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Review

Modeling inflammatory bowel disease by intestinal organoids

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Abstract: Inflammatory bowel disease (IBD) is a chronic and relapsing disease caused by a dysregulated immune response to host intestinal microbiota that occurs in genetically predisposed individuals. IBD encompasses two major clinical entities: ulcerative colitis (UC), which is limited to the colonic mucosa, and Crohn disease (CD), which might affect any segment of the gastrointestinal tract. Despite the prevalence of IBD is increasing worldwide, therapy remains suboptimal, largely because the variability of causative mechanisms, raising the need to develop individualized therapeutic approaches targeted to each individual patient. In this context, patients-derived intestinal organoids represent an effective tool for advancing our understanding on IBD' s pathogenesis. Organoid 3D culture systems offer a unique model for dissecting epithelial mechanisms involved IBDs and test individualized therapy, although the lack of a functional immune system and a microbiota, two driving components of the IBD pathogenesis, represent a major barrier for their exploitation in clinical medicine. In this review we have examined how to improve the translational utility of intestinal organoids in IBD and how co-coltures of 3D or 2D organoids and immune cells and/or intestinal microbiota might help to overcome these limitations.

Keywords: Organoids; IBD; Inflammation; Target therapy; microbiota; immune system

1. Introduction

Inflammatory Bowel diseases (IBD) is growing healthcare burden worldwide with a steadily increasing incidence [1]. IBD is chronic, relapsing, inflammatory disease of the gastrointestinal tract, encompassing two major clinical entities: *Chron's Disease* (CD) and *Ulcerative Colitis* (UC), although a third condition, known as an *indeterminate colitis*, might affect up 15 % of IBD patients [2]. The IBD's pathogenesis is multifactorial with major difference between the two main disease. Generally, CD is associated to a transmural inflammation and affects any part of the gastrointestinal tract (most frequently the terminal ileus, the cecum or the perianal region) in a discontinuous pattern. Histologically, CD shows a thickened submucosa, fissuring ulceration and granulomas. CD, is commonly associated with clinical complication such as abscesses, fistula and stenosis. Unlike CD, UC inflammation affects the rectum or the colon in a continuous pattern and the inflammatory process is limited to the mucosa and submucosa with crypt abscesses [3]. In both diseases, there is a robust inflow of neutrophils and macrophages in the intestinal mucosa, leading to secretion of a variety of pro-inflammatory cytokines that promotes the recruitment of immune cells, which ultimately results in a mucosal inflammation and ulcerations [4,5].

The highest annual incidence of UC is 24.3 per 100.000 habitants -years in Asia and Middle East, and 19.2 per 100.000 habitants in North America. The highest annual incidence of CD is 12.7 per 100.000 habitants in Europe and 20.2 per 100.000 habitants in North

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America, but is lower in Asia and Middle East. The highest reported prevalence values for IBD were in Europe: UC 505 per 100.000 habitants, CD 322 per 100.000 habitants [6]. Since the beginning of 21st century, the incidence rate in developing countries such as Asia, Africa and South America has witnessed a substantial increase, likely as the result of their growing industrialization [6].

More than two thirds of IBD cases are diagnosed before the age of 36 and in 25% before the age of 20. The CD shows the incidence peak between 15 and 40 years, although a second peak in incidence is observed between 50 and 60 years. The UC incidence is higher in individuals of 20-40 years, but it can appear at all ages. Uncommonly, a minor fraction of IBD patients develop intestinal inflammation within the first 6 years of life, this subgroup of patients is generally affect by a more severe disease, characterized clinically by diffuse colon involvement (pancolitis) and severely ulcerating and fistulising disease, reflecting a more robust genetic involvement and specific genetic architecture [7]. Over the last few decades there has been a progressive reduction in the age of diagnosis [8]. To date, IBD treatments have limited efficacy in a large group of patients. The mechanisms of IBD have yet to be elucidated and are difficult to dissect in individual patients. IBD is the result of a complex interaction between environmental factor, the intestinal epithelial barrier (IEB), the immune system and the microbiota on an individual genetic background.

2. Pathogenesis of IBD

IBD is multifactorial disease. A consensus hypothesis is that various combined environmental factors act simultaneously on a genetically predisposed individual leading to an alteration of the intestinal microbiota or a dysregulation of the innate and adaptive immune response to modified microbiota, causing a chronic state of dysregulated mucosal immunity and give rise to a chronic intermittent inflammation of the gastrointestinal tract [9,10] (Figure 1). Environmental factors play an important role in the pathogenesis of IBDs. Numerous environmental factors are established IBD risk factors such as air pollution [11], smoking [12] [13], diet, antibiotics [14] , drugs [15] and social stress [16] [17]. Additionally, early childhood events such as birth (Caesarean or natural birth), breastfeeding and exposure to antibiotics are potential IBD risk factors [18]. Moreover, the quality and composition of food might represent another important causative factor in IBD [19]. Thus, while consumption of a diet enriched in fruits and vegetable has been associated to a reduced risk to develop a CD, the opposite has been noted for those diets enriched in fat and sugar and artificial food additives prevalent in Western diets, which might promote intestinal inflammation interfering with the function of intestinal epithelial barrier. Furthermore, several studies have shown that vitamin D deficiency is common in IBD patients [34].

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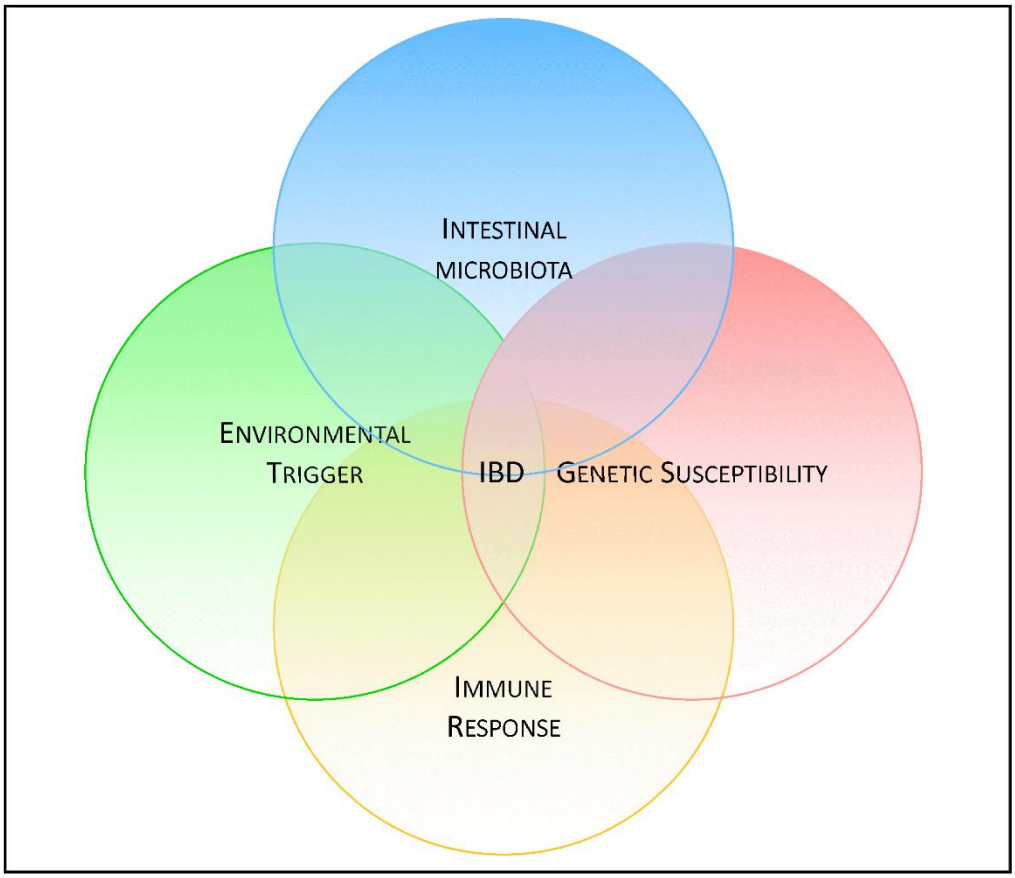

Figure 1

Figure 1. Inflammatory Bowel Disease aetiology. The cause of IBD is complex and multifactorial, a consensus hypothesis is that, in genetically predisposed individuals, various environmental agents (e.g. diet, smoking, pollution, drugs) and exogenous factors (e.g. intestinal microbiota alterations) promote a dysfunctional immune response determining intestinal inflammation.

Physiologically, the innate immune system responses are the first line of defence against potential pathogens. They are mediated by the complex interplay between the intestinal epithelial barrier (IEB), the Pattern Recognition Receptors (PRRs),the Antimicrobial Peptides (AMPs) and a wide variety of cell subset including intestinal epithelium cells (enterocytes and specialized cells), immune cells located in *lamina propria* (dendritic cells (CDs), macrophages, innate lymphoid cells (ILCs) and Natural killer T (NKT) [20]. These rapid responses help to build a bridge to connect primary response to specific immune responses through the induction of the adaptive immune system and effector T cells (Figure 2) [21].

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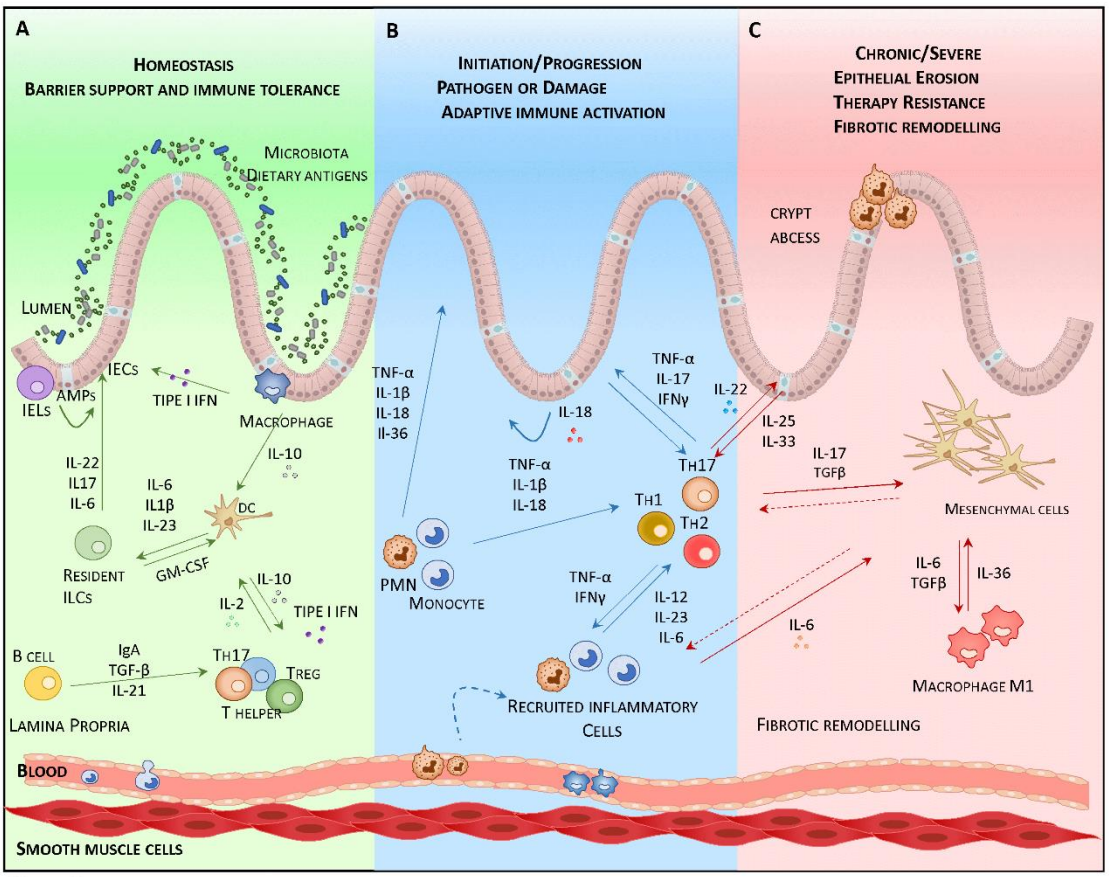

Figure 2

Figure 2. IBD immunopathogenesis. A) The green panel shows in a homeostasis state the cross-talk between IECs and resident innate or adaptive immune cells. Particularly, AMPs-producing IELs, IgA- secreting B cells, macrophages, dendritic cells and T cells strength the epithelial barrier by sampling luminal content and maintaining tolerant state toward bacterial and dietary antigens. **B)** The blue panel illustrates the onset and progression of IBD, the interruption of IECs-immune cells cross-talk leads to the inflammation, driven by secreted pro-inflammatory cytokines. If this initial inflammation is not resolved, pro-inflammatory cells are recruited in situ and secreting cytokines activate pathogenic responses by adaptive Th1 / Th2 and Th17 cells. **C)** In the pink panel, the chronic inflammation is characterized by a substantial pro-inflammatory response driven by adaptive immune, which may evolve over time towards mesenchymal cell activation, thus the imbalanced repair leads to fibrotic remodeling in advanced stage IBD. Abbreviations: DC, dendritic cell; Th, Thelper; ILC, innate linphoid cells; IEL, intraepithelial lymphocyte; PMN, polymorphonuclear leukocyte;GM-CSF, granulocyte-macrophage colony stimulating factor; TNF, tumor necrosis factor IFN, interferon; Ig, immunoglobulins; IL, interleukin; TGF, transforming growth factor.

There is no doubt that IBD has a robust genetic component. The Genome-wide association study (GWAS), the whole genome sequencing (WGS), and the use of customized SNPs array have identified a variety of polymorphisms in gene involved in intestinal function and immune response, that might explain the aberrant and improper immune response that cause a chronic intestinal inflammation in IBD patients. More precisely, 242 non-overlapping genetic susceptibility loci, 30 of which are shared between CD and UC, hve been identified [22,23] and 50 genes are associated with very-earl-onset inflammatory disease (Figure 3). In this regard, meta-analysis of the discovered genes and genetic loci shows an increasing spectrum of human monogenic diseases that can manifest IBD-like intestinal inflammation, and a substantial proportion of patients with those genetic defects present with very early onset of intestinal inflammation [24]. Gene polymorphisms identified hit critical pathways in preserving intestinal function and homeostasis, such as epithelial barrier function and mucosal defence mechanisms (e.g NOD2, CARD9,

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FCGR2A), innate immune response, affecting granulocyte and phagocyte activity, cell migration, autophagy (ATG16L1, IRGM), and also adaptive immune response regulation, disturbing T-cell activation, T-helper-17 (Th17) cell differentiation, and T-cell and B-cell regulation [25].

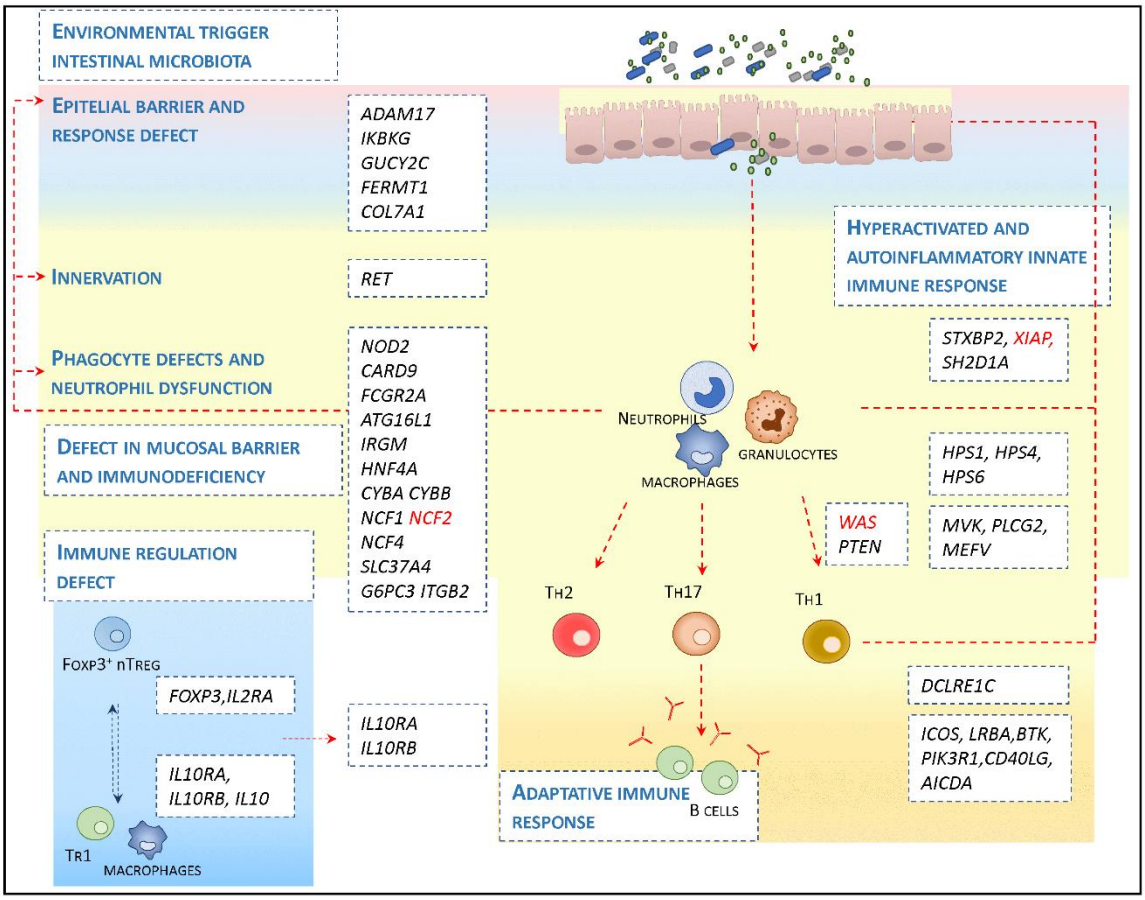

Figure 3

Figure 3. Genetic landscape of IBD. Genetic polymorphisms affect genes involved in intestinal epithelial barrier function and gut innervation, leading to increased intestinal bacterial translocation across the epithelial barrier, and triggering an aberrant immune response. Additionally, polymorphisms can involve genes important for neutrophils activity, phagocytosis and bacterial killing, T and B cell responses, contributing on one hand to intestinal inflammation via autoimmunity on the other hand with an aberrant immune response towards the microbiota bacteria. Furthermore, the defect of FOXP3⁺ Treg cells activation or an alteration in anti-inflammatory interleukin (IL)-10 pathway may produce an improper immune response due to lack of regulation of the pro-inflammatory immune reactions.

The *Nucleotide 2-binding oligomerization domain* (NOD2) also called *caspase recruitment domain 15* (CARD15) is the first gene to be associated with CD [26]. This gene is mutated in over one third of CD patients [27]. Three different single nucleotide polymorphisms (SNPs) have been identified, which role is still controversial. Available research suggests that they are loss-of-function mutations that lead to reduced activation of the innate immune system. Additionally, CD patients associated with the 1007fs mutation in the NOD2 gene show a much more exacerbate disease phenotype than other ones, while the R702W and G908R mutations lead to increase inflammatory cytokines secretion [28]. The NOD2 gene encodes a protein member of the *Nod-like cytosolic receptor* (NLR) family, which together with the membrane *Tool like receptors* (TLR), are the two much important detection systems for microbes [28]. Moreover, it was demonstrated that NOD2 knock-out mice show a compromising of mechanisms of primary defence such as reduced bacterial clear-

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ance, number of goblet cells, anti-microbial molecules and protective mucins and an abundance of non-commensal potential pathogen bacteria, these alterations can lead to intestinal dysbiosis and inflammation [29]. NOD2 has also been implicated in the autophagy initiation [30]. Moreover, following the NOD2-MDP bond, it acts as a molecular scaffold for the arrangement of the autophagy machinery through its interaction *with Autophagy Related 16 Like 1* (ATG16L1). Atg16l1 encodes to a structural protein of immune cell autophagosome, essential for all forms of autophagy. Autophagy is an important process in preserving intracellular homeostasis, in which phagosomes embodies bacteria, microbial antigens, cytosolic and organelle content and all products destined for degradation, fuse with degradative enzyme containing lysosome forming autophagolysosomes [25]. Atg16l1 polymorphisms reduce the phagosome formation and are also linked to CD [31]. The variant encoding the T300A substitution in Atg16l1 increases the susceptibility of the ATG16L1 protein to caspase-3 cleavage and to loss of functions, leading to bacterial and cellular waste products accumulation [32]. *Immunity Related GTPase M* (IRGM) is another important factor in autophagy process. Irgm codifies to a member of immunity-related GTPase M family. Polymorphisms in Irgm lead to reduced protein expression[33,34]. The GWASs have identified several SNPs in Interleukin (IL)-23 Receptor (R), with high association with IBDs [35,36]. The IL23R gene encodes for a receptor subunit of IL-23, a pro-inflammatory cytokine with a key role in driving early response against microbes. IL23 is involved in the T helper (T_H)-17 cells differentiation. Another important aspect is the involvement of the HLA alleles in disease susceptibility. HLA alleles are the strongest genetic component for many autoimmune diseases. Whereas the functional role of some of the most strongly associated genes such as NOD2, ATG16L1 or IL23R becomes increasingly clear, the functional impact of most key candidate genes within IBD loci is currently not understood [37]. Thus, polymorphism of IL23R, IL12B, Janus kinase 2 (JAK2) and Signal Transducer and Activator of Transcription 3 (STAT3) have been identified in both UC and CD [38,39].

Additional genetic factors that might play a role in IBD development are linked to maintenance of the integrity of the intestinal barrier. In the intestine, a monolayer of intestinal epithelial cell (IEC) separates the intestinal microbiota and luminal antigens from immune cells in the lamina propria. Therefore, defects in the epithelial barrier might predispose to intestinal inflammation. In this contest, epithelial dysfunction due to the deficiency of *Adamalysins* (ADAM-17), a disintegrin metalloprotease, causes the neonatal onset of non-bloody later bloody diarrhoea. ADAM17 cleaves different substrates, such as tumour necrosis factor α (TNFα), L-selectin or epidermal growth factor (EGF) [40]. In addition, Hirschsprung's disease patients are affected by enterocolitis, caused by increased bacterial translocation, suggesting a potential role for the germline mutation of RET , a gene involved in neural maturation [41]. Additionally, a recent GWAS study has identified three susceptibility loci related to the epithelial barrier function in IBD patient, such as the *Hepatocyte Nuclear Factor 4 Alpha* (HNF4A) regulating cell junction expression, the *Cadherin 1* (CDH1) encoding E-Cadherin, the principal adherent junction component and finally the *Laminin Subunit Beta 1* (LAMB1) encoding a protein expressed in the intestinal basement membrane [42], *G Protein Subunit Alpha 12* (GNA12), *X-Box Binding Protein 1* (XBP1) or *Collagen Type VII Alpha 1 Chain* (COL7A1) [24].

To highlight the relevance of these genetic factors, several syndromes with hyper- and autoinflammatory defects have been linked to genetic disorders resulting early onset IBD or IBD-like intestinal inflammation. These include either genes encoding for regulatory factors involved in maintenance of neutrophils and intestinal innate barrier functions such as WAS, LRBA, BTK, CD40LG or FOXP3 [43], but also genes such as XIAP, whose defects are the causative factors for the very early onset IBDm, a clinical subset of IBD patients that develop a severe fistulising perianal colonic phenotype affecting up to 20% of patients. XIAP is involved in NOD2-activated Nf-κB signalling and plays a central role in this inflammatory network [44]. The phagocyte NADPH oxidase (phox) complex is another important regulatory factor that mediates the killing of ingested intracellular microbes. Mutations in any of the five phox complex molecules, such as CYBB, CYBA, NCF1,

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NCF2 and NCF4 leads to immunodeficiency and may trigger intestinal inflammation [45]. Interestingly, a missense variant in NCF2 is associated with very early onset IBD [46]. In addition, ICOS, LRBA and CD40LG defects cause alteration in T cell response and BTK, AID, LRBA SKIV2L and TTC37 mutations alter B cell selection and activation [47].

Finally, while the development of IBD is generally linked to excessive inflammation, there is evidence that also a compromised immune function might result in IBD. The later includes patients with neutropenia or neutrophil dysfunction (SLC37A4, G6PC3), or Leukocyte Adhesion Deficiency (ITGB2) show CD-like immunopathology [48]. Finally, an imbalance between pro-inflammatory factors and regulatory T cells including natural regulatory cells (nTreg) and Tr1 cell are present in the inflamed intestine of IBD patients. In this contest the essential function of IL-10 signalling in intestinal immune homeostasis has been widely recognised [49]. It is widely reported that monogenic defects in IL-10 and its receptor genes IL10RA and IL10RB, are involved in intestinal inflammation development and has been associated as well with early-onset UC [50,51]. Another important role in the IBD pathogenesis is contributed by epigenetic modifications. These changes occur in several immune-mediated inflammatory diseases (IMIDs), such as cardiovascular diseases and cancers. In IBD patients, epigenetic modifications induced by environmental factors such as smoking and diet are frequently documented in either DNA methylation and transcription of non-coding RNAs (e.g. microRNAs) [52]. DNA methylation refers to the covalent addition of a methyl group to the cytosine nucleotide, often in the dinucleotide sequence of cytosine phosphate guanine (CpG)-islands. CpG-islands are present in approximately 1-2% of the genome and usually appear to have low transcriptional activity. The increase in DNA methylation has an impact on the risk of onset and on the progression of the disease. MicroRNAs (miRNAs) are a group of non-coding RNAs, which regulate gene expression. They have a role in the T lymphocytes differentiation, in T_H17 signalling pathway and in autophagy (Figure 3).

In conclusion, it is expected that identification of these biomarkers should increase the accuracy in the stratification of IBD patients, helping to predict the clinical course of the disease and define novel therapeutic approaches.

3. Traditional model to study IBD

As mentioned, IECs play a central role in the IBD pathogenesis and several therapies promote epithelial barrier functions, including mucin production or maintenance of barrier integrity. However, only a few therapies specifically and directly target the IECs, and there is a great unmet need for the discovery of new treatment in this area. To this end pre-clinical investigations are largely based on the use transformed intestinal cell lines are used, such as Caco2, enterocyte-like cells, or HT29, that are Goblet-like cells [53]. However, these colture consist of only one cell type and cannot recapitulate neither cell-cell interactions nor interactions with the extracellular microenvironment that occur in a complex organism (Figure 4) [54]. Furthermore, since these cell lines are derived from malignant cells, they have different properties from those of healthy cells, including epithelial integrity, cell polarity and adhesion and cell division. Similarly, while several murine IBD models are currently available, they include chemically induced model (Acetic Acid, TNBS, DSS, Oxazolone), and genetically modified knockout models specific for a gene of interest (e.g Il-10$^{-/-}$, Il-2$^{-/-}$, Ship1$^{-/-}$, Gαi2$^{-/-}$, Runx3$^{-/-}$, TLR5$^{-/-}$, Mdr1a$^{-/-}$). Each model has its own advantages and has contributed over time to elucidate part of the pathogenic mechanisms of IBD. For example, the TNBS model faithfully reproduces the pathogenic characteristics observed in CD. The IL-10$^{-/-}$ model has provided significant contributions to clarify the mechanism of probiotics in IBD. The DSS model is able to recapitulate the human UC and was useful for dissecting the pathogenic mechanism associated to inflammation. However, none of these models can fully reflect human IBD and a unique model capable of reproducing intestinal physiology and the interactions of IELs with the microbial and immune counterparts would be needed (Figure 4). In order to better understand the pathogenic mechanisms of IBD and develop a specific and personalized cell therapy for IBD patients [55]. Because all the above limitations, innovative methods are needed

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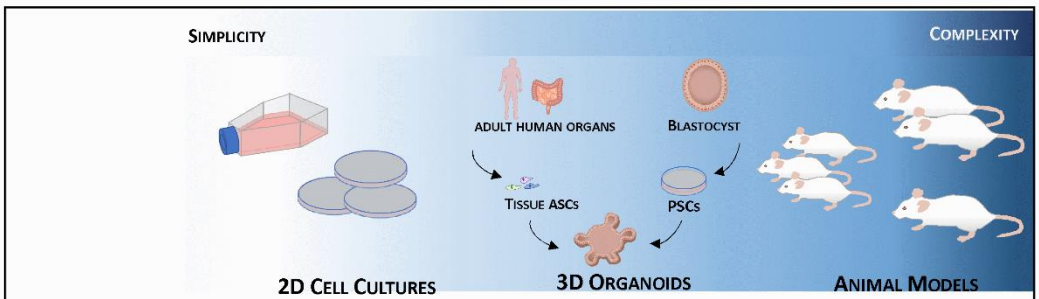
and organoids, 3D cultures systems, giving rise to all the differentiated lineages that populate the intestinal crypt [56], even to the less represented ones, such as enteroendocrine cells, tuft cells or M cells, have gained growing attention [57,58].



	2D Cell Cultures	3D Organoids	Animal Models
Model human physiology and disease	Limited	Partially	Yes
Vascularization and immune system	No	No	Yes
Cell heterogeneity	No	High	High
Manipulability	Excellent	Good (may have experimental variability)	Limited
Reproducibility	High	Low	Low
Genome editing	Yes	Yes	Yes (may require generation of ESCs)
Bio banking	Yes	Yes	Yes (only at cellular level)
High throughput screening	Yes	Yes	No

Figure 4

Figure 4. Comparison of Traditional Models and 3D Organoids cultures. Organoids can be established from adult stem cells (ASCs) or from pluripotent stem cells (PSC). They are a bridge between conventional two-dimensional culture and animal models, organoids have enormous advantages such as experimental manipulability and emulate biologic complexity which make them an important platform modelling human physiology or diseases, and high-throughput screening.

4. Organoids 3D culture systems

Organoids are 3D culture system derived from either embryonic and induced pluripotent stem cells (ESCs or iPSC) or from adult stem cells (ASCs) normally resident in tissues. These cells, seeded in a matrix with specific factor, self-organize and undergo to differentiation, producing epithelial functional cell types, having the ability to undertake some functions of the native organ epithelium and recapping the complex cellular organization observed *in vivo* [59]. *Ex vivo* organoid cultures bridge the gap between *in vitro* models defined by classic monolayer 2D cell cultures and in *vivo* animal systems. Bowel organoids can give rise to all the differentiated lineages that populate the intestine epithelium, even the less represented ones, such as enteroendocrine or tuft cells [60]. Furthermore, they avoid the costs, long-time and xenogeneity problems that may be linked to animal models (Figure 4) [61]. Finally, ASC-derived intestinal organoid cultures can be cryopreserved with high recovery rates upon thawing. This practice facilitates the creation of living biobanks of patient-derived organoids (PDOs) which can be very useful for translational studies since that accessibility to patient samples is often a limiting factor [62].

4.1. Lgr5 $^{+}$ adult intestinal stem cells

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In the physiological state the intestinal epithelia are functionally polarized. Moving from the lumen to the lamina propria several structures are encountered, the first of which is the mucus layer, that protects the IEC from gut microbiota and dietary antigens. On the other side, the mucus layer protects from epithelial damage and intestinal inflammation. It is made up of an outer and an inner mucus layers [63]. The first stratum appears less viscous than the inner one and contains mucin (MUC2), a low concentration of antimicrobial peptides (AMP) and commensal bacteria. The innermost mucus layer is tightly adherent to the IEC, rich in antimicrobial peptides and sterile. In CU patients the inner mucus layer is non-sterile and his structure is altered, and a reduced expression of MUC2 was found in MC patients [64]. On the other side of intestinal barrier, the basolateral side, is located in the *lamina propria,* where various immune cell subsets sense the antigens migrated from gut lumen. The increase in intestinal permeability causes a greater migration of the luminal content into the *lamina propria*, exacerbating the primary immune response [65].

The integrity of the intestinal barrier is preserved by tight junctions, adherent junctions and desmosomes (Figure 5). IECs are constantly exposed to potentially dangerous external agents and they are replaced every 2 or 3 days thanks to adult stem cells (ASC) proliferation and differentiation. ASCs are located in invaginations of the epithelium, known as intestinal crypts. ASCs move along the villi apical side ensuring the IECs turnover [66] [67]. The Leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5 +)-ASCs guarantee this constant renewal. Lgr5 $^{+}$ ASCs are located at the base of the typical tubular invaginations spread in the intestinal epithelium, known as crypts. These structures ensure the protection of epithelial cells from luminal factors. The stem niche is defined by the secretions of the Paneth and mesenchymal cells. These secretions include the Wntβ-catenin factor. Differentiation of crypt cells occurs in response to the inverse gradient relationship between Wnt and Bone morphogenetic proteins (BMP). Wnt concentrations are higher in deep-crypt and the BMP increases in the apical side of the cript towards the lumen. More precisely, in the small intestine epithelium, Wnt induces Ascl2, which promotes the differentiation of ASC Lgr5^{+} in transit amplifying cells (TA). These cells move from the base towards the apical side of the crypt. Since Paneth cells are deep within the crypts, Wnt decreases as the TA cells rise towards the lumen, here the concentration of BMP increases, allowing the TA cells to differentiate into mature cell phenotypes. Factors such as Noggin and Gremlin inhibit bone morphogenetic protein (BMP), inducing TA cell differentiation as illustrated in Figure 5 [56,68]. This system ensures that a specific differentiated cell is generated when required and that old cells are promptly removed.

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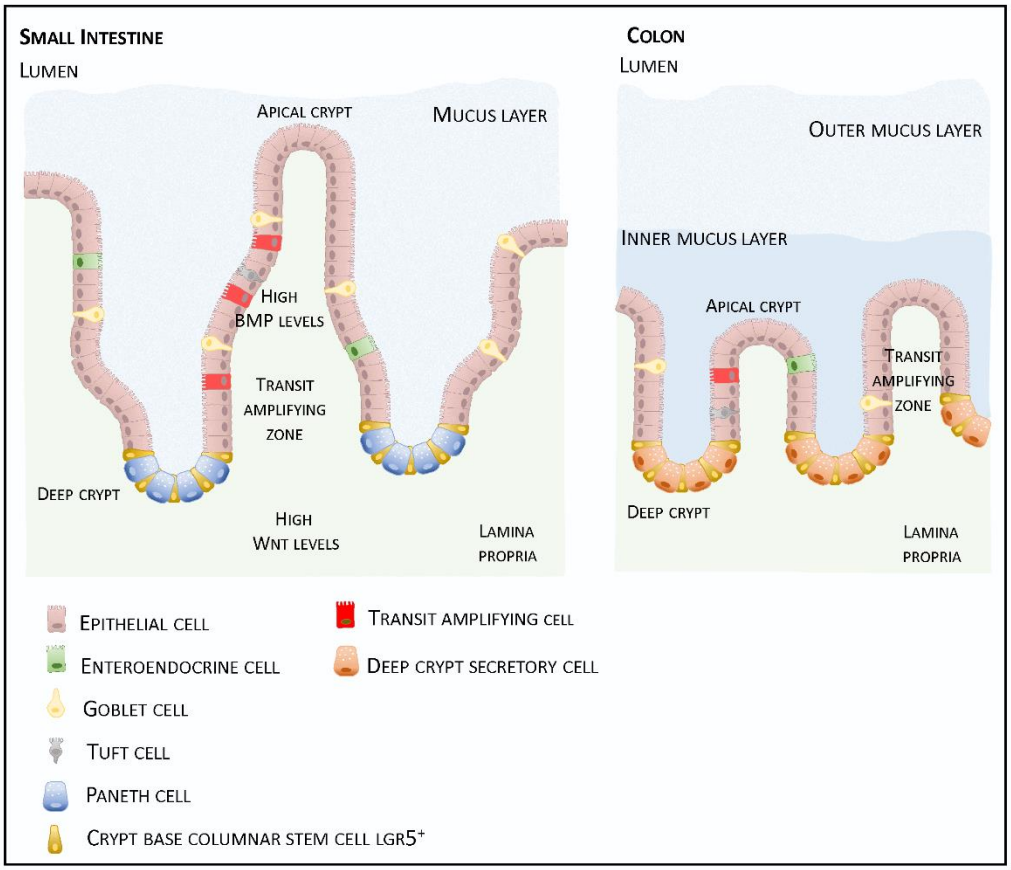

Figure 5

Figure 5. Anatomy of the intestinal epithelium. A single layer of intestinal epithelial cells (IECs) protects intestinal mucosa from environmental antigens, and provides a physical and functional barrier. All of the lineages of specialized IECs originate from the differentiation of the crypt base columnar stem cells LGR5+. Their differentiation occurs in response to Wnt/BMP gradient different in deep or apical side of the crypt. Particularly, levels of Paneth-cells secreted-Wnt are higher in the deep crypt, where cells are located, and decrease in the apical side of the crypt, where BMP levels are high. Thus, in the deep crypt stem cells divide to give rise to more proliferative daughter cells, the transit amplifying cells (TA), which move upwards to the crypt and differentiate owing to the decreasing gradient of Wnt. IECs that reach the tip of the crypt undergo apoptosis and are then waste to the lumen. This entire cycle typically lasts 2–3 days. During their migration, IECs differentiate into absorptive enterocytes, mucus-producing goblet cells, hormone-secreting enteroendocrine cells, antiparasitic tuft cells and Paneth cells (small intestine) or deep crypt secretory cells (colon). Paneth cells migrate downwards to the base of the crypt, where produce essential stem cell growth factors.

4.2. Intestinal organoid culture systems

The first intestinal organoid cultures were established from Lgr5 + ASCs isolated from mouse bowel crypts [56]. To date intestinal organoids are obtained either from single enterocytes, Paneth cells, goblet cells, enteroendocrine and tuft cells (Figure 6).

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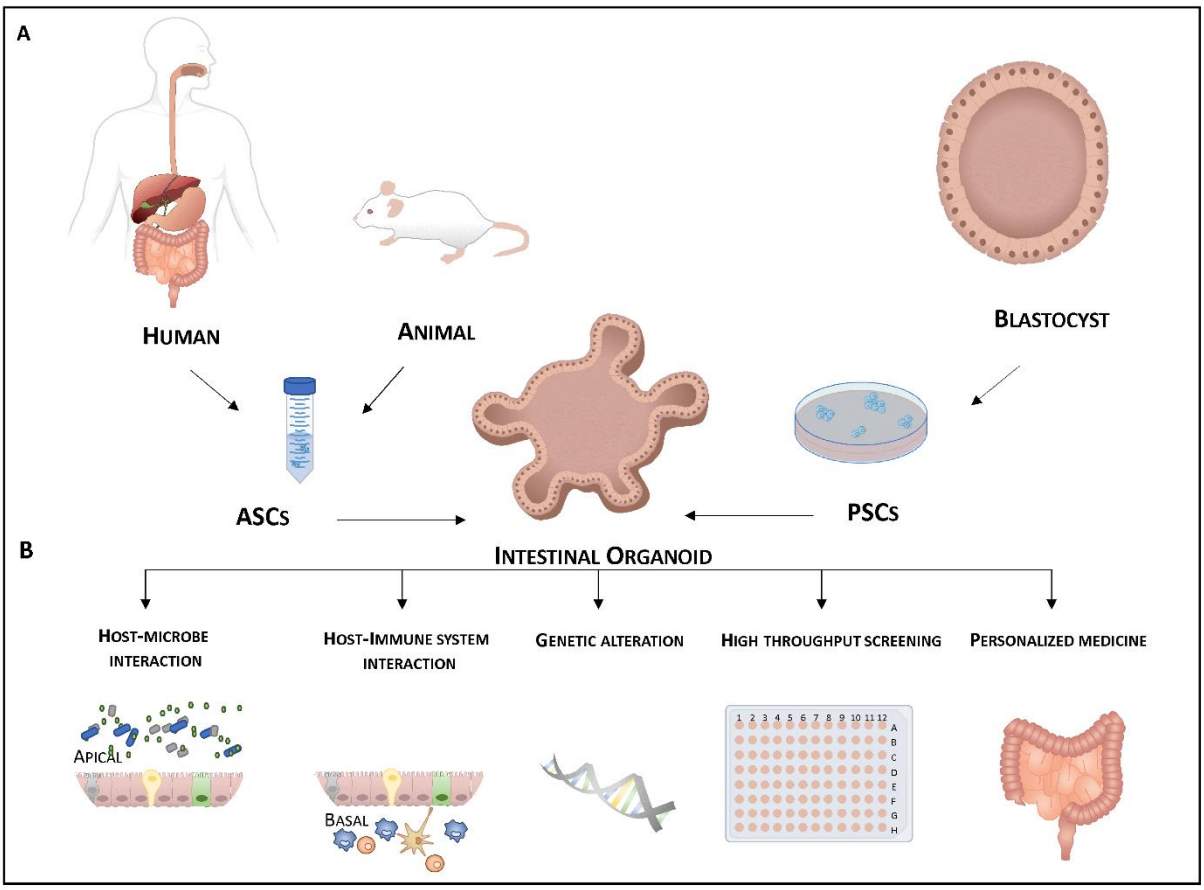

Figure 6

Figure 6. Establishment of intestinal organoid and applications. Human or murine intestinal organoids can be established from ASCs derived by intestinal biopsies or tissue, or from iPSCs. The applications of 3D intestinal organoids are numerous. They are a useful platform to recap and study host-microbe or immune system interaction and modelling inflammatory disease. They allow to study genetic alteration and drug screening directing us moreclose to a personalized medicine.

Lgr5⁺ ASCs or crypts isolated from mouse or human tissue. Crypts or Lgr5⁺ ASCs may be induced to differentiate into organoids holding all cell types of IECs, containing mature iPSC-derived organoids can also produce stromal cell types such as myofibroblasts and smooth muscle cells [69,70]. They summarize the spatial arrangement and polarity observed in the intestinal mucosa, however they can acquire genetic and epigenetic mutations during the culture method, require meticulous maintenance and initially recap fetal tissue. In contrast, ASC-derived intestinal organoids are genetically and epigenetically stable, and they retain the genetic profile and also the transcriptional and epigenetic landscape of the primary tissue from which they are derived [71]. Intestinal organoids are able to perform functions of the native tissue, including endocrine and paracrine secretion, molecular transport, absorption and contraction. They can be easily established by human tissue, offering a more accessible tool for the researchers [69].

The most common and advantageous method for the intestinal organoid establishment is the isolation of the intestinal crypts. Although several protocols exist for crypts isolation [72], the common feature is the incubation of biopsy specimens in a Ca^{2+}-free Ringer solution, followed by mechanical agitation. Isolated intact intestinal crypts, containing Lgr5⁺ ASCs, are seeded in a matrix, such as Matrigel, containing high levels of laminin, characteristic of the stem cell niche, so ASCs can be grown in a media enriched with niche specific growth factors such as Wnt3A, R-spondin, Noggin and EGF [73]. Thus, ASCs survive, avoiding anoikis and proliferate. The crypts then close, form and develop into a sphere-shaped structure, known as spheroid that represents an intermediate differentiation state. Over the next few days, the cells that make up the spheroids proliferate

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and differentiate into functional specialized gut cells. Lgr5+ ASCs migrate to different areas of the spheroid, generating more proliferation sites, thus expanding the culture [72]. Mature organoid structure consists in an apical and a basal side of the intestinal epithelium. The apical side is projected towards the inside of 3D structure, it emulates intestinal lumen, on the contrary the basal side faces towards the outer side of the structure and interacts with matrix components (Figure 7 A). However, there aren't microbiota bacteria in lumen neither the *lamina propria* with immune cells in the basolateral side as in *in vivo* system.

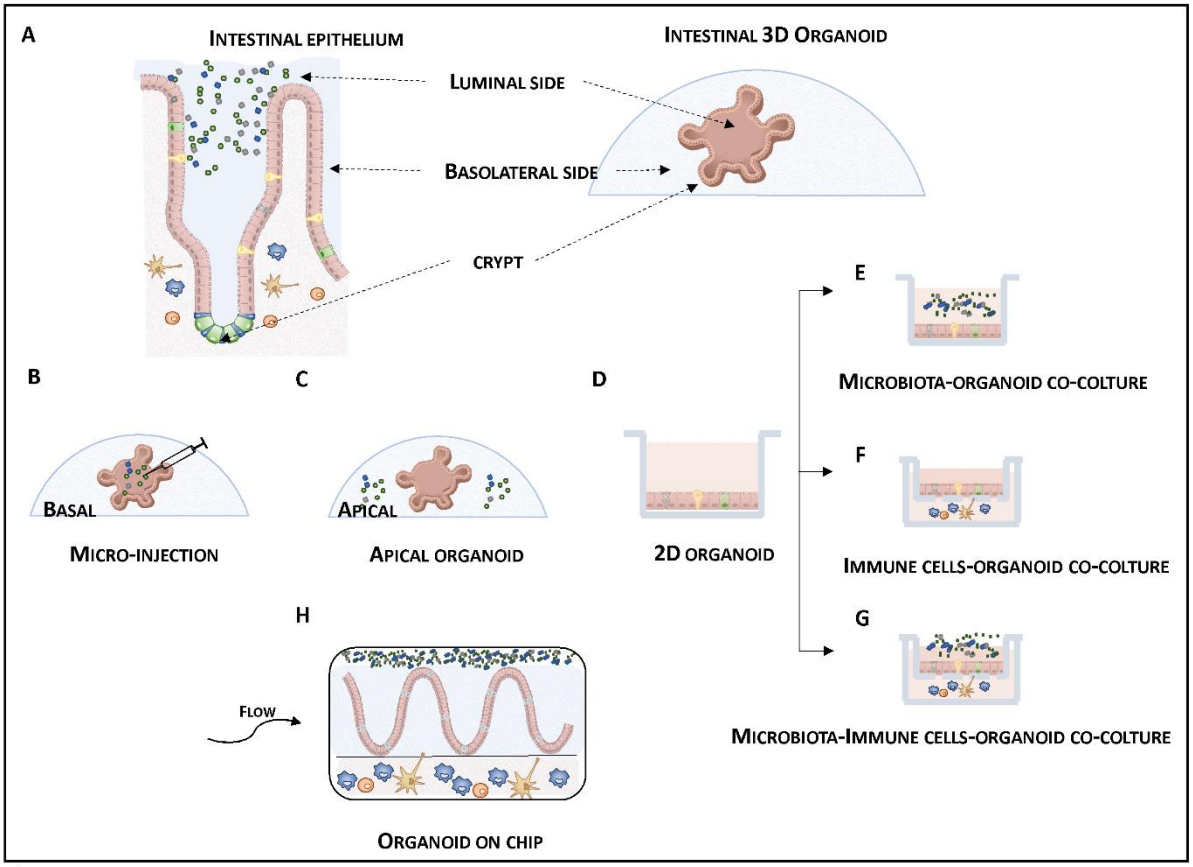

Figure 7

Figure 7. 3D organoids and organoid-derived models. A) The intestinal epithelium and the 3D intestinal organoid model. Organoids can be plated on Matrigel® or collagen-coated surfaces. Organoids can be used as 3D or 2D cultures. **B)** Micro-injection method of 3D organoid **C)** Apical organoids **D)** 2D monolayer cultures **E)** Microbiota-organoid co-colture **F)** Immune cell-organoid co-colture **G)** Immune cell-Microbiota-organoid co-colture **H)** Organoid on chip**.**

Mature organoids can then be mechanically disrupted and passaged and maintain genetic similarity with their cellular counterparts *in vivo*, even after prolonged culture. Particularly, organoids can be set up both from small intestine tissue and it referred to as enteroids, and from colon tissue they can be similarly called colonoids. Colonoid cultures do not contain Wnt-producing Paneth cells, therefore exogenous Wnt is added for the maintenance of the cultures. In small intestine organoids, the development of Paneth cells allows a reduction in exogenous Wnt levels (Figure 5) [74]. Intestinal crypts can be isolated from small surgical resections or endoscopic biopsies [75]. The versatility of the ASC-derived organoid culture system makes it a powerful tool to study the epithelium role in IBD. Organoids can be expanded from healthy mucosal intestinal tissue samples and from both adult and pediatric IBD patients, with comparable growth efficiency. They faithfully retain physiological and pathological characteristics of the intestinal original segment,

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showing tissue-specific, region-specific (colon or small intestine), age and disease transcriptional and epigenetic profiles [71]. In the following sections we will explore the application of human intestinal organoid cultures in IBD-modelling.

5. Intestinal organoid application in IBD modelling

Intestinal organoids provide a more complex *ex vivo* model to elucidate the IBD pathophysiology, allowing the study of the IECs-immune cells-bacteria cross-talk as detailed in Figure 7 [76]. Several approaches have been developed to establish these co-coltures and the principal techniques will be described below.

5.1. Microbiota- host interaction modelling in intestinal organoids

Changes in microbiota composition, specifically a reduction of microbial diversity and an expansion of specific bacterial taxa, a condition known as *dysbiosis*, represent an important risk factor for the onset and progression of IBDs [77]. The gut microbiota establishes within the first 2 weeks of life and usually remains stable throughout life. The intestinal microflora has a great metabolic capacity. The host uses microbiota bacteria to digest indigestible dietary components, important metabolites of this process are short-chain fatty acids (SCFA) [78], and secondary bile acids [79], they are ligands of the GPBAR1 and FXR receptor, expressed respectively on many immune regulatory cell surfaces such as Treg and anti-inflammatory M2 and on intestinal epithelial cells [80][81][82][83]. The intestinal microbiota can be modified by diet, antibiotics, drugs and other environmental factors defined " early-life factor ", to which individuals are exposed to in the first months of life, transforming our microbial allies into potential pathogens [84].

Because the relevance of intestinal microbiota in modulating intestinal physiology and pathology, microbiota-intestinal organoids co-culture have been developed, as a model system to overcome some of the disadvantages associated with the use of mouse models and cell lines, allowing for repeated experiments, conserving the properties of the native organs, recapping the patient and disease's characteristics *in vitro* [72,85]. Furthermore, these co-cultures are useful to the study of the "chain of infection"[76]. For example, the introduction of *Salmonella typhimurium* into intestinal organoids induces the activation of the NF-kB pathway and an upregulation of cytokine-mediated signalling [86]. Similarly, *C. difficile* and the pathogenic *E. coli* have all been cultured in organoid models [87]. Interestingly, enteroid and colonoid cultures from different patients respond differently to infection, due to the unique genetic background of each patient [88].

Organoid culture systems have also been used to study virus infections such as rotavirus and norovirus (HuNoV). These viruses are difficult to grow in single-layer cell cultures. Indeed, the differentiated cells of the organoid better supported rotavirus replication compared to the monolayer cultures, and it has been demonstrated that rotavirus can infect enteroendocrine cell types in addition to enterocytes [89]. These systems would allow to study a large fraction of the individual fecal material species, because, once isolated from faeces, they may be introduced into PDOs, therefore they could represent an important advance in the modeling of IBD and in the study of the interactions between the microbiota bacteria and the patient. Generally, bacteria interact with the apical side of the epithelium, that is projected towards the inside of the 3D structure and therefore it is not easily accessible. Several approaches have been developed to facilitate access to microorganisms, as will be treated below. The 3D organoid microinjection method is a useful technique, but requires extreme precision and accuracy (Figure 7B). Using a fine capillary, the microbe is introduced into the 3D system and contact directly the apical side of the epithelial cells. Secondly, 3D organoids, mechanically dissociated, are mixed with the bacterium and seeded into the matrix, as the organoid fragments grow and aggregate, they incorporate the pathogen inside. Recently, reverse polarity organoids have been established, with the apical side facing towards the medium, known as ''apical organoids'' (Figure 7C). This made the apical side of the epithelium easily accessible, making bacterial-organoid co-cultures possible, without the need for microinjection [90]. In this system

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the apical organoids ASCs can differentiate in the main types of IECs, and so maintain the correct polarity and barrier function. Of interest, ECM proteins are able to regulate epithelial polarity, indeed spheroids, seeded in a matrix, show basal polarity while suspended spheroids without ECM proteins show an apical polarity [91]. Thus, apical organoids were developed from basolateral organoids through digestion of the matrix with EDTA solution. The organoids were removed from the Matrigel without dissociating them into single cells, and then plated in suspension with the growth medium. After only 3 days it has been observed that the polarity of the intestinal organoids was reversed. This model has been used to study entero-pathogenic infections such as Salmonella Typhimurium and Listeria monocytogenes, but could be used to study interactions with commensal microbes. However, the organoids lumen is characterized by hypoxic conditions, allowing the study of facultative or obligate anaerobes. However, due to the presence of small amounts of oxygen in the organoid lumen, obligate anaerobic bacteria cannot be grown for long periods, and this represents a great limitation of the organoids-bacteria culture systems. In this regard, to make the apical side more easily accessible, it has been established 2D monolayer cultures from 3D organoids (Figure 7D). This model scarifying the crypt structures but provide a more physiologically accurate system for study host-microbe interaction. Thus, single cells, derived by the mechanical or enzymatical dissociation of 3D organoids, can bed plated on a matrix-coated-well with the apical side facing up and the basolateral side towards the plate to maintain polarity. The microbes are then introduced into the culture media. However, it is not possible to evaluate the effects on the basolateral surface. Further on, as will be described in the next paragraph, the dissociated cells can also be plated on a Transwell insert, generating a gut polarized cell line that mimic a permeable barrier, separating the apical (lumen) and the basal (lamina propria) compartments of the well, the microbes are then introduced into the culture media and the immune cells plated on the well under the Transwell (Figure 7G). This tool allows the simultaneous assessment of the interactions of the basolateral side of the epithelium with the immune cells and the apical side with the bacteria of the microbiota bacteria. This method represents a more representative and physiologically faithful to in vivo microenvironment, for studying the interactions between the host-microbiota-immune system.

5.2. Modelling of IBD immune system in intestinal organoids

The current ASCs derived-organoid systems models strictly the intestinal structure [92], but they do not contain stromal tissues or *lamina propria*, nor functional immune cells. In the IBD pathogenesis, IECs interact with several cell types including immune cells, mesenchymal cells. Therefore, the co-culture of intestinal organoids and immune cell types may provide a more complex *ex vivo* model to elucidate the IBD pathophysiology more closely. A growing body of evidence demonstrated that the utility of ASC-derived epithelial organoid co-cultures with mesenchymal or immune cells, and the co-culture of patient inflamed bowel tissues with patient's own immune cells is currently the gold standard for IBD research. This system provides the opportunity to investigate the effect that each immune cell subpopulation and how these cells interact with the epithelium cell type. Recently, as described above, research groups have generated 2D monolayers from 3D organoid culture systems (Figure 7E) [93]. This model sacrificing the crypt structure, but provides a more physiologically accurate system for dissecting the pathogenesis of IBD.

Bowel antigen presenting cells (APCs) such as DCs and macrophages sample antigen from the gut lumen via PRR and stimulate T-lymphocyte-mediated response. Physiologically, APCs activate tolerogenic response, maintaining intestinal homeostasis. intestinal macrophages respond to TGF-beta and produce anti-inflammatory cytokines such as IL-10 [94]. In CD patients, they infiltrate inflammation sites and produce excessive amounts of IL-23, TNFα and IL-6 [95]. Recently, this innovative system was established using human small intestine organoids co-cultured with macrophages and pathogenic *Escherichia coli*. This study shows that macrophages (M0) extend their dendrites across the monolayer

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to interact with E. coli in the "lumen" improving the physical barrier function of the organoid monolayer. Co-culture with macrophages also provided resistance to permeability induced by pathogenic E. coli. The results obtained helped to clarify how the epithelium and innate immune cells coordinate the response to enteric pathogens[96].

Within mesenteric lymph nodes (mLN) and Gut-associated lymphoid tissue (GALT), antigen presentation by DCs and macrophages induce the maturation and differentiation of naïve T cells into Th-1 for the clearance of intracellular pathogens, Th-2 mediate responses against parasites, Th-17 are directed against bacteria and extracellular fungi or Tregs which play an important role in the maintenance of cell homeostasis. Generally, gut-resident T lymphocytes exist in a tolerogenic state. The IBD pathogenesis is strictly connected to T lymphocytes, and abnormally activated cells lead to inflammation through the excessive release of cytokines and chemokines which have further effects on both the innate and adaptive immune systems, perpetuating an exacerbated inflammatory process [97]. The cause of this T cell dysregulation is largely unknown, it is largely reported CD is characterized by abnormal Th1 immune response, while UC has been considered as a Th2-mediated disease. Recently, the role of Th9 in the pathogenesis of CU has been identified, the amount of IL-9^{+} Th9 cells are increased in the lamina propria of patient with active disease [98]. In the same way Th17 cells are involved in both CU and CD pathogenesis. A recent model of intestinal murine organoids co-coltured with and intraepithelial lymphocytes (IELs), has provided a further provided characterization of the IELs survival and motility mechanisms, which are altered in the intestinal mucosa of IBD patients [99]. Another study refers to the generation of M cells, critical in regulating immune response in the gut, in murine organoid models using the recombinant RANKL protein, an NF-κB ligand that induces the expression of the transcription factor SpiB, that drives M cell differentiation [100]. Induction of functional M cells in human organoid models provide insights into antigen presentation at the intestinal barrier. Recent reports have focused on the role exerted by innate lymphoid cells (ILCs) in maintaining the integrity of the intestinal barrier and in triggering the immune response against pathogens [101]. ILC3s play an important role in maintaining IEC, in defence against extracellular bacteria and fungi, and in the modulation of inflammatory bowel disease, thanks to their interaction with DCs and macrophages. Specifically, ILC3 can be classified on the natural cytotoxicity receptor (NCR) expression. More specifically, NCR^{+} ILC3 produce IL-22 which stimulates the production of AMPs. NCR^{-}ILC3 produce both IL-17 and IL-22. It has been widely shown that in chronic diseases ILC3s are able to downregulate RORγτ expression and over-regulate T-bet expression, inducing IFNγ production, so they are defined ex-ILC3. ILC3s are therefore considered to be important inflammation mediators and their depletions result in colitis resolution [102]. In this context, IL-22^{+}-ILC3s were co-cultured with intestinal organoids [103]. It was observed that ILC3 significantly increased the bulk of intestinal organoids and this phenomenon was inhibited by an anti-IL-22 antibody. Therefore, this suggests that ILC3 are also able to activate ASCs, promoting their proliferation. ILC3 have a critical role in CD, as IL-22^{+}-ILC3s are decreased while IFN-γ^{+}-IL-17^{+}-ILC3 are increased in the intestine of CD patients [103]. This co-culture model could be useful for studying the ASCs-ILC3 interaction in IBD.

5.3. Organoids and ECM

In addition to an immune dysfunction , IBD particularly CD, is characterized by altered ECM function [104]. Thus, while a defect in ECM deposition might lead to the ulcers or fistulas formation, the uncontrolled overproduction of extracellular matrix ECM is the cause of excessive fibrosis and stenosis of the intestine that typically occurs in CD patients. ECM generation is promoted by cross-talk between activated macrophages and mesenchymal cells. Adequate models of fibrosis are lacking and animal models are less biologically relevant. The intestinal organoids derived from ASCs do not include a mesenchymal component. In a study [105], human intestinal ESCs derived-organoids are established. Mature organoids with a high number of mesenchymal cells were selected and used to

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evaluate the efficacy of the antifibrotic drug, spironolactone, showing the pre-clinical and research potential of intestinal organoids as a future validate model of intestinal fibrosis.

6. Organoids-on a chip

Aside from intestinal anatomy, intestinal motility and luminal flow are physiological functions of the intestine that are also important to reproduce to study intestinal environment but are very difficult to recap ex vivo. Recently, these proprieties have been simulated via microfluidic platforms lined with epithelial cells, and known as "gut-on-a chip" [106]. These platforms enable flow luminal patterns, mechanical strain, shear stress, and peristaltic activity to be reproduced with greater accuracy than previously possible. In recent years, microfluidic devices have been developed incorporating organoid-derived cells with active perfusion of growth medium components, which allow simulation of intestinal homeostasis and cell turnover (Figure 7H) [107]. These provide a more representative and physiologically relevant model for studying host-microbiota interactions, by inoculating with bacterial cultures [108]. A gut-on-chip device consists of two channels that simulate the intestinal lumen and a blood vessel, separated by a membrane lined with ECM [109]. In the organoid-on chip model, fragments of human bowel organoids are plated onto a porous ECM-coated membrane and human intestinal microvascular endothelial cells are seeded on the opposite side of the same membrane within a parallel channel. Fluid flow and peristalsis-like movements promote epithelial multilinear differentiation, including goblet cells and accumulation of a bilayer structure of mucus, while maintaining a subpopulation of proliferative epithelial cells [110]. A disadvantage of organoid-on-chip devices is the presence of an aerobic environment within the epithelial chamber, which prevents the introduction of strictly anaerobic bacteria [109]. This limitation has been addressed thanks to the development of a novel microfluidic device with a transluminal hypoxia gradient, which allows studying also the effect of obligate anaerobes on the epithelium[108]. Overall, these systems are useful for studying the pathophysiology of inflammatory bowel diseases [111]. However, these models are expensive, require special training to use, and do not allow for high-throughput experiments.

7. Intestinal organoids and personalized medicine

Current therapeutic strategies for IBD treatment suppress the expression of pro-inflammatory cytokines, immune cell activation, their proliferation, migration and their resistance to apoptosis [112][113]. IBDs are currently treated with immunosuppressive drugs, including local and systemic corticosteroids, anti-inflammatory agents, anti-cytokine therapies, biological agents and probiotics [114][115].

However, there is still a large subset of patients who do not respond or have an incomplete response to treatment or relapse at the end of treatment.

ASCs-derived organoids retain the genetic profile and the transcriptional and epigenetic landscape of the primary tissue from which they are established [71].

IBD is a polygenic disease, determined by specific genes polymorphisms. Mutations usually involved epithelial barrier, oxidative-stress, immune tolerance and autophagy genes [24]. IBD-patient-tissue-derived-organoids carriers of specific mutations so represent a useful model for the study of these diseases , contributing to the molecular stratification of the disease [116].

Indeed, the discovering of a link between specific mutations and clinical IBD phenotypes, such as disease course, response to treatment, risk of progression to colitis-associated carcinoma (CAC) may improve the conventional clinical classification [117]. PDOs from patient with active IBD is established [118]. In a recent study, IBD-PDOs had a distinct phenotype from those derived from control tissue, with a smaller size, greater cell death, abnormal cell polarization, and less ability to budding. Furthermore, they showed a reduced of the tight junction proteins ZO1, Occludin and Claudin-1 expression, and alterations in the adherent junction and desmosomal proteins expression [118]. These alterations may due to permanent changes in ASCs genome [14]. Furthermore, CD-patients-

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derived-ASCs exhibited a distinct gene expression pattern from the classical stem cell markers such as LGR5 and SMOC2 [119].

A recent study shows that the mouse-derived colonoids exposed to chronic inflammatory stimuli (TNFα, IL-1β, IL-6, LPS, flagellin) undergo an over-regulation of the NFκB pathway, which persists after removal of the stimuli [120]. Also, these organoids underwent a transformation into an undifferentiated state, along with the upregulation of genes related carcinogenesis, suggesting their potential utility as a model for studying epithelial changes that occur in CAC.

Other studies using pediatric-IBD-patients-PDOs showed alterations in DNA methylation and transcription profiles that may be related to therapy responses [121].

Growing evidence supports the idea that epigenetic modifications are not only present primarily in the intestinal epithelium but they may also acquire during the disease progress[122]. Alterations in the DNA methylation profile and non-coding RNA (e.g. miRNA) has been identified in the mucosa of IBD patients [123]. A recent report shows that the CD or UC derived-ACSs have specific stable changes in DNA methylation and transcription that are maintained in cultures of intestinal organoids [121].

Several epigenetic mechanisms regulate epithelial function, both in homeostasis and in IBD and they are regulated by the *lamina propria* cells and microbial signals [124]. Organoid cultures may be useful for understanding the role of stable epithelial changes induced by disease-dependent stromal and bacterial factors.

Therefore, IBD-PDO appear an important model for the study of epithelial and mucosal abnormalities occurring in IBD.

In particular, healthy individual derived organoid cultures serve to explore the specific biological role played by IBD pro-inflammatory factors in the integrity of the epithelial barrier. In contrast, IBD-PDO are useful to explore the cumulative effect of genetics and a pro-inflammatory environment on epithelial permeability. This feature may help to understand clinical cases in which genetic and epigenetic variants haven't a direct effect on epithelial behaviour or phenotype.

8. Conclusions

The pathogenesis of IBD involves a complex interaction between the environmental, genetic or microbial factors and the aberrant immune responses. Treatment strategies available in IBD aim to suppress the inflammatory response. However, current treatments often fail. In this review the role of IECs in maintaining intestinal homeostasis, in epithelial regeneration, has been extensively treated. However, none of the currently available therapies target the epithelium. Therefore, a more adequate system is needed for the study of the pathogenic mechanisms underlying IBD and for the identification of new therapeutic targets.

The PDO system has unique advantages and opportunities. Indeed, they retain the specific characteristics of the site and patient from which they derive and thus mimic cellular behaviour in an extremely versatile ex vivo model. The establishment of co-cultures with immune cells and microbiota and the development of new platforms such as organoid on chip, allow to reproduce a system closer and closer to the physiology observed in vivo by reproducing the complex cross-talk between IELs and the microbial or immune counterparts, and movements peristaltic, shear stress and flow movements are extremely difficult to reproduce in traditional models but essential to be able to study and fully understand the mechanisms underlying these complex inflammatory pathologies. Intestinal organoids represent a very promising tool to personalize medicine and to overcome the problem of patient-resistance to current therapies. **Authors' contribution:** The authors equally contributed to the writing of the manuscript.

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