. Several potential key factors, such as immune events, toxic environmental agents, genetic predisposition and abnormal microbiota have been established to play a role in the development of Crohn's disease, but the mechanism behind it is still under investigation. Intestinal mucosa homeostasis relies on a delicate balance between self-renewal and differentiation, which is guaranteed by intestinal stem cells turnover within the local crypts. Recent studies suggested that an impaired differentiation of intestinal stem cells (ISCs) may facilitate the onset of Crohn's disease via formation of a defective antimicrobial barrier, which enables intestinal bacteria to cause inflammation. The aim of our study is to explore whether ISCs are disrupted and their regenerative properties are altered in Crohn's disease, thus representing a novel player in the disease onset and a potential therapeutic target to restore intestinal mucosa self-renewal abilities.

Methods. A transcriptome analysis has been conducted on intestinal samples (both marginal and inflamed area with respect of the disease location) obtained from patients with and without Crohn's disease to assess the expression of ISC markers. ISC regenerative properties have been tested by taking advantage of the mini-guts assay, where purified crypts were cultured as tridimensional organoids and their morphology/development were evaluated. Finally, mini-guts were grown from healthy subjects in serum of patients with Crohn's disease to prove whether circulating factors/cytokines may exert a significant effect in abrogating organoids development and affect the expression of ISC markers.

Results. ISC markers, particularly LGR5 and EPHB2, are poorly expressed in crypts isolated from intestinal samples of patients with Crohn's disease. Interestingly, ISC markers are more preserved in the healthy zone, while their expression is nearly absent in the inflamed area. More importantly, ISC capability of self-renew tested in the mini-guts assay, was significantly abrogated in patients with Crohn's disease, with only 40% of mini-guts developing from crypts obtained from the marginal area and less than 5% from those isolated from the inflamed portion of the intestinal sample. When cultured in the presence of serum of Crohn's disease patients, mini-guts obtained from crypts of controls failed to generate normal self-renewed organoids and showed a reduced expression of EPHB2 and LGR5 ISC markers.

Conclusion. We have demonstrated that the ISCs pool is reduced in Crohn's disease and this is associated with a loss of regenerative properties of ISCs within the local crypts. When cultured in vitro with serum of patients with Crohn's disease, mini-guts growth is fully abrogated thus suggesting that a defect in ISCs pool and function exist in the context of Crohn's disease.

Riassunto

Introduzione. Numerosi fattori, tra cui eventi immunitari, agenti ambientali tossici, predisposizione genetica e microbiota anormale svolgono un ruolo chiave nella patogenesi della malattia di Crohn, ma il principale meccanismo alla base è ancora oggetto di studio. L'omeostasi della mucosa intestinale si basa su un delicato equilibrio tra auto-rinnovamento e differenziazione, che è garantita dal ricambio delle cellule staminali intestinali all'interno delle cripte. Recenti studi suggeriscono che una differenziazione alterata delle cellule staminali intestinali (ISC) può facilitare l'insorgenza della malattia di Crohn attraverso la formazione di una barriera antimicrobica difettosa, che consente ai batteri intestinali di provocare l'infiammazione. Lo scopo del nostro studio è quello di esplorare se le ISC sono danneggiate e le loro proprietà rigenerative sono alterate nella malattia di Crohn, rappresentando così un nuovo attore nell'insorgenza della malattia e un potenziale bersaglio terapeutico per ripristinare le capacità di auto-rinnovamento della mucosa intestinale.

Metodi. Su campioni intestinali (sia area marginale che infiammata rispetto alla sede della malattia) ottenuti da pazienti con e senza malattia di Crohn è stata condotta un'analisi del trascrittoma per valutare l'espressione dei marcatori per ISC. Le proprietà rigenerative delle ISC sono state studiate utilizzando il test del mini-guts, in cui le cripte purificate sono state coltivate come organoidi tridimensionali e la loro morfologia / sviluppo sono stati valutati. Infine, mini-guts sono stati coltivati a partire dalle cripte di soggetti sani con siero di pazienti affetti da malattia di Crohn per dimostrare se i fattori circolanti / citochine potessero esercitare un effetto significativo nell'abrogazione dello sviluppo di organoidi e influenzare l'espressione dei marcatori per ISC.

Risultati. I marcatori per ISC, in particolare LGR5 e EPHB2, sono scarsamente espressi in cripte isolate da campioni intestinali di pazienti con malattia di Crohn. È interessante notare che i marcatori per ISC sono più preservati nella zona sana, mentre la loro espressione è quasi assente nell'area infiammata. Ancora più importante, la capacità delle ISC di auto-rinnovamento testata nel saggio mini-guts, è stata trovata significativamente ridotta nei pazienti con malattia di Crohn, con solo il 40% dei mini-guts che si sviluppano da cripte ottenute dall'area marginale e meno del 5% da quelle isolate dalla parte infiammata del campione intestinale. Quando i mini-guts ottenuti dalle cripte dei controlli sono stati coltivati in presenza di siero di pazienti affetti da malattia di Crohn non riuscivano a generare organoidi normali e mostravano una ridotta espressione dei marcatori per ISC EPHB2 e LGR5.

Conclusione. Abbiamo dimostrato che il pool delle ISC è ridotto nella malattia di Crohn e che questo si associa a una perdita di proprietà rigenerative delle ISC all'interno delle cripte. La crescita di mini-gut in vitro in presenza del siero di pazienti affetti da morbo di Crohn, è quasi completamente annullata, suggerendo quindi che nel contesto della malattia di Crohn esiste un difetto del pool e della funzione rigenerativa delle ISC.

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CHAPTER 1

Introduction

1.1 Background

Inflammatory bowel disease (IBD) is a group of diseases characterized by a chronic inflammation, which affects the digestive tract, in particular the intestine and encompass Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis (IC)[1].

The incidence and prevalence of IBD have increased worldwide in the past 50 years, especially in industrialized countries, with Europe, United Kingdom and North America having the highest incidence[1,2]. In North America the incidence of UC varies from 2.2 to 14.3 cases per 100.000 person-years, whereas that of CD varies from 3.1 to 14.6 cases per 100.000 person-years[1]. The prevalence of UC ranges from 37 to 246 cases per 100.000 person-years, whereas that of CD from

26 to 199 cases per 100.000 person-years. In Europe, the incidence of UC varies from 1.5 to 20.3 cases per 100.000 person/years and from 0.7 to 9.8 cases for CD, while the prevalence of UC ranges between 21.4 and 243 cases and between 8.3 and 214 cases per 100.000 person/years for CD[1]. IBD is more rare in the other geographic areas, with the exception of Israel, Australia and South Africa. A higher incidence (2 to 6 times) of IBD has been observed in the Jewish population of USA, Europe and South Africa. The prevalence decreases progressively in non-Jewish white populations, in African-Americans, Hispanics and Asians. The highest socio-economic classes tend to be more affected. IBD is a familiar disease in 5-10% of patients[1,2]. Regarding the age of the onset there are two peaks: between 15 and 30 years and between 60 and 80 years. The pediatric cases of IBD are gradually increasing, 25% of new patients are under 20 years of age and there were cases of early onset even in the first years. The male to female ratio is 1 for UC, whereas 1.1 to 1.8:1 for CD[1,2]. The highest mortality occurs during the first year of illness and in long-term illness due to the risk of colorectal cancer.

The pathogenesis of IBD is not yet fully understood. According to the most recognized hypothesis, IBD is a multifactorial disorder with a complex interaction between genetic factors, immune system, intestinal microbiota and environmental factors, which all lead to an abnormal inflammatory response against the intestine[1,2]

1.2 Genetic predisposition

The first studies carried out for the research of genetic determinants of CD have used linkage mapping, which enabled the discovery of the nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*), located on the chromosome 16, as the major gene of susceptibility of the disease. NOD2 is one of the leucine rich repeat/nucleotide-binding site (NBS/LRR) protein family that together with toll like receptors (TLR) are involved in the detection of invasive pathogens. Unlike seconds, the NBS/LRR are cytoplasmic "sensors", both recognize conserved molecules of microbes such as lipopolysaccharides and peptidoglycans[3]. The identification of allelic variants of single nucleotide polymorphisms (SNP) most frequently represented in patients affected by IBD through genoma wide association studies (GWAS) was extremely important. To date, thanks to GWAS, which involved Caucasian population but also other ethnic groups, 241 susceptibility loci for IBD are confirmed[4]. Among the identified loci there are ATG16L1 and IRGM, referring to the autophagy pathway. Autophagy is particularly important for the intracellular clearance of microorganisms ("xenophagia"), but also in the recognition of infected cells by the immune system through the generation of bacterial antigenic peptides[5]. SNP in ATG16L1 at risk is a missense mutation which involves the substitution

of threonine with alanine, which increases the degradation of the protein by the caspase 3[3,6]. Moreover preliminary studies on mice have revealed that reduced levels of ATG16L1 are associated with an altered function of exocytosis of the antimicrobial peptides by the Paneth cells, thus facilitating the invasion of the intestinal mucosa by microbial agents and the onset of the inflammatory process[7]. GWAS, with the identification of numerous susceptibility variants within or near genes implicated in the biology of Th17 (for ex. RORC, IL-23R, JAK2, STAT3), have supported the increasingly relevant role of these cells in the pathogenesis of IBD. GWAS also highlighted the important role played by regulatory T cells (T reg) in the pathogenesis, identifying IBD-associated loci containing IL-2, IL-2RA (part of IL-2R) and STAT5[8].

1.3 Abnormal immune response

The altered immune response results from altered mechanisms of both adaptive and innate immunity[9]. The innate immune response is the first line of defence against any aggressor. Unlike the adaptive one, it is nonspecific and does not confer long-term immunity. It includes different cell types: immune cells (neutrophils, macrophages, dendritic cells)[10-12] and non-immune cells (epithelial

cells, endothelial cells and myofibroblasts)[9]. The first physical barriers encountered by pathogens and food antigens are represented by the mucous layer and the intestinal epithelium, which consists of enterocytes and specialized epithelial cells such as mucous cells and Paneth cells (these are present only at the base of the crypts of the small intestine). Barrier defects and increased intestinal permeability were found in both UC and CD subjects. However, it is not yet clear whether these changes are the cause or the consequence of the chronic inflammatory response. Within the cross-talk between innate and adaptive immunity, cytokines are also involved, and IL-23 above all.[9] Cytokines whose levels are significantly increased in individuals affected by CD compared to healthy individuals include: IL-2, IL-12, IL-18 (the latter two induce the increase in INF-γ), while IL-5 level was mainly increased in UC. In both diseases there is an increase in pro-inflammatory cytokines IL-1β, IL-6 and TNF-α[13]. Until a decade ago there was undisputed the Th1/Th2 paradigm, according to which Crohn's disease was a Th1-associated disease based on the production of INF-γ and IL-12, while ulcerative colitis was an atypical Th2 condition based on the elevated production of IL-5, IL-13, but low levels of IL-4. This paradigm has been re-evaluated with the discovery of the fundamental role played by Th17 and the crosstalk between Th1, Th2, Th17 and Treg cells. Thus Crohn's disease is a mixed Th1/Th17 condition[14]. Regulatory T cells, on the other hand, play a key role in inducing tolerance towards self and

non-self antigens. In Crohn's disease, given the exaggerated inflammatory response underway, they are likely to be dysfunctional, but this remains to be defined[15].

1.4 Alteration of intestinal microbiota

Intestinal microbiota refers to the numerous micro-organisms residing in niches adjacent to intestinal epithelial surface[16]. *Firmicutes* and *Bacteroides* represent 90% of intestinal bacteria, whereas *Actinobacteria* and *Proteobacteria* are less than 5%[17]. There is a real symbiotic relationship between intestinal flora and human organism: supply of undigested material as nourishment to the bacteria and in return they perform different functions. Among these, the main one is the trophic function for the epithelial cells of the colon, to which short-chain fatty acids are supplied (acetic, propionic and butyric acid) starting from the fermentation of undigested material (in particular plant-based polysaccharides)[16,18].

Dysbiosis, the alteration of the autochthonous bacterial flora, is one of the main factors implicated in the alteration of the immune response and therefore in the

pathogenesis of CD. One of the evidences is the efficacy of antibiotic therapy in the treatment of IBD. The main alterations observed are a reduction in the diversity of the bacterial species present, a reduction in the *Firmicutes* and a concomitant increase in *Proteobacteria* (*E. coli* and *Bacteroides*), or shift from species predominantly "symbionts" to those potentially "pathobionts"[19,20]. To date there is no evidence that a single microorganism could be the cause of IBD.

1.5 Environment

Smoking, diet, vitamin D deficiency and antibiotic therapy are among the environmental factors that may play a role in the pathogenesis of IBD[21]. Tobacco smoking has, surprisingly and in an unclear manner, different effects in the two types of IBD. In fact, the protective effect of smoking in UC is well known in the literature both in the onset and in the clinical course, and the opposite action in CD[2]. Several studies demonstrate the role of another environmental factor, diet, in the pathogenesis of IBD, in particular in modeling the intestinal bacterial flora, in influencing its composition and growth. Some dietary components may alter the composition of the microbiota in negative[18]. Antibiotics can alter the intestinal microbial ecology. For example, a short cycle of cyclosporine induces

a reduction in the diversity of bacterial species, while amoxicillin taken for 10 days induces a shift from *Bacteroides*, *Clostridia* towards *Enterobacteriacae*[22].

1.6 Intestinal Stem Cells

According to several studies, the alterations of the compartment of intestinal epithelial stem cells play an important role in the pathogenesis of inflammatory bowel diseases. Gersmann et al.[23] hypothesize that the bacterial invasion of the mucosa and the consequent inflammatory process result from an altered differentiation of intestinal stem cells in Paneth cells in CD, in goblet cells in ulcerative colitis. In particular, the Authors detected reduced levels of the transcription factor TCF4, which is fundamental in the differentiation process of Paneth cells, in CD affecting ileum compared to CD affecting colon or ulcerative colitis. Furthermore, this alteration was associated with a reduction in the number of these secretory cells and a reduced expression of the HD5 and HD6 alpha defensins (released by these cells) both in ileal biopsies of Crohn-affected individuals and in TCF4 knock-out mice. The Authors also confirmed the impaired differentiation of ISCs in goblet cells in UC as compared to CD, by demonstrating reduced levels of transcription factors Hath1 and KLF4, which

regulate the differentiation of goblet cells. This could explain the thinning of the mucous layer observed in the UC (normally between 100 and 300 μm), which facilitates the invasion of microbial agents. Also Kini et al.[24] showed the role of stem cells in the pathogenesis of IBD, by demonstrating a significant reduction in the number of ISCs in the marginal areas to the inflamed ones in UC patients. This loss was associated with a concomitant increase in the number of mature colonocytes and a reduced expression of the signaling pathways Wnt and Notch. This demonstrates the presence of altered signals in stem cell niche and the following loss of the balance between proliferation and differentiation. This defect in intestine areas macroscopically unaffected by the disease, adjacent to the inflammatory process, underlines the role played by the ISCs in the early stage of the pathogenesis of UC. Dotti et al.[25] showed that permanent alterations of the intestinal epithelium found in patients with UC in remission may result in a persistent alteration of the epithelial stem compartment. Therefore, the ISCs were not simply spectators of the ongoing pathological process but they were "reprogrammed" impacting on the progression of the disease. In their study[25], which included patients with active UC, UC in remission and controls (with colorectal cancer), the Authors observed significant differences in gene expression of epithelial organoids developed from crypts of patients with UC as compared to those of controls. Genes associated with antimicrobial function and those coding for the components of the mucus were downregulated

compared to controls, not only in the mini-guts of patients with active UC, but also in those with UC in remission, suggesting a possible role of permanent alterations in ISCs in the pathogenesis of the disease. The relevant role of inflammatory environment on the regenerative properties of intestinal stem cells has been recently confirmed by Suzuki et al.[26], who showed that mini-guts developed from the crypts extracted of CD patients had an altered expression profile of intestinal stem cells OLFM4 and SLC12A2, as compared to non-IBD controls.

CHAPTER 2

Aims of the study

The growing interest in the regenerative properties of the intestinal mucosa of patients with CD, the recent evidence about intestinal epithelial changes (reduction in the number of goblet cells and Paneth cells) and the main signaling pathways regulating ISC in CD patients suggest the primary role that an alteration of the stem cell compartment may have in the onset and recurrence of the disease. Also, given that the current conventional therapy, based mainly on immunosuppression, does not seem to be effective in treating CD but rather is often associated with a high incidence of recurrence and surgical complications, an alternative target beyond the immune system seems to be relevant for developing new therapeutic strategies for CD treatment. Our study aims to analyze from a quantitative and functional point of view the ISC pool in active Crohn's disease. The ultimate goal is to identify the damage to

ISCs in the course of CDs, which may represent a new therapeutic target in the treatment of the disease.

CHAPTER 3

Methods

3.1 Patiens and study desing

Healthy subjects (CTRL) were enrolled (n=10) among patients undergoing colonscopy or intestinal surgery for diverticulosis, colon cancer, irritable bowel syndrome. Age: 41.3 ± 2.2 (mean±SEM); Sex (M/F): 7/3.

IBD individuals had a 5-year history of Crohn's disease and were enrolled at the moment of surgery procedure for disease complications (strictures, fistulas) or during an endoscopy routine examination before undergoing surgery. Age: 47.1 ± 3.1 (mean±SEM); Sex (M/F): 4/6. All subjects provided informed consent before study enrollment.

3.2 Pathology and immunofluorescence

Colorectal endoscopy procedure was performed in healthy subjects and in Crohn's disease patients using a Welch Allyn optic sigmoid scope. Intestinal mucosal samples were fixed in buffered formalin (formaldehyde 4% w/v and acetate buffer 0.05 M) and routinely processed in paraffin wax. 3μm-thick sections of each enrolled case were stained with Hematoxylin & Eosin (H&E) for morphological evaluations.

Immunofluorescence samples obtained from intestinal biopsies were observed using a confocal system (LSM 510 Meta scan head integrated with the Axiovert 200 M inverted microscope; Carl Zeiss, Jena, Germany) with a 63x oil objective. Images were acquired in multitrack mode, using consecutive and independent optical pathways. The following primary antibodies were used: mouse vimentin (1:80, monoclonal, clone V9 Dako) mouse aldheyde (1:1000, monoclonal, clone 44, BD), mouse cytokerain 20 (1:100, monoclonal, clone Ks20.8, Dako).

3.4 Crypts isolation and mini-guts development

Crypts were extracted from mucosa and sub-mucosa of intestinal samples of healthy subjects (healthy controls) or obtained from patients with established Crohn's disease undergoing surgery for disease complications (strictures, fistulae) or colonscopy as routinely clinical exam. Mucosa was incubated with a mixture of antibiotics Normocin, [Invivogen, San Diego, California 92121, USA], Gentamycin [Invitrogen, Carlsbad, CA, USA] and Fungizone [Invitrogen]) for 15 minutes at room temperature, and then tissue was minced into small pieces and incubated with 10 mM Dithiothreitol (DTT) (Sigma) in PBS 2-3 times for several minutes. Samples were then transferred to 8 mM EDTA in PBS and incubated for 30 minutes at 37°C. After this step, vigorous shaking of the sample yielded supernatants enriched in colonic crypts. Fetal bovine serum (FBS, Sigma 12103C-500ML) was added to a final concentration of 5%, and single cells were removed by centrifugation 40×g for 2 minutes. Crypts were mixed with 50 µl of Matrigel (BD Biosciences 354234) and plated on pre-warmed culture dishes. After solidification, crypts were overlaid with complete crypt culture medium: Wnt3a-conditioned medium and Advanced DMEM/F12 (Life Technologies 1263010) 50:50 , supplemented with Glutamax, 10 mM (Life Technologies 35050038) HEPES (Life Technologies 15630080), N-2 [1×] (Life Technologies 17502048), B-27 without retinoic acid [1×](Life Technologies

12587010), 10 mM Nicotinamide (Sigma N0636), 1 mM N-Acetyl-L-cysteine (Sigma A965), 50 ng/ml human EGF (Life Technologies PHG0311), 1 μg/ml RSPO1 (Sino Biological 11083-H08H), 100 ng/ml human Noggin (Peprotech 12010C), 1 μg/ml Gastrin (Sigma-Aldrich SCP0152), 500 nM LY2157299 (Axon MedChem 1491), 10 μM SB202190 (Sigma S7067) and 0.01 μM PGE2 (Sigma P6532). Medium was replaced every 3 days. Percentage of developed mini-guts with at least one crypt domain was assessed as already described [27,28].

3.5 In vitro mini gut generation study

Crypts were isolated from healthy subject samples and cultured as described above to generate mini-guts. To culture isolated crypts with crypt culturing medium containing human serum of individuals with Crohn's disease (CD), namely "CD" medium, in place of regular FBS, L-Wnt3 cells were grown in 10% "CD" serum to generate conditioned medium that was further added 50:50 to Advanced DMEM/F12 medium in order to obtain the crypts culture medium as already described. After 8 days, crypts were collected, and the morphology, mini-gut growth, expression of intestinal signature markers (EPHB2, LGR5), and Caspase 8 (Life Technologies) were examined using RT-PCR.

3.6 Immunoblotting

Total proteins of intestinal samples were extracted in Laemmli buffer (Tris–HCl 62.5 mmol/l, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol) and their concentration was measured[29]. 35 μg of total protein was electrophoresed on 7% SDS-PAGE gels and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany). Blots were then stained with Ponceau S. Membranes were blocked for 1 h in TBS (Tris [10 mmol/l], NaCl [150mmol/l]), 0.1% Tween-20, 5% non-fat dry milk, pH 7.4 at 25° C, incubated for 12 h with 200 mg/ml of a polyclonal anti-goat EphB2 antibody or polyclonal anti-goat LGR5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with a monoclonal mouse anti-β-actin antibody (Santa Cruz Biotechnology) diluted 1:1000 in TBS–5% milk at 4° C, washed four times with TBS–0.1% Tween-20, then incubated with a peroxidase-labeled rabbit anti-goat IgG secondary antibody (or rabbit anti mouse for β-actin) diluted 1:1000 (Santa Cruz Biotechnology) in TBS–5% milk, and finally washed with TBS–0.1% Tween-20. The resulting bands were visualized using enhanced chemiluminescence (SuperSignal; Pierce, Rockford, IL, USA).

3.6 Flow cytometry

The expression of the ISC markers EphB2 (BV711 anti-human EphB2 antibody, BD Biosciences) and LGR5 (PE anti-human LGR5, Origene, Rockville, MD) was

determined by flow cytometry by excluding CD45- and CD11b-positive cells (V450 anti-human CD45 and CD11b, BD Biosciences, San Jose, CA). Propidium iodide (PI) was added (10 μg/ml) to exclude dead cells. Samples were run on a BD FACS CELESTA and analyzed by FlowJo.

3.7 Morphology imaging analysis

The images of mini-guts were taken at day 0 and at day 8 by inverted microscopy Leica DH/RB and acquired with Axio Vision AC Release 4.3. Pictures reported in figures represent mini-guts at day 8, 10X magnification.

3.8 Transcriptome profiling

Quantitative PCR arrays are the most reliable and accurate tool for analyzing the expression of a focused panel of genes relevant to a pathway or a disease state. PCR arrays allow gene expression analysis with the sensitivity, dynamic range, and specifi city of a real-time PCR as well as the multi-gene profi ling capability of a microarray.

Total RNA was isolated from purified intestinal crypt suspension using the RNeasy Mini Kit (Qiagen, Valencia, CA) with on-column DNase I digestion. Next, 3 μg total RNA from each sample was reverse-transcribed using the RT2 First Strand kit (C-03; SABiosciences, Frederick, MD). We used the Human Stem Cell RT2 Profiler PCR Arrays (PAHS-405Z) and a custom array with the following genes: AXIN2, OLFM4, BMI1, RNF43, CDCA7, SLC12A2, CDK6, SOX9, DKC1, ZNRF3, ETS2, EPHB2, FAM84A, LGR5, GPX2, ACTB (SABiosciences). The Profiler PCR Arrays measure quantitatively the expression of a panel of genes using SYBR Green-based real-time PCR.

3.9 qRT-PCR analysis

RNA from purified intestinal crypts was extracted using Trizol Reagent (Invitrogen), and qRT-PCR analysis was performed using TaqMan assays (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The normalized expression values were determined using the ΔCt method. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) data were normalized for the expression of ACTB, and ΔCt values were calculated. Analysis was performed in technical and biological triplicates.

Gene Symbol	UniGene #	Refseq Accession #	Band Size (bp)	Reference Position
LGR5	Hs.658889	NM_003667	91	1665
EPHB2	Hs.523329	NM_004442	68	2908
ACTB	Hs.520640	NM_001101	174	730

3.10 Luminex cytokine measurement

Levels of cytokines were assessed in serum of patients with established Crohn's disease and healthy subjects using the Bio-Plex Pro human cytokine 17-plex panel (M5000031YV, Bio-Rad, Hercules, CA) according to the manufacturer's protocol. A fold change analysis has been conducted and a heat map has been generated using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA).

3.11 Statistical analysis

Data are presented as mean and standard error of the mean (SEM) and were tested for normal distribution with the Kolmogorov-Smirnov test and for homogeneity of variances with Levene's test. The statistical significance of differences was tested with two-tailed *t*-test and the chi-square ($\chi 2$) tests. Significance between the two groups was determined by two-tailed unpaired

Student's *t* test. For multiple comparisons, the ANOVA test with Bonferroni correction was employed. All data were entered into and analyzed using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA). All statistical tests were performed at the 5% significance level.

CHAPTER 4

Results

4.1 Intestinal crypts morphology is disrupted in CD

We first analyzed the intestinal morphology in samples obtained from patients with Crohn's disease and underwent diagnostic evaluation or surgery and compared it with samples obtained from patients without CD. The clinical and demographic characteristics of the patients enrolled in the study are summarized in Table 1.

Table 1. Clinical and demographic characteristics of patients enrolled.

	CD (n=30)	CTRL (n=30)	*p value*
Gender (M/F)	23/7	11/19	0.004
Age (y, mean±SD)	45.5 ± 15.3	52.3 ± 13.3	ns
Disease Duration (y, mean±SD)	14.2 ± 10.9	-	
Disease Phenotype (n of cases)		-	
Inflammatory	1		
Stenosis	17		
Fistulas	1		
Fistulas and stenosis	11		
Surgery		-	
n=1	16		
n<1	14		
Extra intestinal Symptoms (Y/N)	7/23	-	
CD therapy		-	
5-ASA	5		
Steroids	2		
Azathioprin	5		
Infliximab	4		
Adalimumab	5		
Vedolizumab	1		

Abbreviations: *Y/N, yes/no; n, number; y, years; SD, standard deviation.*

Hematoxylin and eosin staining of the intestinal samples examined showed a slight distortion of the mucosal architecture in patients with CD, which was not detected in samples of CD patients where crypts structure was well-preserved. In particular, the crypts normally straight and parallel assumed unusual orientations with respect to each other and assumed an anomalous

morphology. Elongated and ramified crypts with wide lumen were visible side by side to others in which the lumen was almost absent. This alteration was associated with the presence of aftoid ulcers, areas of common epithelial loss in the CD, together with the presence of a lympho-plasm-granulocytic neutrophil infiltrate and eosinophilic infiltrate in the lamina propria. The abnormalities found in intestinal crypts in patients with CD may result from repeated cycles of destruction induced by the intense inflammatory process and regeneration (Figure 1).

Figure 1. Mucosa morphology in CD and CTRL.

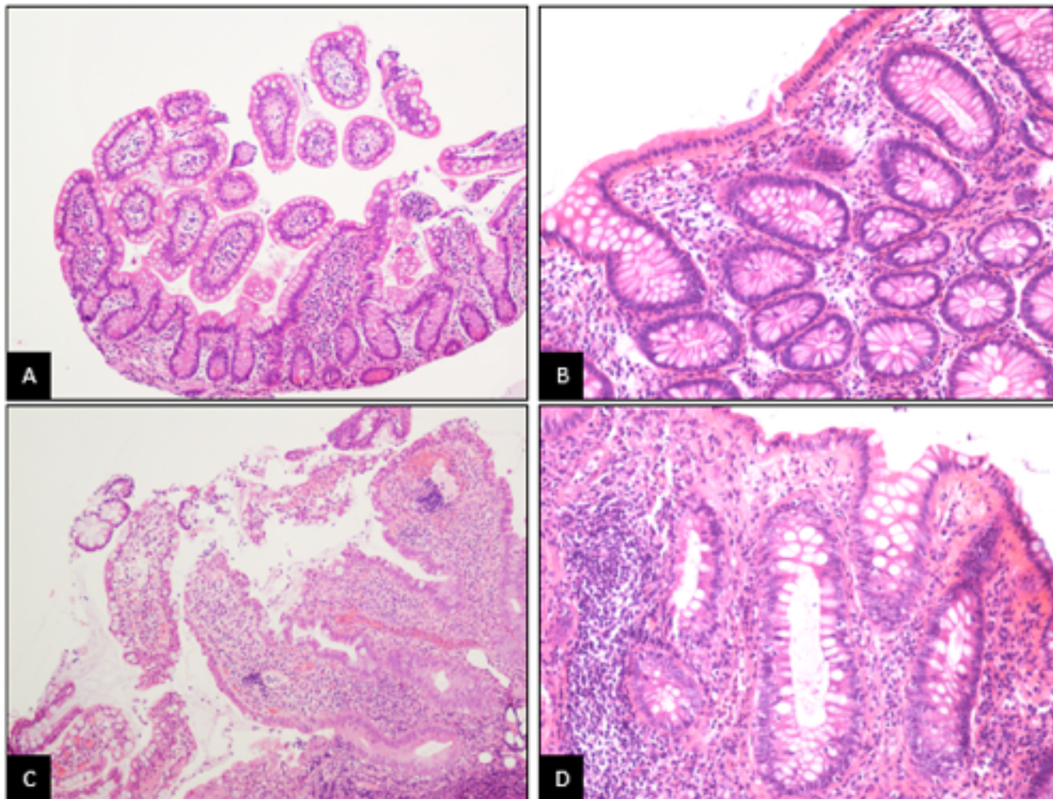

Figure 1. A. CTRL: normal ileal mucosa; 20x magnification. **B**. CTRL: normal colon mucosa; 20x magnification. **C**. CD: Ileal mucosa with slight architectural alteration and site of inflammatory pattern with moderate inflammatory infiltrate lymph-plasm-granulocytic neutrophilic and eosinophilic infiltrate in the lamina propria; 20x magnification. **D**. CD: colon mucosa with inflammatory infiltrate; 20x magnification.

4.2 ISCs pool is reduced in CD

To demonstrate if the alteration of the mucosal architecture in the course of CD was also due to an altered stem cell turnover, we first defined the specific transcriptomic profile of the intestinal crypts extracted from the samples of patients with CD and compared it with that of crypts purified from non-CD patients. After isolating the crypts, we extracted the total RNA. We therefore used a specific array already designed to detect the most important markers that characterize intestinal stem cells. Through this assay screening we demonstrated that some of the stem cell markers related to intestinal stem cells are altered and that in particular the two that showed the greatest reduction of expression were LGR5 and EPHB2 (Figure 2). We further analyzed whether the

inflamed area of the intestinal sample obtained from CD patients, where the disease effect is more evident, showed a different profile as compared to the marginal area, where the crypts structure was still detectable and preserved. Interestingly, we found a the more pronounced reduction of ISC markers, particularly EPHB2 and LGR5, in the inflamed area as compared to the marginal area.

Figure 2. **Intestinal transcriptome profile in CD patients and CTRL**.

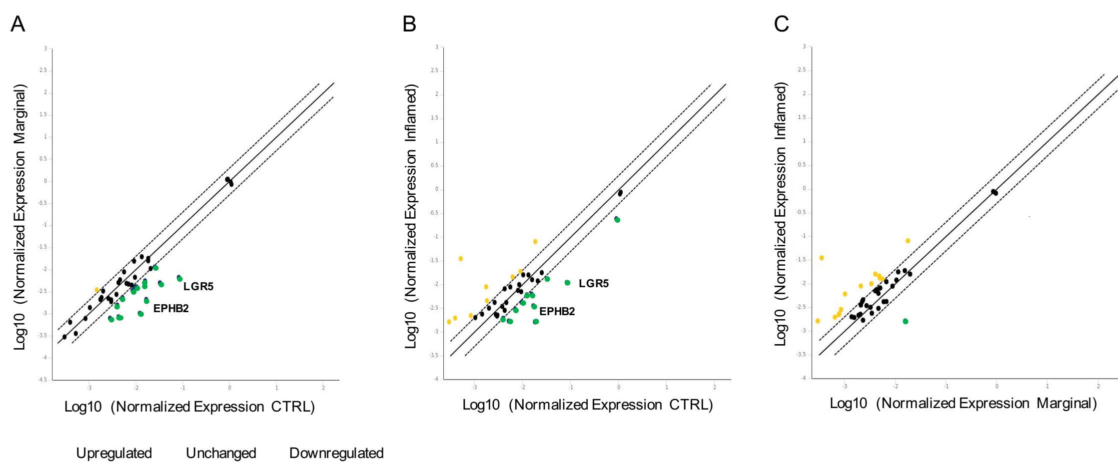

Figure 2. Transcriptomic profile of the ISCs examined on intestinal crypts isolated from CTRLs (n = 4) and from the marginal and inflamed areas of intestinal specimens obtained from patients with CD (n = 4). The scatter plots report the different gene expression in the marginal area versus CTRL (**A**), in the inflamed area versus CTRL (**B**) and marginal area versus inflamed area (**C**).

The reduced expression of LGR5 and EPHB2 found in samples of patients with CD, both in the marginal and inflamed area, as compared to CTRL, suggested a possible alteration of the intestinal stem cell pool that resides at the base of the crypts. To confirm this alteration, we conducted an immunofluorescence analysis that allowed us to demonstrate a reduced expression of the EPHB2 protein in crypts isolated from patients with CD, both from the marginal and the inflamed area, while the expression of other non-stem cell markers such as cytokeratin 20, epithelial marker, was preserved (Figure 3).

Figure 3. **ISC (EPHB2) and non-ISC (CKT20) markers expression in CD**.

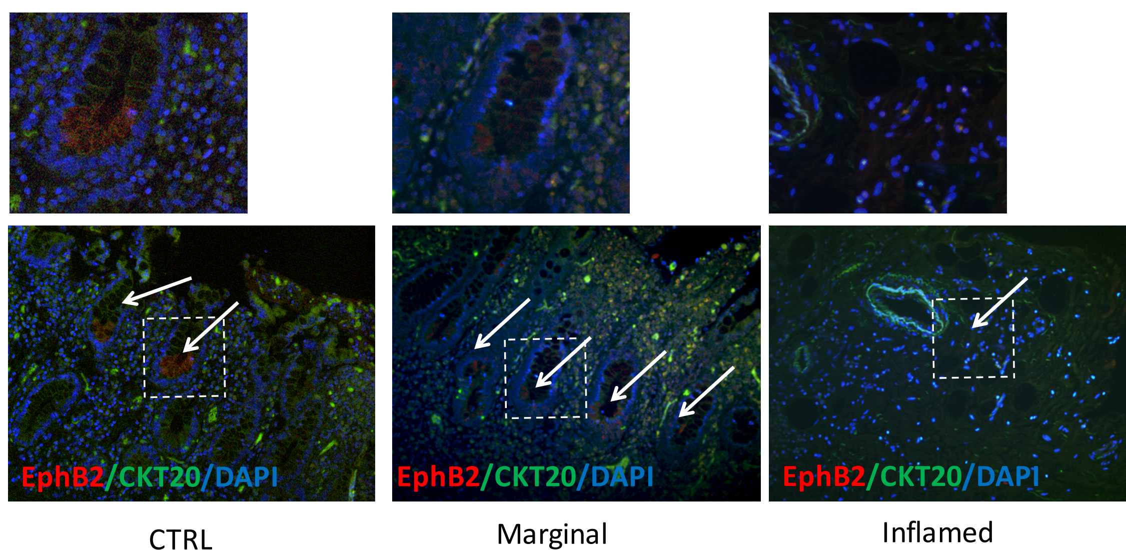

Figure 3. Immunofluorescence of the epithelial cytokeratin 20 marker (CKT20) and the EphB2 intestinal stem marker in the samples obtained from controls and in the marginal and inflamed areas of the samples obtained from patients with CD.

Next, we performed some confirmatory analysis to detect the expression of the ISC markers we found altered in our trascriptome profile, EPHB2 and LGR5, in crypts obtained from patients with CD and from controls. First, we confirmed that mRNA levels of both EPHB2 and LGR5 were significantly decreased in the crypts from the marginal and inflamed area of the CD patients as compared to non-IBD patients, with that in the inflamed area being almost undetectable (Figure 4: A-B). With regard to protein expression, we first performed a western blot analysis which demonstrated a more evident reduction in the expression of EPHB2, while that of LGR5 was only slightly affected (Figure 4C).

Figure 4. **LGR5 and EPHB2 mRNA and protein expression in CD**.

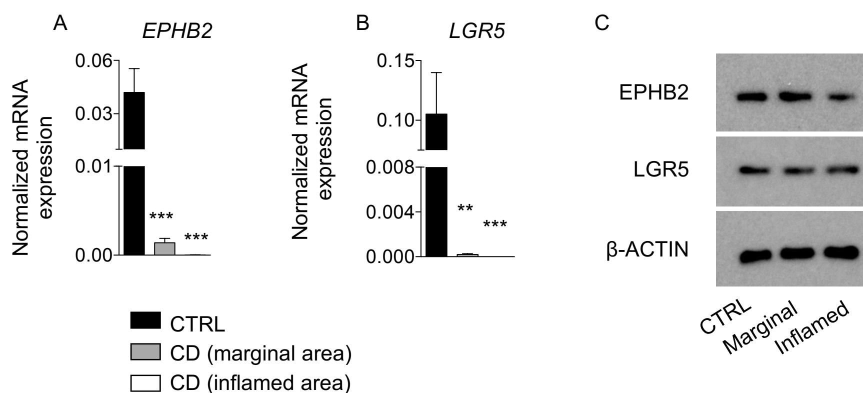

Figure 4. **A-B**. Bar graphs representing the normalized mRNAs expression of *EPHB2* and *LGR5* proteins measured with the qRT-PCR on the isolated crypts of CD patients (marginal and inflamed area) as compared to non-IBD patients

(CTRL). All samples were normalized for the expression of the housekeeping gene ACTB (ΔCt). **C**. Representative image of the EPHB2 and LGR5 protein expression on isolated intestinal crypts by western blot. (n = 3 CTRL, n = 3 marginal areas, n = 3 inflamed areas). Data are expressed as mean ± standard mean error (SEM). * P <0.01; ** p <0.001; *** p <0.0001.

Finally, we used flow cytometry to confirm the reduced expression of LGR5 and EPHB2 in crypts isolated from samples of patients with CD. Interestingly, we found an increased lymphoid infiltrate in CD samples as compared to non-IBD patients, particularly in the inflamed area (Figure 5A). Upon gating on $CD45^{-}CD11c^{-}$ live (PI^{-}) cells, we observed a significant decrease in $EPHB2^{+}$ cells, particularly in $EPHB2^{hi}$ cells both in the marginal and inflamed area of CD samples as compared to CTRL, and a reduction in $LGR5^{+}$ cells although to a lesser degree, which paralleled our observations at western blot (Figure 5: B-D).

Figure 5. **EPHB2+ and LGR5+ cells in CD by flow cytometry**

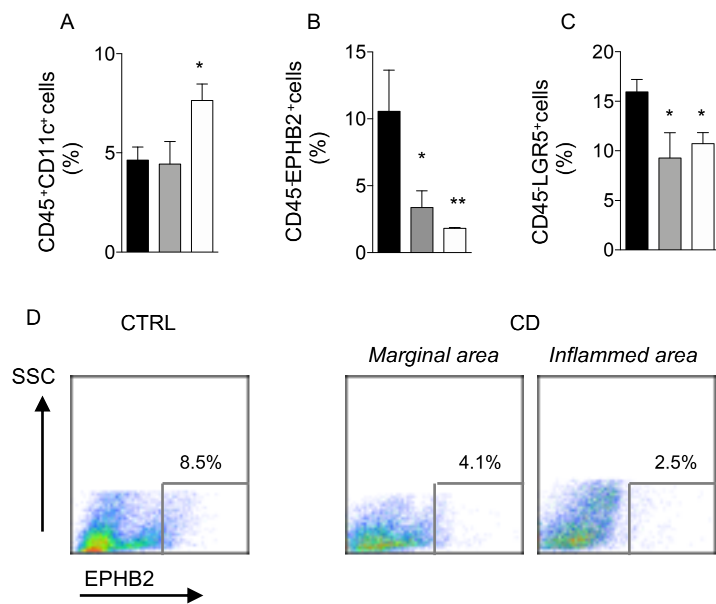

Figure 5. **A-B**. Quantification and representative histograms of the flow cytometric analysis of EPHB2+ cells in crypts isolated from the marginal and inflamed areas of patients with CD compared to healthy subjects (n = 4 CTRL, n = 4 marginal areas, n = 4 inflamed areas). Data are expressed as mean ± standard mean error (SEM). * P <0.01; ** p <0.001; *** p <0.0001.

4.3 ISCs regenerative properties are abrogated in CD

Given that the intestinal stem cell pool is depleted in CD samples, we assessed whether ISCs regenerative properties were also altered thus preventing their ability to self-renew the intestinal mucosa upon inflammation.

To do this we used a new organ-on-a-chip generation method and isolated the intestinal crypts obtained from samples of patients with CD, marginal area and inflamed area, and from non-CD patients. The crypts obtained were grown on a three-dimensional matrigel support with appropriate culture medium in order to develop intestinal mini-guts. At a distance of 8 days, we evaluated the number of intestinal organoids formed and their morphological characteristics. The organoids developed from the crypts of non-CD patients were about 85% and showed a well-preserved morphology with numerous crypts domains (Figure 6). The crypts isolated from the marginal area of CD patients developed a significant lower percentage of organoids, less than 40%, and from the morphological point of view the latter appeared similar to small spheroids (Figure 6). Finally, the intestinal crypts isolated from the inflamed area failed to develop organoids and remained small and fragmented, thus suggesting that the ability to self-renew in vitro was completely abrogated (Figure 6). Together with our gene expression studies, these results show that there in CD patients a

defect of the intestinal stem cells pool exists and it is associated with altered regenerative properties of the mucosa.

Figure 6. **In vitro generation of mini-guts in CD**.

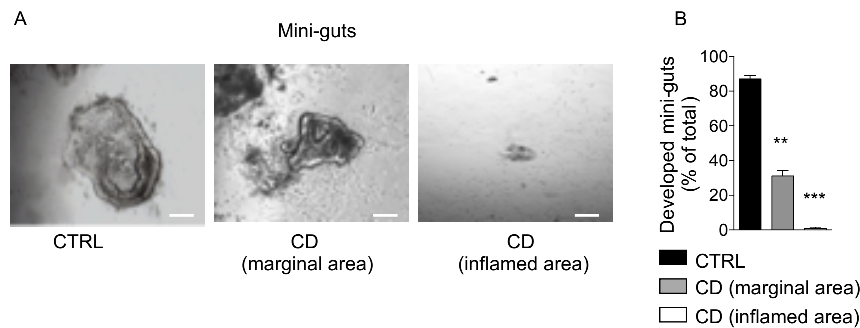

Figure 6. **A**. Representative pictures of mini-guts developed by crypts isolated from the marginal and inflamed areas of patients with CD and CTRL cultured for 8 days in vitro; 20x magnification. **B**. Bar graphs reporting the percentage of mini-guts developed at day 8 (n = 4 CTRL, n = 4 marginal areas, n = 4 inflamed areas). Data are expressed as mean ± standard mean error (SEM) ** p <0.001; *** p <0.0001.

4.4 Circulating factors play a key role in ISCs disruption in CD

The observation that the intestinal stem cells are altered both as a pool and as regenerative capacity, lead us to investigate more in details the mechanisms behind this alteration. Given the relevant role that the inflammatory pathways and immune system play in the pathogenesis of CD, we first assessed by Luminex the peripheral cytokines pattern of the patients enrolled in our study. Analysis of the serum obtained from patients with CD and non-CD (CTRLs, who were not suffering from other inflammatory or immune-mediated pathology) demonstrated a significant increase in the levels of the following cytokines: IL-8, TNF-α and IFN-γ (Figure 7).

Figure 7. Peripheral cytokines profile in CD.

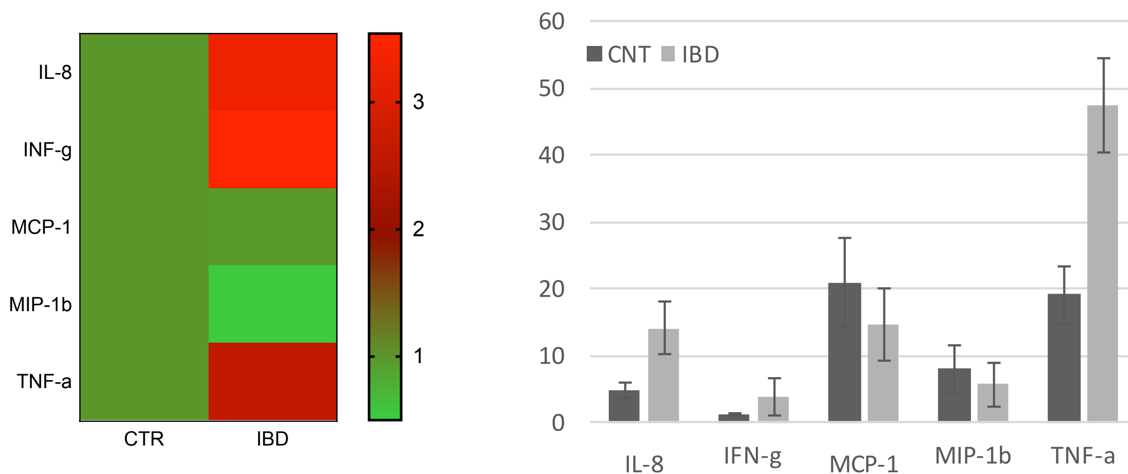

Figure 7. Heat-map and bar graphs depicting cytokines detected in serum samples obtained from patients with CD and CTRL (n = 10). Data are represented as fold change in the heat-map and as mean ± standard deviation in the bar graphs as compared to CTRL.

To further demonstrate whether cytokines found increased in the serum of CD patients may play a role in the onset/progression of the disease and affect the intestinal stem cells regenerative properties, we isolated intestinal crypts from samples obtained from non-CD patients (CTRL) and cultured them in vitro in the presence of a pool generated from the sera of 10 patients with CD for 8 days. Interestingly, the serum of patients with CD prevented the full development of the intestinal organoids, which appeared to be collapsed, and the percentage of developed mini-guts was significantly reduced (Figure 8). This may suggest that the regenerative capacities of the ISC are altered when exposed to serum of CD patients enriched of pro-inflammatory cytokines. In particular, the effect obtained by the patients' serum on the percentage of growth of the intestinal organoids was very similar to that observed for the mini-gut of patients with CD.

Figure 8. **In vitro generation of mini-guts upon CD serum exposure**.

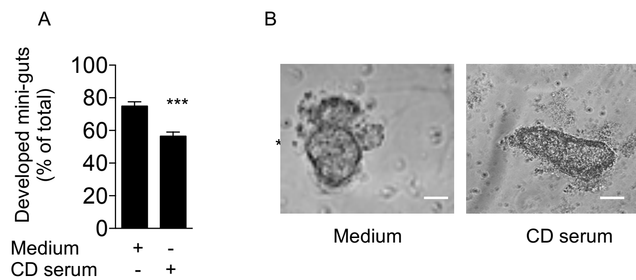

Figure 8. **A**. Bar graphs reporting the percentage of mini-guts developed from intestinal crypts isolated from CTRL samples after 8 days of culture under normal conditions or with the addition of a pool generated from the sera of 10 patients with CD. **B**. Representative pictures of mini-gut developed by intestinal crypts isolated from samples of CTRL and cultured for 8 days under normal conditions or by adding a pool generated from the sera of 10 patients with CD; 40x magnification.Data are expressed as mean ± standard mean error (SEM) ** p <0.001; *** p <0.0001.

More importantly, expression of ISC markers EPHB2 and LGR5 was significantly reduced in the mini-guts cultured with serum of patients with CD, thus emphasizing the relevant effects that the peripheral cytokines detected may have in targeting the ISCs in this disease (Figure 9).

Figure 9. **ISC markers expression in miniguts cultured with CD serum**.

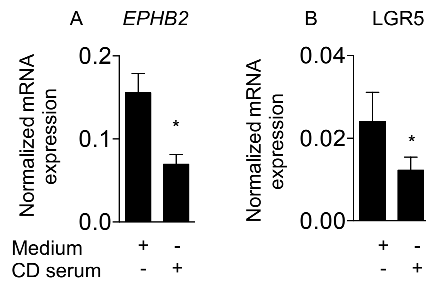

Figure 9. **A-B**. Bar graphs depicting the mRNA expression of the EPHB2 (A) and LGR5 (B) ISC markers in the mini-guts of non-CD patients (CTRL) grown in the presence of the serum of patients with CD as compared to that detected in medium. All samples were normalized for the expression of the housekeeping gene ACTB (ΔCt) (n = 3). Data are expressed as mean ± standard mean error (SEM). * P <0.01; ** p <0.001; *** p <0.0001.

CHAPTER 5

Discussion and Conclusion

5.1 Discussion

Crohn's disease is an inflammatory bowel disease that is associated with a significant reduction in quality of life due to cyclic and disabling symptoms such as abdominal pain, diarrhea, and severe complications that require hospitalization [1,30].

While several factors have been described to be associated with the onset of Crohn's disease, the mechanism behind its pathogenesis is still poorly understood. A complex interaction among genetic factors, immune system, intestinal microbiota and environmental factors may results in an abnormal inflammatory response that targets the gastrointestinal tract [1,20,31].

The current available therapeutic approaches, which mainly consist of anti-inflammatory and immunosuppressive drugs, aim to halt the excessive local inflammation, thus favoring the remission of the disease in the long-term [32-34].

More recently, conventional therapy has been associated with the use of biological drugs such as TNF-α inhibitors[32]. Despite the pharmacological advances, more than 80% of patients with CD still undergo surgery during their life due to a failure of the medical therapy or to the development of intestinal complications (stenosis, fistula, abscesses) [35].

Unfortunately, surgery is not a definitive cure for CD, with about 70% of patients showing an endoscopic recurrence of the disease at 1 year after surgery, which is of a severe degree in 25% of cases. Furthermore, about 20% of patients undergoing surgery shows a clinical relapse within 1 year from the intervention and from 30 to 70% of the patients require a new surgery within 10 years from the first procedure [36].

The high rate of recurrence and low rate of remission suggest that both surgery and pharmacological therapy available are not able to control the disease and prevent the relapse of it and further highlight the need to develop new therapeutic strategies aiming to target new players involved in the onset/progression of CD disease. Recent studies have highlighted the possible role that intestinal stem cells can play in the onset and reactivation of chronic inflammatory bowel diseases. In particular, Dotti et al. [25]and Gersmann et al. [23]

showed that a defect in the differentiation of intestinal stem cells exists in Crohn's disease and ulcerative colitis, which results in disrupted regenerative properties of the intestinal mucosa and in a reduction of Paneth and of the goblet cells, key-plyer in creating a first barrier against invading pathogens.

With the aim of unveiling the role of ISCs and their disruption in the onset of Crohn's disease, we performed a quantitative and functional analysis of the ISCs pool in a population of patients with CD who underwent surgery for intestinal complications and in a group of non-IBD controls.

5.1.1 ISC markers expression is reduced in CD

First, we performed a transcriptomic analysis of intestinal crypts extracted from samples of patients with CD, both from the marginal and from the inflamed area, and from non-IBD controls. This analysis revealed that markers related to ISCs were under-expressed as compared to non-IBD controls, in particular LGR5 and EPHB2, two membrane receptors belonging to the Wnt signaling pathway, the main regulatory pathway for the proliferation of ISC [37].

While the more pronounced reduction in ISC expression markers was evident in the inflamed area of CD patients' samples, where the mucosa was extremely

damaged, it was detectable also in the marginal zone, which was more preserved from the disease attack and inflammation, thus suggesting that the intestinal stem cell pool may be altered already at an early stage of the disease. Immunofluorescence analysis also confirmed a reduced expression of the EPHB2 protein at the level of the crypts isolated from the marginal and inflamed areas of CD patients. Analysis of LGR5 and EphB2 ISC markers in the crypts isolated from controls and patients with CD by Western blot, RT-PCR and flow cytometry also demonstrated a reduced expression of those markers in patients with CD, and in particular their near absence in the inflamed areas. Therefore, our study provides strong evidence of a significant reduction in the ISCs pool both in the inflamed and marginal areas of the intestine of patients with CD, which was, to the best of our knowledge, only detected for ulcerative colitis [24].

5.1.2 ISCs function is altered in CD

To evaluate whether ISCs were also altered in terms of function, the regenerative properties of these cells were tested using a new organ-on-a-chip generation method. The intestinal crypts obtained from the marginal and inflamed areas of patients with CD and controls were cultured on a three-dimensional matrigel support with a specific

culture medium for the development of intestinal mini-organoids, so called mini-guts. After 8 days of culture, crypts obtained from non-IBD patient showed normal self-renewal abilities to develop mini-guts, while those of CD patients fail to grow histologically normal mini-guts, particularly if grown from the inflamed area. This data reveals that in the CD the reduced ISCs pool is paralleled with a functional disruption, may be responsible for the inability of the mucosal barrier to respond to the attack of intestinal pathogens.

5.1.3 Circulating factor/cytokines mediate reduction in ISCs pool in CD

It has been already established that circulating factors may play a role in mediating ISCs damaging in intestinal disorders[27]. Moreover, systemic and local inflammation plays a significant role in the onset and progression of the CD disease, thus suggesting that peripheral cytokines may represent a trigger in promoting ISCs injuries.

We first demonstrated by using Luminex that patients with CD have a peculiar peripheral cytokines profile with increased levels of IL-8, TNF-α and IFN-γ as compared to non-IBD patients, as already shown in other studies [38,39]. However, the link between those increased levels of cytokines and regenerative properties of intestinal mucosa have never been explored in CD. Therefore, to evaluate the

effect of these inflammatory mediators and other molecules contained in the serum of patients with CD on the regenerative properties of ISC, intestinal crypts extracted from non-IBD samples were cultured in vitro in presence of a pool generated from the sera of 10 patients with CD for 8 days. The deleterious effects of circulating factors and cytokines on ISCs pool and regenerative properties was demonstrated by a significant reduction in mini-guts development when exposed to CD serum. This was further confirmed by a reduced expression of EPHB2 and LGR5.

5.2 Conclusion

Our study revealed that a defect of the intestinal stem cell pool and of its regenerative properties exists in CD, as demonstrated by the reduced expression of ISC markers and failure in mini-guts growth. ISCs are profoundly disrupted in the inflamed area, where ISCs are almost absent and mini-guts development is completely abrogated, but it is also detectable in crypts obtained from the marginal area, thus suggesting that the ISCs defect may be present at early stages of the disease. Interesting circulating factors/cytokines play a significant role in altering the ISCs pool as mini-guts grown with serum

of CD patients is prevented. We may thus conclude that ISCs are altered in CD and inflammatory response may trigger this defect, thus halting regeneration of the mucosa and enabling pathogens entrance to maintain the inflammation.

Finally, our data also confirm that ISCs may become a new therapeutic target of Crohn's disease in the future by further investigating the mechanisms by which inflammatory mediators, and other molecules, significantly increased in the serum of patients with CD induce ISCs damage.

5.3 Study limitations and future perspectives

The results obtained need to be confirmed on a larger sample of patients, as the small sample size may have limited the statistical power of our analysis, making it more difficult to arrive at strong conclusions. A larger sample could also include subjects with Crohn's disease in remission phase, in addition to the Crohn's disease subjects in the active phase enrolled in our study. This would allow us to assess whether the intestinal stem cell pool defect and the impairment of their regenerative capacity is preserved in absence of inflammation, or if the latter "reprograms" the ISC inducing permanent alterations.

Current approaches to treat Crohn’s disease such as anti-inflammatory/immunotherapies and surgery are limited in their ability to prevent relapses and have questionable long-term effects. Here, we discover that protecting the ISCs pool in Crohn’s disease may be of clinical relevance, thus improving clinical outcomes and reducing patients’ hospitalization.

CHAPTER 6

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